Hypothyroidism increases Fos immunoreactivity in cholinergic neurons of brain medullary dorsal vagal complex in rats

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Yuan, Pu-Qing, and Hong Yang. Hypothyroidism increases Fos immunoreactivity in cholinergic neurons of brain medullary dorsal vagal complex in rats. Am J Physiol Endocrinol Metab 289: E892–E899, 2005—Hypo- or hyperthyroidism is associated with autonomic disorders. We studied Fos expression in the medullary dorsal motor nucleus of the vagus (DMV), nucleus tractus solitarii (NTS), and area postrema (AP) in four groups of rats with different thyroid states induced by a combination of drinking water and daily intraperitoneal injection for 1–4 wk: 1) tap water and vehicle; 2) 0.1% propylthiouracil (PTU) and vehicle; 3) PTU and thyroxine (T4; 2 μg/100 g); and 4) tap water and T4 (10 μg/100 g). The numbers of Fos immunoreactive (IR) positive neurons in the DMV, NTS, and AP were low in euthyroid rats but significantly higher in the 4-wk duration in hypothyroid rats, which were prevented by simultaneous T4 replacement. Hyperthyroidism had no effect on Fos expression in these areas. There were significant negative correlations between T4 levels and the numbers of Fos-IR-positive neurons in the DMV (r = −0.6388, P < 0.008), NTS (r = −0.6741, P < 0.003), and AP (r = −0.5622, P < 0.004). Double staining showed that Fos immunoreactivity in the DMV of hypothyroid rats was mostly localized in choline acetyltransferase-containing neurons. Thyroid hormone receptors α1 and β2 were localized in the observed nuclei. These results indicate that thyroid hormone influences the DMV/NTS/AP neuronal activity, which may contribute to the vagal-related visceral disorders observed in hypothyroidism.

thymloid hormone; dorsal motor nucleus of the vagus; nucleus tractus solitarii; vagus nerve; area postrema

HYPOTHYROIDISM IS A PREVALENT DISEASE worldwide and has a high incidence in elderly patients with nonthyroidal disease (3, 39). Extensive cardiovascular, gastrointestinal (GI), and metabolic disorders, such as sinus bradycardia, altered GI secretion and motility, and hyperlipidemia are the main clinical symptoms of hypothyroidism. These symptoms indicate autonomic dysfunction with a relative vagal dominance (43, 44) and correlate to increased mortality from cardiovascular and cerebrovascular diseases in those with a history of overt or subclinical thyroid disorders (29, 39). How thyroid hormone regulates vagal activities, particularly the central mechanism, is still poorly understood.

On the basis of solidly established physiological role of medullary thyrotropin-releasing hormone (TRH)-containing vagal regulatory pathways in the central regulation of GI functions (47), we recently revealed that feedback regulation of TRH gene expression by thyroid hormone in the medullary raphe pallidus (Rpa), raphe obscurus (Rob), and parapyramidal region (PPR) may contribute to hypothyroidism-induced autonomic disorders (49–52). TRH-containing projections arising from these nuclei innervate the dorsal vagal complex (DVC) (20) and the sympathetic motor neurons in the intermediolateral cell column of the spinal cord (40). Our previous studies showed that hypothyroidism increases TRH mRNA levels in the Rpa, Rob, and PPR and induces Fos expression, a widely accepted parameter indicating brain neuronal activation, in the TRH-synthesizing neurons in these nuclei (49, 51). In contrast, hyperthyroidism decreases TRH gene expression in the Rpa, Rob, and PPR (50). The increase of Fos expression in the Rpa and Rob negatively correlated with serum thyroid hormone levels ≤4 wk after the induction of hypothyroidism (51). In addition, the thyroid hormone receptor subtype-β2 (TRβ2), which mediates the feedback regulation of TRH gene expression by thyroid hormone (11, 16, 19), is localized in these nuclei (52). These findings demonstrate that the TRH-containing neurons in the Rpa, Rob, and PPR are medullary targets of thyroid hormone with regard to its regulation of autonomic functions.

The DVC, downstream from the Rpa/Rob/PPR in the medullary TRH-containing vagal regulatory circuits, is composed of the dorsal motor nucleus of the vagus (DMV), which contains somata of parasympathetic efferents projecting to the visceral organs (4, 46) and the nucleus tractus solitarii (NTS), with neurons receiving vagal afferent input from the viscera (13). The nearby area postrema (AP) shares a synaptic relationship with the NTS. The NTS and AP are the first relays of the central nervous system receiving sensory stimuli from the viscera and play a key role in integrating neuronal and hormonal responses to internal and external environmental changes (7). Dense TRH-containing nerve terminals and TRH receptors are located in the DMV and NTS (22, 38). Electron microscopic studies reveal that TRH terminals make direct synaptic contact with dendrites of gastric motoneurons throughout the DMV and medial NTS (37, 38). TRH acts as an excitatory neurotransmitter on the vagal motor neurons in the DMV to elevate vagal efferent discharge and stimulate gastric and pancreatic secretory/motor functions (23, 26, 47, 48). On the basis of these anatomic and functional findings, we investigated in the present study whether altered thyroid states influence the neuronal activity of the DMV, NTS, and AP using immunohistochemistry of Fos, the protein product of the immediate early gene c-fos, which is a commonly accepted parameter indicating brain neuronal activation in the medullary

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marker of neuronal activation in the brain and peripheral enteric plexuses and is widely used to reveal neural circuits involved in a specific response to a stimulus (25). The neuronal phenotype of hypothyroidism-activated DMV cells and the localization of TRα1 and TRβ2 in the DMV, NTS, and AP were also studied.

MATERIALS AND METHODS

Animals and treatments. Male Sprague-Dawley rats weighing 270–320 g (Harlan, Indianapolis, IN) were maintained on Purina rat chow and tap water ad libitum and housed under controlled temperature (22 ± 2°C) and illumination (light on 0600–1800 h) for ≥7 days before any treatment. Rats were then divided into four groups and treated for 4 wk: 1) euthyroid, injected intraperitoneally daily with vehicle (0.02 N NaOH-saline); 2) hypothyroid, induced by 0.1% 6-n-propyl-2-thiouracil (PTU; Sigma Chemical, St. Louis, MO) in the drinking water and a daily intraperitoneal injection of vehicle; 3) hypothyroid with thyroxine (T3, Sigma) replacement, 0.1% PTU in the drinking water and daily intraperitoneal injection of T3 (2 μg/100 g), and 4) hyperthyroid, induced by a daily intraperitoneal injection of a higher dose of T3 (10 μg/100 g). At the end of the 4-wk treatment, the body weights of rats in the four groups were: euthyroid, 379 ± 6 g; PTU-treated, 291 ± 6 g; PTU plus T3 replacement, 338 ± 9 g; and high-dose T3, 358 ± 7 g. In the time course study, four groups of rats (4–6 rats/group) drinking 0.1% PTU without daily intraperitoneal injections were killed after 1, 2, 3, or 4 wk of treatment. The corresponding control group received no treatment. At the end of treatments, all rats were fasted for 24 h and then euthanized by transcardial perfusion under deep pentobarbital sodium (70 mg/kg ip; Abbott Laboratories, North Chicago, IL) anesthesia. Blood samples (0.5 ml/rat) were collected from the left ventricle before perfusion, and sera were kept at −75°C before total T4 levels were measured. Brain stems were collected and fixed in 4% paraformaldehyde in 0.1 M PB buffer for Fos and ChAT immunohistochemistry. All animal protocols were approved by the Veterans Affairs Greater Los Angeles Healthcare System Research Service Animal Committee.

T4 radioimmunoassay. Serum aliquots (10 μl) were used to measure total T4 levels with a commercial radioimmunoassay kit (ICN Biomedicals, Costa Mesa, CA). The sensitivity of the assay ranged from 0 to 25 μg/dl. All samples were measured in duplicate.

Fos-like immunohistochemistry and quantitative analysis. Fos immunoreactivity was detected as previously described (5, 51). Rats were deeply anesthetized with pentobarbital sodium, and a transcardial perfusion fixation technique was used that has been proven to be a suitable preservation technique for nervous tissue (8). After the thoracic cavity was opened, each animal was transcardially perfused with 100 ml of isotonic saline, followed by 500 ml of 4% paraformaldehyde in 0.1 M phosphate buffer (PB, pH 7.4) via a blunt needle inserted into the aorta and drained through the right atrium of the heart. After the perfusion, the rat brains were removed, postfixed for 3 h at 4°C in the same fixative, and subsequently cryoprotected overnight in 20% sucrose in 0.1 M PB. Frozen coronal sections (30 μm) of the brain stem were cryostat cut (IEC, Needham Heights, MA) at the interalatal levels of −4.24 to −5.30 mm, according to the atlas of Paxinos and Watson (32). This included the main rostral-caudal length of the DMV and NTS and the entire AP in the dorsal medulla. Free-floating sections were incubated for 24 h at 4°C with the primary antibody (Fos Ab-5 rabbit polyclonal antibody; Oncogene Research Products, Cambridge, MA; dilution 1:10,000 in 0.01 M PBS containing 0.3% Triton X-100) followed by 1 h at room temperature with a biotinylated secondary antibody (goat anti-rabbit; Jackson ImmunoResearch Laboratories, West Grove, PA; dilution 1:1,000). Sections were finally processed for avidin-biotin-peroxidase by using diaminobenzidine as the chromogen and then mounted on slides (Superfrost/Plus; Fisher Scientific, Pittsburgh, PA), dehydrated in ethanol, cleared in xylenes, and coverslipped. The presence of Fos immunoreactivity was detected with bright-field microscopy as a dark brown reaction product in the cell nuclei. The numbers of Fos-IR-positive neurons in the DMV, NTS, and AP were counted and quantified as the average number from 18 sections per nucleus in each rat. Both the left and right DMV were added together and taken as one region. Double-counting errors were corrected by the following formula proposed by Abercrombie (1) to estimate nuclear populations from microtome sections: A = P × [M/(L + M)], with P being the corrected cell count, A the total cell count, M the section thickness (μm), and L the average diameter of the nucleus (μm). To determine the average diameter of the nuclei, 10 randomly selected Fos-IR-positive nuclei were measured with a microruler in each section, and ≥5 sections per region were measured.

Double immunostaining for Fos and Chat. After incubations with Fos antibody and biotinylated goat anti-rabbit IgG in Fos-like immunohistochemistry as described above, sections were processed for avidin-biotin-peroxidase by using diaminobenzidine enhanced with nickel ammonium sulfate as the first chromogen. Sections were then rinsed with phosphate-buffered saline (PBS) for 3 h at room temperature and incubated with a polyclonal goat anti-ChAT serum (no. AB144p; Chemicon International, Temecula, CA; 1:500 in PBS containing 0.3% Triton X-100. Finally, sections were processed by an avidin-biotin-peroxidase procedure using diaminobenzidine as the second chromogen. Fos immunoreactivity was detected as a dark blue reaction product in the nuclei, and Chat immunoreactivity appeared as a brown reaction product in the cytoplasm.

TRα1 in situ hybridization using digoxigenin-labeled cRNA probe. Brains were removed after decapitation and immediately frozen with dry ice. Cryostat-cut brain stem sections (10 μm) were collected at the levels from interaural −4.24 to −5.30 mm. The in situ hybridization histochemistry was performed with digoxigenin-labeled TRα1 probe, based on the method of Panoskaltsis-Mortari and Bucy (31). The 1.2-kb EcoRI TRα1 fragment was subcloned in plasmid PCMVTNT (kindly provided by Dr. G. Brent, University of California, Los Angeles). The antisense and sense TRα1 probes were synthesized by T7 or SP6 RNA polymerase using a Dig RNA labeling kit (Boehringer Mannheim, Indianapolis, IN) with 1 μg of XhoI- and NotI-linearized plasmid DNA or PvuII-linearized pSPT18-Neo plasmid DNA (provided with the Boehringer Mannheim kit) as templates, respectively. The amount of labeled probes was estimated with a spot test (24). Sections were fixed in 4% paraformaldehyde in 0.1 M PB (pH 7.4) prepared with 0.1% diethyl pyrocarbonate sterile water, washed in 2× SSC, and acetylated in 0.25% acetic anhydride. Digoxigenin-labeled probes (1 μg/ml) were added to a hybridization mixture, which consisted of 50% deionized formamide, 1× Denhardt’s solution, 4× SSC, 0.5 mg/ml herring sperm DNA, 0.25 mg/ml yeast tRNA, and 10% dextran sulfate. Hybridization was carried out in the hybridization buffer at 45°C for 16 h. After hybridization, sections were washed twice in 2× SSC for 10 min each time, treated with ribonuclease A (20 μg/ml, Boehringer Mannheim) for 30 min at 37°C to digest the unhybridized RNA, and then desalted in SSC solutions of decreasing concentrations. The immunohistochemical detection was performed with anti-digoxigenin antibody conjugated with alkaline phosphatase (1:500, Boehringer Mannheim). Nitroblue tetrazolium/salt phosphate (Boehringer Mannheim) was used as the chromogen. TRα1 mRNA signals appeared as a dark blue reaction product in the cytoplasm.

TRβ2 immunohistochemistry. The primary antiseraum was raised in a New Zealand White rabbit against a synthetic peptide corresponding to amino acids 111–142 of TRβ2 and was kindly provided by Dr. Harold L. Schwartz (Department of Medicine, University of California, Irvine, CA). The specificity of the antiseraum had been tested in previous studies (18, 42) The immunohistochemistry was performed...
as in our previous studies (52) with the antibody dilution of 1:4,000. The presence of TRβ2 immunoreactivity was detected as a dark blue reaction product in cell nuclei. The control staining was performed using the preimmune serum from the same rabbit that produced the primary antibody (kindly provided by Dr. Harold L. Schwartz).

Statistical analysis. Quantitative data are expressed as the mean ± SE of each group. Comparisons between two groups were analyzed by unpaired Student’s t-test and multiple groups were compared by two-way or one-way analysis of variance using a statistics program (SigmaStat 2.03). Correlations between serum T4 levels and the numbers of Fos-IR-positive neurons in specific brain stem regions were analyzed by linear correlation. P values of <0.05 were considered statistically significant.

RESULTS

Serum T4 levels in different thyroid states. Serum T4 levels after 4 wk of treatment in euthyroid rats were 3.0 ± 0.5 μg/dl (n = 4). Rats drinking 0.1% PTU had 83% lower serum T4 levels (0.5 ± 0.0 μg/dl; n = 6) compared with the euthyroid rats. Daily injection of T4 (2 μg/100 g) in PTU-treated rats prevented this decrease and brought serum T4 levels to 4.9 ± 1.9 μg/dl (n = 6). Animals with hyperthyroidism induced by daily intraperitoneal injection of a higher dose of T4 (10 μg/100 g) showed a sixfold higher level of serum T4 (19.2 ± 0.7 μg/dl; n = 6) compared with the euthyroid rats.

Numbers of Fos-IR-positive neurons in the DMV, NTS, and AP in different thyroid states. Rats that drank tap water and were injected intraperitoneally with vehicle for 4 wk had only a few Fos-IR-positive cells in the DMV, NTS, and AP. Hypothyroidism selectively induced remarkable increases of Fos-IR-positive neurons in the DMV, NTS, and AP but not in the surrounding areas or in other nuclei within the caudal dorsal medulla, including the cuneate nucleus, gracile nucleus, and hypoglossal nucleus. Simultaneous T4 replacement (2 μg/100 g) in PTU-treated rats completely prevented the induction of Fos in the DMV, NTS, and AP. A daily high dose of T4 injection (10 μg/100 g/day) did not significantly increase the numbers of Fos-IR-positive neurons in these nuclei (Figs. 1 and 2).

Negative correlations between serum T4 levels and numbers of Fos-IR-positive neurons in the DMV, NTS, and AP. Correlations between serum T4 levels and the numbers of Fos-IR-positive neurons in the DMV, NTS, and AP were observed during the development of hypothyroidism. Serum T4 levels gradually decreased as the PTU treatment continued and reached a significantly low level of 16% compared with the control value after 2 wk of treatment. Thereafter, T4 levels remained low until the end of the 4-wk treatment period (Fig. 3, top left). During the progression of the hypothyroidism, there were significant negative correlations between serum T4 levels and the numbers of Fos-IR-positive neurons in the DMV, NTS, and AP (Fig. 3).

Colocalization of Fos and ChAT in the DMV of hypothyroid rats. ChAT-IR-positive neurons were localized within the DMV and the ventrally adjacent hypoglossal nucleus. There were few Fos-IR-positive neurons in the ChAT-IR-containing neurons in euthyroid rats (Fig. 4A). In contrast, ~50% of ChAT-containing neurons in the DMV of hypothyroid rats were Fos-IR-positive, and almost all the Fos expression in the DMV was in ChAT-IR-positive neurons. No Fos expression was seen in the hypoglossal nucleus (Fig. 4B).

TRα1 mRNA signals and TRβ2 immunohistochemistry in rat dorsal medulla. TRα1 mRNA signals were detected in the cytoplasm of many nuclei in the rat dorsal medulla, most remarkably in the DMV and moderately in the NTS and AP (Fig. 5). TRα1 mRNA signals were not observed in brainstem sections incubated with the sense probe (Fig. 5) or pSPT 18-Neo probe (data not shown).

TRβ2-IR-positive cells were widely distributed throughout the gray matter in the medulla. No immunostaining was present in white matter fiber tracts. Within the dorsal medulla, many neuronal groups contained some TRβ2-IR-positive cells with different immunostaining density. Dense accumulations of TRβ2 immunoreactivity were particularly apparent in the cells of the DMV and NTS and extended throughout the entire rostral-caudal extent of the nuclei. The reaction product was

![Fig. 1](http://ajpendo.physiology.org/)

Fig. 1. Number of Fos-immunoreactive (IR)-positive cells detected by immunohistochemistry in the medullary dorsal motor nucleus of the vagus (DMV), nucleus tractus solitarii (NTS), and area postrema (AP) of rats with different thyroid states after 4 wk of treatment. Hypothyroidism induced remarkable increase of Fos-IR-positive cells in these nuclei. Simultaneous thyroxine (T4) replacement (2 μg·100 g·day⁻¹) in propylthiouracil (PTU)-treated rats completely prevented Fos induction. Daily high dose of T4 injection (10 μg·100 g·day⁻¹) did not significantly increase the numbers of Fos-IR-positive neurons in these nuclei. Each column represents mean ± SE of 18 sections/rat, and the number of rats is indicated in parenthesis for each group. *P < 0.05 compared with all other groups.
primarily confined to the cell nuclei. No immunostaining was seen in the control sections incubated with preimmune serum of the same rabbit that produced the primary antibody (Fig. 6).

**DISCUSSION**

Results obtained from the present study clearly show that hypothyroidism induced by PTU results in a remarkable induction of Fos expression in the brain medullary DMV, NTS, and AP, the nuclei that directly control vagal functions. In 24-h-fasted euthyroid rats, only a few Fos-IR-positive neurons were observed in the dorsal medulla, consistent with previous observations (5). In contrast, PTU added to the drinking water gradually decreased serum thyroid hormone levels and increased the number of Fos-IR-positive neurons in the DMV, NTS, and AP. The increase of Fos expression in hypothyroid rats was not caused by circadian variations or altered environmental temperature, because all the rats were kept under the same illumination and temperature controls. In addition, the Fos induction was not related to the feeding and digesting process, since the rats were fasted for 24 h, which empties the stomach and does not induce Fos expression in the DMV, NTS, and AP (present study) (5). Our data indicate that the increase in the numbers of Fos-IR-positive neurons in the DMV, NTS, and AP in PTU-treated rats most likely resulted from the reduction of circulating thyroid hormone levels. This was supported by the time course study showing negative correlations between the numbers of Fos-IR-positive neurons in these nuclei and serum T4 levels. Although the size of some experimental groups was small, statistical analysis of the data showed that the differences in Fos expression among groups of different thyroid states were striking. Furthermore, simultaneous T4 replacement completely inhibited PTU-induced Fos expression in these nuclei. This result excludes the possibility that PTU treatment induces Fos expression independent of hypothyroidism. The increases of Fos expression in the DMV, NTS, and AP in PTU-treated rats were clearly confined to these nuclei and not in the surrounding areas, indicating that the induction is highly nucleus selective. In addition, the time course of the Fos induction in the dorsal medulla of PTU-treated rats showed that the increase of Fos expression became significant after 1 wk of treatment, which was similar to the onset of Fos induction in the paraventricular nucleus (PVN) of the hypothalamus after thyroidectomy (15). Taken together, these findings suggest that, in addition to the neurons in the hypothalamic PVN and medullary Rpa, Rob, and PPR (15, 51), neurons in the medullary DMV, NTS, and AP are responsive to decreased levels of circulating thyroid hormone.

In vitro and in vivo studies have provided evidence that c-fos expression is regulated by thyroid hormone. The nuclear thyroid hormone receptor represses transcription activation by transcription factor activator protein-1 (AP-1) in a thyroid hormone-dependent fashion (41). Thyroid hormone receptors suppress c-fos by binding to the response elements on the promoter and acting as transcriptional silencers (53). Triiodothyronine (T3) decreased c-fos mRNA levels and the mRNA response to other stimuli, reducing the abundance of nuclear proteins that bind to an AP-1 binding site and the levels of c-fos protein (33). T3 also strongly decreased basal and stimuli-induced c-fos promoter activity (33). Our previous and present results that Fos was induced in the ventral medullary
Rpa, Rob, PPR, DMV, NTS, and AP by reduced circulating thyroid hormone levels are consistent with these in vitro findings.

On the other hand, there are possibilities that the increase of Fos expression may not represent an effect of hypothyroidism per se. Indirect mechanisms mediated by thyroid state-influenced neurotransmitters and/or peptides, which innervate the DMV, NTS, and AP neurons, cannot be excluded. Hypothyroid-induced metabolic disorders, hyperthermia, and other complications may also significantly or partly contribute to the Fos induction in the DMV, NTS, and AP. Likewise, we cannot completely exclude the possibility of Fos induction by fasting-related metabolic changes, which could be different between the euthyroid and hypothyroid rats. PTU treatment in rats altered preference taste behavior independent of hypothyroidism (6). In the present study, the body weight increase of the PTU-treated rats was smaller than control rats. However, we do not think that it was caused by a specific effect of PTU on eating behavior because reduced body weight was also observed in thyroidectomy-induced hypothyroid rats (49) and commonly attributed to decreased food intake due to hypothyroidism-induced low metabolic rate.

Fig. 3. Left: time courses of serum T4 levels and number of Fos-IR-positive cells in DMV, NTS, and AP during progress of hypothyroidism. Four groups of rats (4–6 rats/group) were killed after 1, 2, 3, or 4 wk of drinking 0.1% PTU. Corresponding control group received no treatment. Each column represents mean ± SE of 4–6 rats as indicated at bottom of column. *P < 0.05 compared with week 0 levels. Right: significant negative correlations between serum T4 levels and numbers of Fos-IR-positive neurons in the DMV, NTS, and AP; r, correlation coefficient.

![Fig. 3](image_url)
In contrast to hypothyroidism, hyperthyroidism induced by a high dose of T₄ injection (10 μg·100 g⁻¹·day⁻¹) did not influence the numbers of Fos-IR-positive neurons in the DMV, NTS, and AP. The T₄ replacement dose in PTU-treated rats selected in the present study (2 μg/100 g) was based on previous reports (45). However, it brought serum T₄ of PTU-treated rats to higher levels than in euthyroid controls. Previous studies have documented that thyroid hormone-dependent reversal of hypothyroidism-induced changes, such as a rise in hypothalamic TRH gene expression (12, 17), heart rate (9), and nuclear thyroid hormone receptor levels in the anterior pituitary (17), required high doses of thyroid hormone administration, which induced supraphysiological and hyperthyroid circulating levels. This phenomenon is consistent with the results obtained from our previous study showing that, when serum T₄ levels in PTU-treated rats with T₄ replacement were 2.6-fold higher than the euthyroid T₄ levels, the prevention of the Fos induction in medullary Rpa, Rob, and PPR of PTU-treated rats was still incomplete (51). In the present study, T₄ replacement completely prevented the Fos increase in the DMV, NTS, and AP but only partly reversed the decrease in body weight in PTU-treated rats (338 ± 9 vs. 379 ± 6 g of euthyroid and vs. 290 ± 6 g of PTU only).

It has been well established that increased Fos expression in the ventral medullary Rpa, Rob, and PPR of hypothyroid rats correlates with feedback regulation of thyroid hormone on TRH gene expression in these nuclei (49–52). The dorsal medullary DMV, NTS, and AP, unlike the Rpa, Rob, and PPR, do not contain TRH-synthesizing neurons and are downstream nuclei in these medullary vagal regulatory circuits, receiving dense innervation of TRH-containing fibers arising from the Rpa, Rob, and PPR and have receptors that bind TRH, which acts as a neurotransmitter to activate DMV neurons and inhibit NTS neurons (20, 23, 47). Therefore, the mechanism for the hypothyroidism-induced neuronal activation in the DMV, NTS, and AP could be different from that in the Rpa, Rob, and PPR. One of the possible mechanisms is that increased TRH synthesis in the Rpa, Rob, and PPR results in more TRH stimulation on DVC neurons in the hypothyroid state. This is supported by our previous and present observations that increased TRH gene expression in the Rpa, Rob, and PPR in hypothyroid animals was coupled with neuronal activation in

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**Fig. 5.** Medullary coronal sections (interaural −4.80 mm) showing TRα1 mRNA signals generated by in situ hybridization with a digoxigenin-labeled probe in the dorsal caudal medulla. **Top:** lower magnification of TRα1 mRNA signals detected in the cytoplasm of cells in section treated with antisense probe, most remarkably in DMV and moderately in NTS and AP. TRα1 mRNA signals were not observed in section incubated with the sense probe. **Bottom:** magnified parts of antisense probe-treated section showing TRα1 mRNA in DMV, NTS, and AP, respectively. Bar = 100 μm.

**Fig. 6.** A: medullary coronal section (interaural −4.80 mm) showing that TRβ2 immunoreactivity was distributed in the dorsal caudal medulla, particularly in DMV, NTS, and AP, and localized in the cell nuclei. B: no immunostaining was seen in control section incubated with preimmune serum instead of TRβ2 antibody. Bar = 100 μm.
the DVC. Studies aimed at selective removal of TRH innervation of the DMV, NTS, and AP are the next approaches to further reveal the role of TRH-containing Rpa, Rob, and PPR projections in hypothyroidism-induced DVC/AP neuronal activation.

Compared with the Fos induction in the Rpa, Rob, and PPR (51), the outbreak of Fos expression in the dorsal medullary DMV, NTS, and AP was more prompt. It reached levels close to their peaks in the first week and remained at these levels during the entire 4-wk observation period. In addition, the increase of Fos expression in the DMV, NTS, and AP in PTU-treated rats was completely prevented by T4 replacement, which resulted in incomplete prevention in the Rpa, Rob, and PPR (51). These findings suggest that the DMV, NTS, and AP neurons are more susceptible to serum thyroid hormone change and indicate that there is a mechanism(s) for hypothyroidism-induced neuronal activation in the DMV, NTS, and AP in addition to the above-mentioned secondary effect resulting from the elevation of TRH synthesis in the Rpa, Rob, and PPR.

In supporting the possible direct action of thyroid hormone on neurons in the DMV, NTS, and PPR, our results showed that thyroid hormone receptor subtypes α1 and β2 localized in these nuclei. Previous studies using genetic and pharmaceutical approaches found that TRα1 is mostly involved in regulating sympathetic action during thermogenesis and heart function, especially the heart rate and rhythm (21, 36), whereas TRβ2 is mostly involved in elevating the metabolic rate (10). The mechanism through which thyroid hormone regulates neuronal functions in medullary DMV, NTS, and AP at the cellular level has yet to be studied.

ChAT, the biosynthesizing enzyme for acetylcholine, is presently the most specific indicator for monitoring the functional state of cholinergic neurons and is contained in vagal preganglionic motor neurons of the DMV (27, 34). Changes in ChAT mRNA levels have been used to evaluate enzyme response to stimuli (28, 30). Hypothyroidism induces Fos expression in about 50% of ChAT neurons in the DMV, suggesting the possibility of elevated ChAT gene expression. This is supported by previous observations that the ChAT gene contains a sequence responsive to c-fos (2). ChAT mRNA increases after Fos induction in brain sections exposed to stimuli (21, 36), and pretreatment of cultured rat embryonic brain neurons with anti-c-fos blocked nerve growth factor-mediated increases in ChAT activity by 67% (35). Activation of ChAT-containing neurons indicates an increase of acetylcholine synthesis in the DMV neurons that is in accordance with the parasympathetic preponderance in hypothyroidism (43, 44).

In summary, hypothyroidism-induced Fos expression in the dorsal medulla was selectively localized in neurons of the DMV, NTS, and AP, negatively correlated with serum T4 levels, prevented by T4 replacement, and, in the DMV, mainly localized to ChAT-synthesizing neurons. Although the mechanism needs to be further studied, these data, together with the well-established autonomic regulatory function of these nuclei and the previously reported neuronal activation of TRH-synthesizing neurons in the Rpa, Rob, and PPR of hypothyroid rats (51), clearly indicate that the functional change of the medullary TRH-containing Rpa/Rob/PPR-DCV/AP pathway is an important constituent of the mechanisms through which hypothyroidism alters autonomic nervous system function.

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