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Hypophysiotrophic TRH-Producing Neurons Identified by Combining Immunohistochemistry for Pro-TRH and Retrograde Tracing

HITOSHI KAWANO, YOSHIHIRO TSURUO, HIROSHI BANDO, AND SHIGEO DAIKOKU
Departments of Anatomy (H.K., Y.T., S.D.) and Internal Medicine (H.B.), School of Medicine, The University of Tokushima, Tokushima 770, Japan

ABSTRACT

To determine hypophysiotrophic thyrotropin-releasing hormone (TRH)-producing neurons in the rat hypothalamus, we employed a combination of the immunohistochemistry for TRH prohormone (pro-TRH) and the retrograde tracing of neurons that project to the median eminence (ME) by injecting biotinylated wheat germ agglutinin (WGA) into the ME. In intact rats, immunoreactive pro-TRH-positive neurons occurred in the parvicellular paraventricular nucleus (parvi-PVN), basal part of the anterior and lateral hypothalamus, perifornical area and dorsomedial nucleus, especially accumulating in the parvi-PVN. Twenty-four hours after injection of the WGA into the middle portion of the ME, we found neurons that incorporated the lectin in the anterior periventricular area, the PVN, and the arcuate nucleus. When we examined serial sections consecutively stained with anti-WGA, anti-pro-TRH, and anti-WGA, most of the pro-TRH-labeled neurons in the medial parvi-PVN and a part of the neurons in the anterior periventricular area and in the anterior, lateral, and dorsal parvi-PVN appeared to incorporate WGA. These neurons may correspond with the hypophysiotrophic TRH-synthesizing neurons in the rat hypothalamus.

Key words: hypothalamus, localization, wheat germ agglutinin, rat

Since the antiserum for thyrotropin-releasing hormone (TRH) became available in immunohistochemistry (Hökfelt et al., '75a,b), many studies have demonstrated immunoreactive TRH neurons in the hypothalamus (Jackson and Reichlin, '77; Johansson and Hökfelt, '80; Brownstein et al., '82; Lechan and Jackson, '82; Nishiyama et al., '85). However, TRH was also found in other locations of the central nervous system, including the brainstem and spinal cord (Jackson and Lechan, '83; Lechan et al., '83; Ishikawa et al., '84). In these regions, TRH does not regulate thyrotrophs. Even in the hypothalamus, TRH immunoreactivity occurred in various locations, and much effort was concentrated on clarifying the locations of hypophysiotrophic TRH-containing neurons in various experimental ways (Martin and Reichlin, '72; Brownstein et al., '82; Lechan et al., '82; Palkovits et al., '82; Nishiyama et al., '85). However, substantial evidence is lacking to assess the neurons projecting to the median eminence, because of the methodological difficulty in determining the neurons that project to the median eminence to release the hormonal TRH. Recently, it was shown that an antiserum raised to a theoretical TRH progenitor sequence (pro-TRH-SH) stains neurons whose distribution corresponds to cells immunolabeled for TRH in the brain (Lechan and Jackson, '82; Jackson et al., '85; Lechan et al., '86; Merchenthaler et al., '89a). The serum revealed neurons in animals without colchicine pretreatment, which is commonly used to arrest axonal transport, and in animals fixed with conventional Bouin's fixative, in which anti-TRH serum did not reveal immunoreactive neurons. This conventional tissue preparation was also used in the immunohistochemical demonstration of wheat germ agglutinin (WGA) retrogradely transported from the nerve terminals in the median eminence to the cell bodies (Kawano and Daikoku, '87). Hence it became possible to identify hypophysiotrophic TRH neurons by applying a combination of immunohistochemistry for pro-TRH and retrograde tracing by injecting WGA to the median eminence.

MATERIALS AND METHODS

Preparation of anti-pro-TRH serum

The antiserum for pro-TRH was generated in rabbits against synthetic decapeptide, Cys-Lys-Arg-Gln-His-Pro-Gly-Lys-Arg-Cys (pro-TRH-SH; kindly supplied by Dr. F.

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Address reprint requests to Dr. Shigeo Daikoku, Dept. of Anatomy, School of Medicine, The University of Tokushima, Tokushima 770, Japan.

Shimizu, Fujii Memorial Research Institute, Otsuka Pharmaceutical Co. LTD), conjugated to bovine thyroglobulin with glutaraldehyde according to the method of Reichlin et al. ('68). Two female New Zealand White rabbits were immunized with the antigen (250 μg/ml saline) emulsified with Freund's adjuvant at 3–4 week intervals, and the specificities of the serum were tested by blood samples collected at appropriate intervals from the auricular vein.

Specificities of the serum

We examined the immunological specificity of the antiserum by radioimmunoassay using ^{125}I-labeled pro-TRH-SH. A sufficiently high titer and affinity of the serum were determined (ED_{90} = 15 pg/100 μl and ED_{50} = 220 pg/100 μl, respectively, at a final dilution of 1:56,000), while cross-reactivities with TRH, luteinizing hormone releasing hormone (LHRH), somatostatin, rat corticotropin-releasing factor (CRF), rat hypothalamic growth-hormone releasing factor (GRF), oxytocin, and arginine vasopressin were less than 0.1% at 50% displacement of the radiolabeled tracer.

The staining specificity of the anti-pro-TRH serum was examined in paraffin sections of the rat hypothalamus. Animals were killed by perfusing Bouin's fixative or modified Zamboni's fixative (Hisano et al., '86) through the ascending aorta under Nembutal anesthesia. Some animals received an intraventricular administration of colchicine (100 μg/10 μl saline) while under ether anesthesia 24 hours before sacrifice. The hypothalamus excised was further fixed overnight in the same fixative, dehydrated by an ascending ethanol series, embedded in paraffin in vacuo, and cut serially at 5 μm thickness in the frontal plane.

Adjacent serial paraffin sections were stained sequentially with various combinations of anti-pro-TRH serum, anti-TRH serum, and one of those sera preabsorbed with corresponding antigens. Their staining specificities were tested by comparing immunostainability of the corresponding histological structures. Immunostaining was carried out according to the double-bridge peroxidase-anti-peroxidase method (Vacca et al., '80). In this study, it appeared that the immunoreaction for anti-TRH was not evident in Bouin's-fixed tissues but that for anti-pro-TRH was shown in the tissues fixed with either Zamboni's or Bouin's fixatives, as shown by other authors (Merchenthaler et al., '89a). Anti-TRH was kindly supplied by Prof. M. Suzuki (Institute of Endocrinology, Gunma University). The characterization and staining specificity of this antiserum was reported previously (Nishiyama et al., '83).

Retrograde tracing

Adult Sprague-Dawley rats weighing 200–250 g were used. Animals were anesthetized with an intraperitoneal injection of urethane (100 mg/100 g body weight), and 1 μl of 5% biotinylated WGA was gently injected into the central portion of the median eminence exposed by the parapharyngeal approach, the details of which have been described previously (Kawano and Daikoku, '87). After the surgery, animals were kept at 26°–28°C maintaining spontaneous respiration (40–70/min) and sacrificed 24 hours later by perfusing Bouin's fixative. WGA conveyed from the nerve terminals in the median eminence to the perikarya was determined immunohistochemically by using anti-WGA (E.Y Laboratories), because, as mentioned previously (Kawano and Daikoku, '87, '88; Kawano et al., '88), we found that anti-WGA gives a more intense stain than does avidin-peroxidase.

To identify the neurons containing both pro-TRH and WGA, we cut three consecutive frontal sections (5 μm thick) of the hypothalamus at intervals of 100 μm rostrocaudally from the preoptic area to the premammillary nucleus. The middle of the three serial sections was immunostained for pro-TRH and the other two sections for WGA. Immunostaining was carried out according to the double-bridge peroxidase-anti-peroxidase method (Vacca et al., '80), the details of which were previously described (Nishiyama et al., '85). The neurons immunoreactive for WGA or pro-TRH were separately drawn on white paper with the aid of a camera lucida and identified. The numbers of immunoreactive pro-TRH neurons with or without WGA immunoreactivity were estimated and are indicated in Table 1.

Immunostaining

Anti-TRH, anti-pro-TRH, and anti-WGA were diluted with 0.1 M phosphate buffer containing rat liver extract at 1:1,000, 1:1,000, and 1:250, respectively. The specificity of the staining for WGA was confirmed by preabsorption tests in which the reaction of anti-WGA was completely eliminated in the presence of 10 μg WGA/ml. The immunoreaction for pro-TRH was completely blocked by addition of 10 μM pro-TRH-SH, whereas even 100 μM TRH did not alter the immunoreaction for anti-pro-TRH.

RESULTS

In the hypothalamus fixed with Bouin's solution the presence of immunoreactive substance for the pro-TRH antiserum was confined to neuronal cell bodies, as mentioned by Jackson et al. ('85). Although the immunoreactive cell bodies were widely distributed throughout the hypothalamus, they appeared to accumulate in the PVN (Fig. 1), in

Abbreviations

AH	anterior hypothalamus
am	anterior magnocellular part of the PVN
ap	anterior parvicellular part of the PVN
APA	anterior periventricular area
AR	arcuate nucleus
DM	dorsomedial nucleus
dp	dorsal parvicellular part of the PVN
F	fornix
lp	lateral parvicellular part of the PVN
mm	medial magnocellular part of the PVN
mp	medial parvicellular part of the PVN
OC	optic chiasm
OT	optic tract
OVLT	organum vasculosum of the lamina terminalis
pm	posterior magnocellular part of the PVN
POA	preoptic area
pv	periventricular part of the PVN
PVN	paraventricular nucleus
SC	suprachiasmatic nucleus
SO	supraoptic nucleus
V	third ventricle
VM	ventromedial nucleus

TABLE 1. Number of Neurons Immunoreactive for Pro-TRH and WGA in the Parvicellular Subdivisions of the PVN (Mean ± SE, n = 4)

Subdivision of the PVN	Number of neurons positive for		Double-labeled cells (%)
	pro-TRH[1]	pro-TRH/WGA	
ap	125.0 ± 5.7	2.5 ± 0.9	1.6
mp	146.3 ± 6.3	99.5 ± 6.2	68.0
pv	50.8 ± 4.7	29.5 ± 2.3	58.1
dp	5.8 ± 0.8	3.0 ± 0.6	51.7
lp	6.3 ± 0.9	0.3 ± 0.3	4.8

[1]Numbers of cells were counted in sections at 100 μm intervals.

which the cells were located in all the parvicellular subdivisions (Swanson and Kuypers, '80), concentrating in the anterior parvicellular, medial parvicellular, and periventricular subdivisions (Fig. 2). In other parts of the hypothalamus, the cells occurred in the basal portion of the anterior hypothalamus from the preoptic area to the level of the anterior extremity of the median eminence, in the perifornical area, in the dorsomedial nucleus, in the lateral hypothalamus, in the posterior portion of the arcuate nucleus, and in the premammillary region. A faint immunoreaction was evident in a few magnocellular neurons in the PVN and the supraoptic nucleus. No immunoreaction was found in the suprachiasmatic and ventromedial nuclei. The same intrahypothalamic localization of anti-TRH-positive neurons was found in animals fixed with Zamboni's fixative. The anti-TRH serum did not show immunoreactive neurons in the sections of Bouin's-fixed hypothalamus. The reason may be that Zamboni's fixative has as a constituent glutaraldehyde, which stabilizes the tripeptide in situ. The pro-TRH antiserum also stained cells in the Zamboni's-fixed hypothalamus, as shown by Merchenthaler et al. ('89a). The localization of immunoreactive pro-TRH was essentially identical in tissue sections fixed with Bouin's and Zamboni's fixatives, although the former tissue sections gave much more intense reaction than the latter. When the cells immunoreactive for pro-TRH and for TRH were compared in serial sections from colchicine-treated rats fixed with Zamboni's fixative, all pro-TRH-positive cells were virtually identical with TRH-positive ones, and vice versa (Fig. 3).

Retrograde tracing

We used four animals that showed no indication of leakage into the third ventricle of the tracer injected into the median eminence. In the median eminence the tracer spread over the subependymal, internal and external layers; in the external layer massive TRH axons appeared abutting on the portal capillaries (Fig. 4).

The tracer incorporated from the axonal terminals and retrogradely transported to the perikarya was found to be

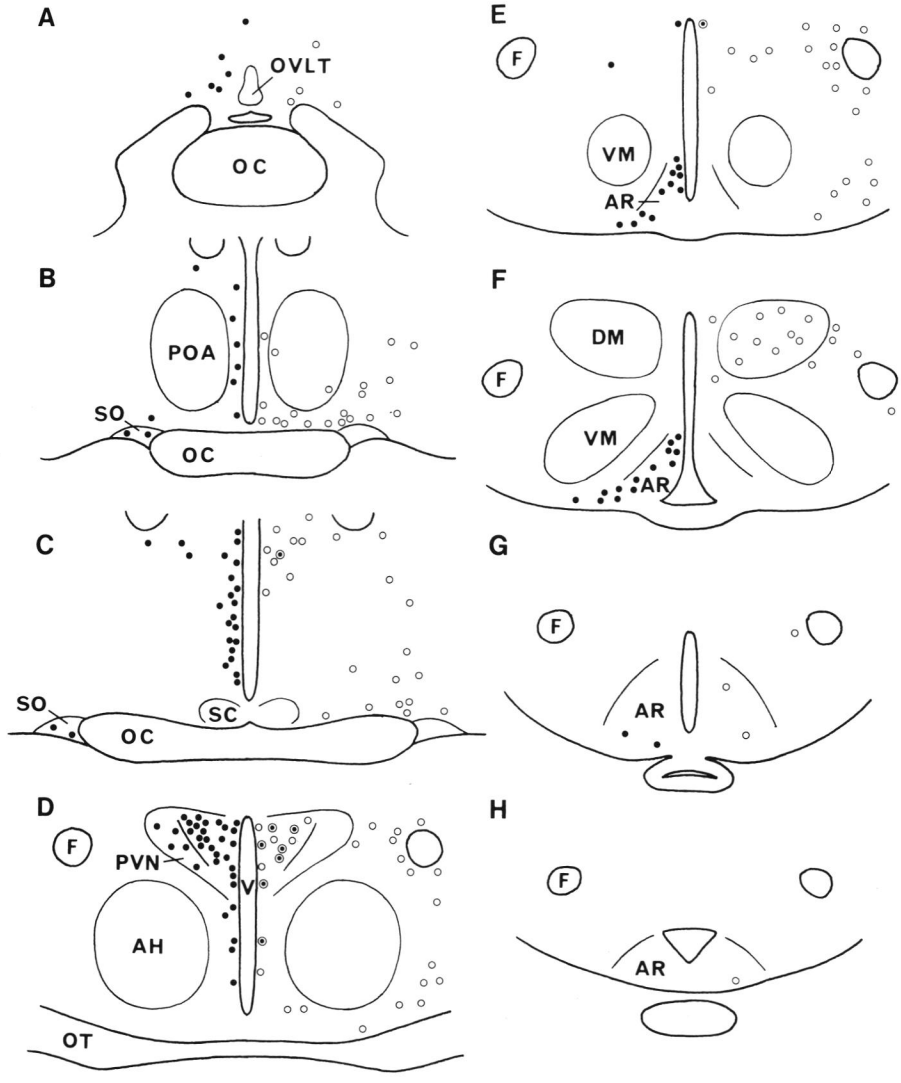

Fig. 1. Schematic illustrations (**A–H**) of the frontal sections of the hypothalamus arranged rostrocaudally from the OVLT at intervals of 0, 0.6, 1.2, 1.8, 2.4, 3.2, 4.0, and 4.4 mm. Localizations of neurons containing WGA (dots), pro-TRH (circles), and both WGA and pro-TRH (dotted circles) are shown.

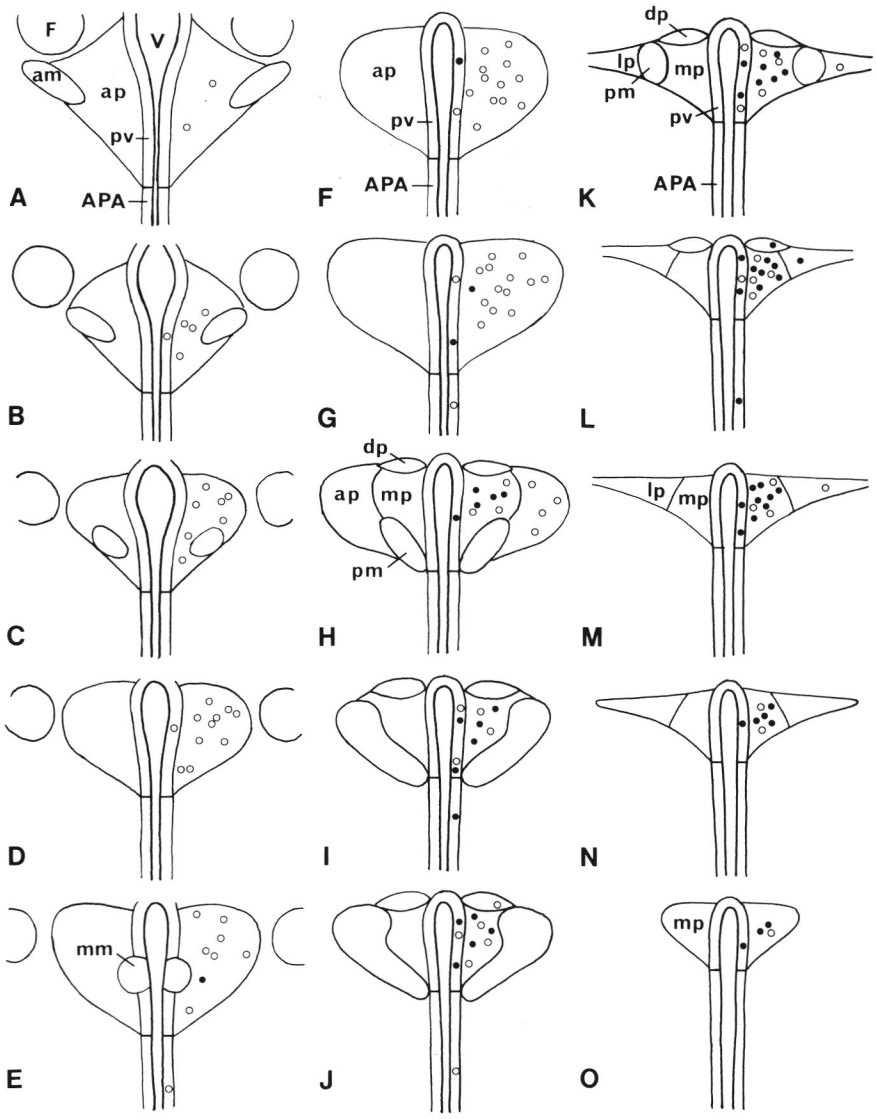

Fig. 2. Schematic illustrations of the frontal sections of the PVN cut at 100 μm intervals are arranged anteroposteriorly in alphabetical order (**A–O**). Localizations of neurons containing pro-TRH (circles) and both pro-TRH and WGA (dots) are shown in the subdivisions of the PVN (Swanson and Kuypers, '80).

accumulated in neuronal perikarya localized in the arcuate nucleus, in the anterior periventricular area, and in the parvicellular division of the PVN (Figs. 1, 2). In addition, neurons incorporating the tracer were also found scattered in the medial septum, diagonal band of Broca, and medial preoptic area. All these neurons were small in size (7–15 μm in diameter). The tracer was also incorporated into some large neurons in the PVN and supraoptic nucleus. The tracer may be incorporated by the fibers of passage lying in the internal layer of the median eminence, the fibers of which derive from the large neurosecretory neurons and project to the posterior pituitary.

Some of the pro-TRH-labeled neurons located in the anterior periventricular area and most of the neurons located in the parvicellular PVN were found to incorporate WGA (Figs. 2, 5). In other hypothalamic regions WGA-labeled neurons did not show immunoreactivity for pro-TRH (Fig. 1). In the parvi-PVN, WGA-labeled neurons with pro-TRH immunoreactivity were concentrated in the medial parvicellular (68.1%) and in the periventricular (58.1%) subdivisions (Table 1). In the anterior parvicellular subdivision in which a number of pro-TRH-positive neurons exist, however, only a few cells were double labeled (Fig. 6). The dorsal and lateral parvicellular subdivisions contained a small number of pro-TRH neurons, and in the former about half of the cells were retrogradely labeled with WGA (Fig. 2, Table 1).

DISCUSSION

In a combination of retrograde tracing with WGA and immunohistochemistry for pro-TRH, we determined neurons containing hypophysiotrophic TRH in the rat hypothalamus.

Although TRH was isolated from the hypothalamus (Bøler et al., '69; Burgus et al., '69) as a hormone having

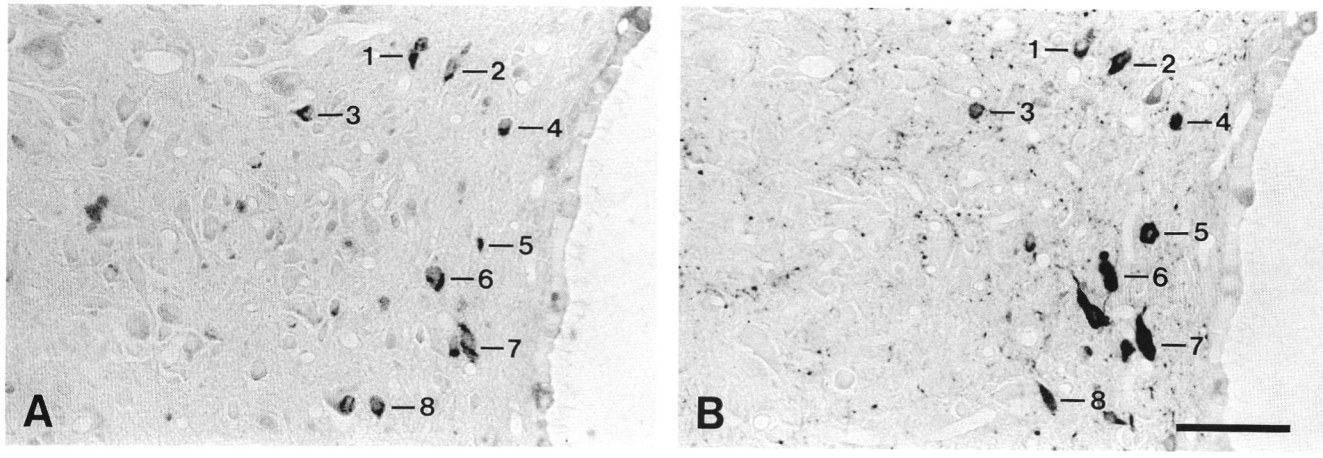

Fig. 3. Two serial sections (A, B) cut at the level of the PVN of a modified Zamboni's-fixed hypothalamus. Staining with anti-pro-TRH (**A**) and anti-TRH (**B**). Numbered immunoreactive cells in A and B are identical. Scale bar = 50 μm.

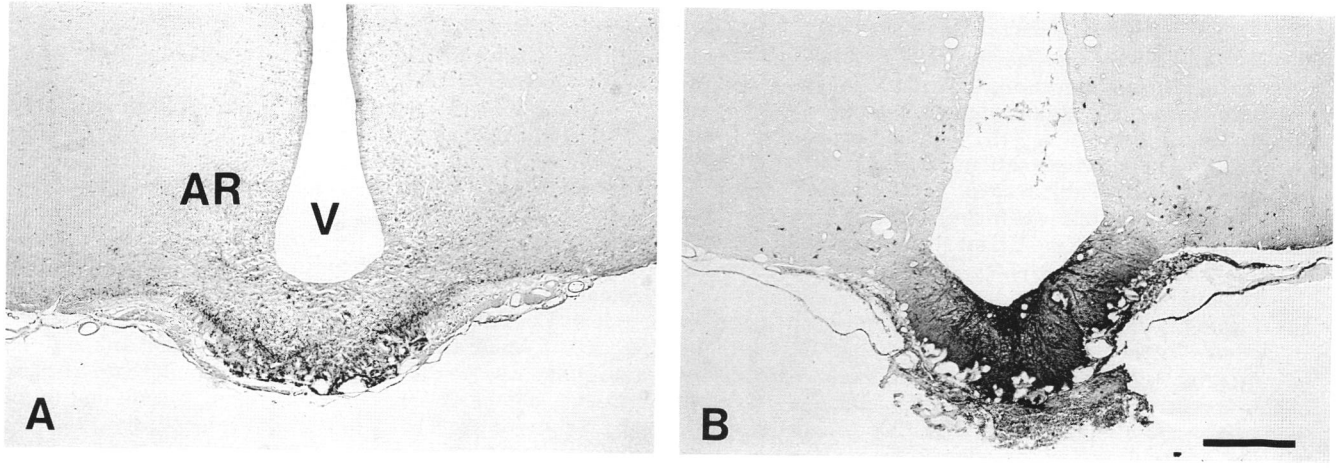

Fig. 4. The preinfundibular portions of the hypothalamus of an intact rat fixed with modified Zamboni's (**A**) and a Bouin's-fixed rat injected with WGA into the median eminence (**B**). The extent of WGA (B) covers that of TRH fibers (A) in the median eminence. Scale bar = 200 μm.

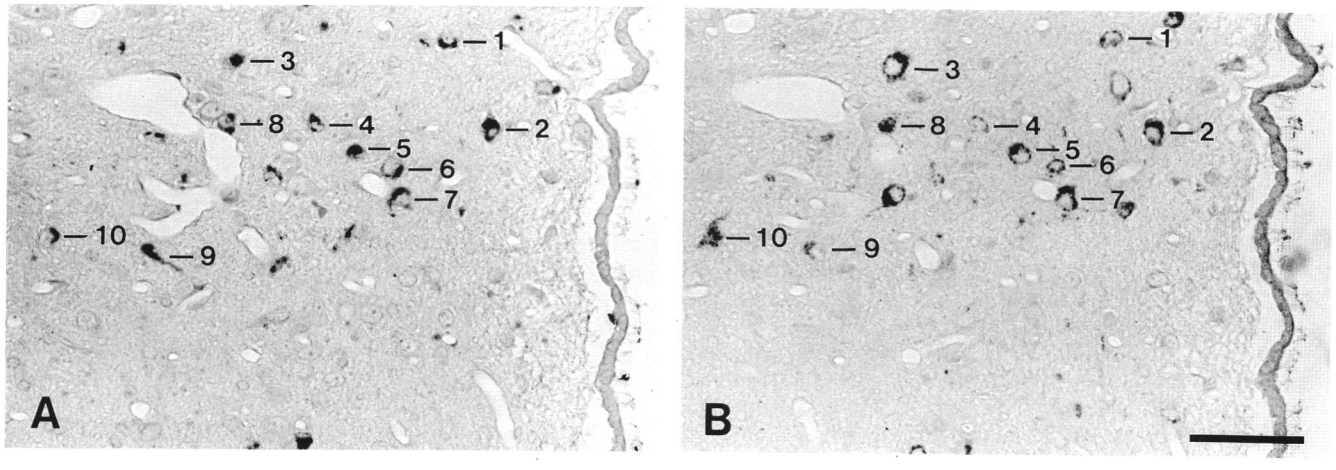

Fig. 5. Two sequential serial sections (A, B) cut at the level of the most caudal part of the medial parvicellular subdivision of the PVN from a WGA-injected, Bouin's-fixed rat. Staining with anti-pro-TRH (**A**) and anti-WGA (**B**). Most of the pro-TRH immunoreactive cells numbered in A are identical to the same numbered WGA-labeled cells in B. Scale bar = 50 μm.

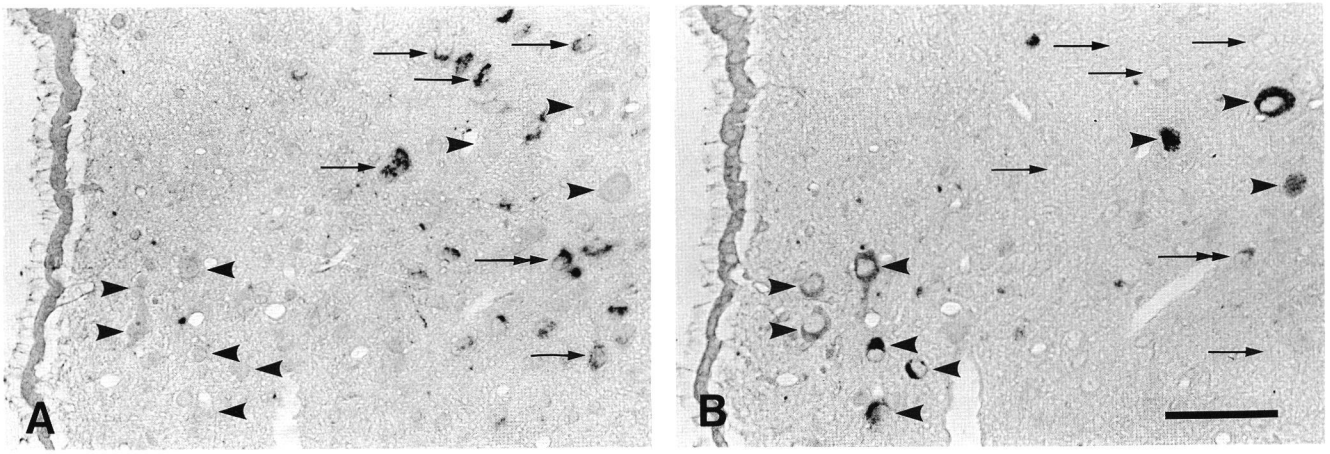

Fig. 6. Two sequential serial sections (A, B) cut at the level of the anterior parvicellular subdivision of the PVN from a WGA-injected animal. Staining with anti-pro-TRH (**A**) and anti-WGA (**B**). Neurons immunoreactive for only pro-TRH (arrows), for only WGA (arrowheads), and for both pro-TRH and WGA (double arrow) are shown. Scale bar = 50 μm.

thyrotrophic hormone-releasing activity, its widespread distribution throughout the central nervous system and the digestive system (Leppäluoto et al., '78; Morley, '79) induced the concept that TRH has divergent functional significance. The multiple actions of TRH have been shown by many authors (Tashjian et al., '71; Plotnikoff et al., '72; Brownstein et al., '74; Jackson and Reichlin, '74, '77; Oliver et al., '74; Winokur and Utiger, '74; Renaud et al., '75; Braitman et al., '80; Kreider et al., '81). Even in the hypothalamus, its widespread distribution and its physiological roles in thermoregulation, behavior, stress, and homeostasis have further defined the concept that TRH acts not only as a hypophysiotrophic agent but also as a neurotransmitter or neuromodulator (Brownstein et al., '74). Hence it seems to be essential in neuroendocrinology to discriminate neurons containing hypophysiotrophic TRH from those containing nonhormonal TRH in the hypothalamus (Brownstein et al., '75; Hefco et al., '75).

Morphologically, it is certain that hypophysiotrophic TRH-containing neurons must project to the external layer of the median eminence. In fact, immunohistochemistry demonstrated that a massive accumulation of fibers immunolabeled for TRH in the external layer of the median eminence release the hormone (Hökfelt et al., '75b; Choy and Watkins, '77; Lechan and Jackson, '82). Retrograde labeling is a powerful tool in demonstrating the original neurons projecting to the median eminence (Lechan et al., '82). As the retrograde tracer, WGA and horseradish peroxidase (HRP) (Wiegand and Price, '80) were commonly used. Using WGA injection into the median eminence in rats, Lechan et al. ('82) found labeled neurons in the dorsomedial and basolateral portions of the arcuate nucleus, the periventricular nucleus, the septo-preoptic region, the diagonal band of Broca, and the brainstem. Only rare cells were labeled in the ventromedial nucleus of the hypothalamus. Similar results were obtained by HRP injection into the median eminence (Wiegand and Price, '80). In previous studies, we employed WGA injection into the median eminence of rats and obtained similar distribution of WGA-labeled hypothalamic neurons; we further demonstrated immunoreactive tyrosine hydroxylase (Kawano and Daikoku, '87), somatostatin (Kawano and Daikoku, '88), and CRF (Kawano et al., '88) in those neurons. The present study was performed in the same line.

To determine the neurons with hormonal TRH, Ishikawa et al. ('88) employed a combination of immunohistochemistry for TRH and retrograde labeling with HRP in rats treated with intraventricular colchicine to determine TRH-containing neurons storing the peptide within the perikarya by arresting the axonal transport. However, they found only a few TRH neurons containing HRP. Although colchicine is useable in demonstrating peptide-containing cell bodies (Merchenthaler et al. '89b), the application of stable signals to know the synthesis of the peptides seems to be advantageous. We did not use colchicine and found a larger number of cells doubly labeled by using a combination of antiserum for pro-TRH and retrograde labeling with WGA. Pro-TRH is a precursor sequence included in the TRH molecule; it expresses the presence of TRH and also TRH mRNA. The immunostainability of pro-TRH may not be influenced by colchicine treatment (Lechan et al., '86).

The localization of pro-TRH has been studied extensively in the hypothalamus (Jackson et al., '85; Koller et al., '87; Lechan et al., '87; Segerson et al., '87a; Merchenthaler et al., '89a). Cells double labeled with anti-pro-TRH and WGA accumulated in the medial parvicellular and periventricular subdivisions. Only a few double-labeled cells were detected in the anterior parvicellular subdivision, in which many pro-TRH neurons exist. In a previous study (Nishiyama et al., '85) we have mentioned that only the TRH neurons localized in the medial parvicellular and periventricular subdivisions responded to thyroid hormone deficiency. Recently, it has been proposed that TRH mRNA in the PVN is affected by thyroid hormone deficiency (Koller et al., '87; Segerson et al., '87b) and administration (Dyess et al., '88), responding with an increased and decreased amount, respectively. As the conclusion, we gave substantial evidence that, in the rat, neurons containing hormonal TRH exist in the parvi-PVN and are concentrated in the medial parvicellular and periventricular subdivisions.

In this study, we examined neurons using three consecutive 5 μm thick frontal sections at intervals of 100 μm. The sections were stained consecutively with anti-WGA, anti-pro-TRH, and anti-WGA. Hence we were easily able to identify

the labeled neurons. However, this study is incomplete for estimating the absolute number of labeled neurons even in the PVN, since the WGA injected did not cover the whole extent of the median eminence. The subpial application of WGA proposed by Merchenthaler et al. ('89b) might be a considerable improvement in quantitative studies.

Delineation of the PVN has been proposed by Swanson and his coworkers (Swanson and Kuypers, '80; Swanson et al., '80), and eight distinct subdivisions (three magnocellular and five parvicellular) based on cytoarchitectonic analysis and the cellular projections were generated. In a retrograde tracing they found that, although neurons existing in all the parvicellular subdivisions project to the median eminence, those in the lateral and dorsal parvicellular subdivisions and the ventral and posterior portions of the medial parvicellular division also project to the spinal cord and dorsal medulla (Swanson and Kuypers, '80; Swanson et al., '80). The present findings accord with their data. It would be of interest to examine how such functional divergence occurs in neurons with the same phenotypical properties.

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