DISTRIBUTION OF THYROTROPIN RELEASING HORMONE RECEPTOR TYPE 2 IN RATS: AN IMMUNOHISTOCHEMICAL STUDY

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Objective. To investigate the organ distribuion of thyrotropin releasing hormone receptor (TRHR) type 2 in rats by immunohistochemical method.

Methods. TRHR type 2 was identified immunohistochemically in the rat tissues using specific anti-TRHR antiserum raised in New Zealand white rabbits immunized with a conjugate of synthetic TRHR type 2 (5-23) with bovine serum albumin. Immunohistochemical analysis was performed by avidin-biotin complex method.

Results. TRHR type 2 immunoreactivity was visualized in the central nervous system, anterior pituitary, gastric mucosa, Auerbach's and Meissner's nervous branch of the stomach, small intestine and colon, retina amd testis. Significant stain was detected in neural perikarya, axons and dendrites. When using antiserum preincubated with synthetic TRHR type 2(5-23) or anterior pituitary homogenates, no significant stain of anterior pituitary was detected.

Conclusions. These findings suggest that TRHR type 2 is widely distributed and that the method used is valuable in studying the distribution of TRHR type 2 in rats.

Key words: TRH receptor type 2 – TRH – Immunohistochemistry – Organ distribution – rat

The action of thyrotropin releasing hormone (TRH) is mediated via its receptor (Gershengorn et al. 1996). A specific receptor gene for TRH receptor (TRHR) was cloned and its mRNA and protein were found widely distributed in rats (Staub et al. 1990; Duthie et al. 1993). However, since several synthetic analogues with weak TRH activity were found, the presence of another TRH receptor has been suggested (Metcalf 1982). We previously reported that TRHR type 1 was widely distributed in the rats (Mitsuma et al. 1995). In 1998, a new subtype of TRHR was cloned and characterized as TRHR type 2 (Itadami et al. 1998). This promptly motivated us to study its distribution in rats. We investigated TRHR type 2 distribution in rats using a specific antiserum to TRHR type 2 which was raised in rabbits immunized with a conjugate of synthetic TRHR type 2 protein fragment, TRHR type 2 (5-23) with bovine serum albumin (BSA).

Materials and Methods

Animals. Male Wistar rats weighing 250-280 g were obtained from Shizuoka Animal Laboratory Co. (Shizuoka, Japan) and housed in temperature (22 °C) and humidity (60 %) controlled room under 12 h illumination cycle. They were fed with laboratory chow and water *ad libitum*.

Drugs. BSA, glutaraldehyde, paraformaldehyde, sodium pentobarbital and Bouin's fluid were obtained from Katayama Chem. Co. (Osaka, Japan), all reagents being of analytical grade. Complete Freund's adjuvant was obtained from Wako Chemical Co. (Osaka, Japan).

Preparation of anti-TRH type 2 antibody. Peptide corresponding to TRHR type 2 (5-23) was synthesized with solid phase method using an automated peptide synthesizer, followed by purification with HPLC: SNVSLIHGDTTLGLPEYKV. Syntheic TRHR type 2 (5-23) was conjugated on an equal basis to BSA by the method previously described for anti-GHRH antibody, using glutaraldehyde (Mitsuma et al. 1996).

New Zealand white rabbits weighing 3 kg were obtained from Nakajima Animal Laboratory (Gifu, Japan). They were immunized with the emulsion of one mg of this conjugate in one ml water in complete Freund's adjuvant (1:2, v/v) which was injected into food pad at intervals of three weeks. Blood was drawn one week after each injection and the presence of anti TRHR type 2 was checked. This technique was essentialy similar to that used previously for the preparation of antibody against sodium iodide symporter (Mitsuma et al. 1997a), somatostatin receptor type 2 (Mitsuma et al. 1996), type 3 (Mitsuma et al. 1997b) and type 4 (Mitsuma et al. 1997c) or calcium sensing receptor (Mitsuma et al. 1999)

Preparation of tissue for TRHR type 2 estimation. TRHR type 2 fraction was obtained by the method previously described (Mitsuma et al. 1995). In brief, the anterior pituitaries were removed from the rat and a pool of anterior pituitary weighing 100 mg was homogenized in 5 ml ice-cold 20 mM phosphate buffer (pH 7.4). The homogenates were centrifuged at 4 °C for 20 min at 30,000 x g. The pellet was resuspended in 5.0 ml cold phosphate buffer and used as TRHR type 2 fraction.

Perfusion method and immunohistochemical method. The rats were anesthetized with intravenous administration of sodium pentobarbital (30 mg/kg) and transcardially perfused with 0.01 % glutaraldehyde and 4 % paraformaldehyde in Bouin's solution (pH 7.2). The brain, spinal cord, pituitary galnd, lung, heart, kidney. liver, thyroid gland, parathyroid gland, stomach, small intestine, colon, adrenal gland, pancreas and testis were removed and post-fixed for an additional hour at 4 °C, then cut into 4 µm slices using a vibratome. Immunohistochemical treatment was performed by avidin-biotin complex (ABC) method, using Vecstatin kits (Vector Laboratories, Burlingame, CA, USA). Primary antibody was used after dilution (1:50). To confirm the specificity of anti TRHR type 2 antibody, the following methods were used in the anterior pituitary: 1. omission of the primary antiserum or second antiserum in the peroxidase-antiperoxidase technique; 2. reabsorption of the antiserum prior to the incubation of experimental tissues with synthetic peptide TRHR type 2; specific immunohistochemical stain could not be seen in any of these control paradigm (Fig. 1); 3. serial dilution of primary antiserum was used. Specific stain disappeared at 1:500 dilutionm. The brain nuclei were determined using the map by Pellegrino and Cushman (969).

Results

Specific staining appeared in several organs and tissues (Tab. 1) such as central nervous system (Fig.

Table 1
Distribution of TRHR type 2 in rats

1. **Telencephalon**
   Olfactory system: anterior olfactory nucleus, olfactory tubercles, olfactory bulb
   Amygdaloid and related areas: lateral nucleus, medial nucleus, basomedial nucleus, anterior cortical nucleus, endopiriform nucleus
   Cortex: pyriform, frontal-orbital, frontal-parietal, entorhimal
   Hippocampal formation: cornus Ammonis, dentate gyrus, stratum pyramidale, subiculum
   Septal region: lateral septal nucleus, medial septal nucleus,
   Basal ganglia and related area: nucleus accumbens globus pallidus, claustrum, striatum, nucleus striae terminalis
2. **Diencephalon**
   Thalamus: perithalamic and subthalamic areas, centralmedial nucleus, paratential nucleus, ventromedial nucleus, renien nucleus, posterior nucleus
   Hypothalamus: paraventricular nucleus, arcuate nucleus, ventromedial nucleus, anterior hypothalamic area, lateral hypothalamic area, periventricular hypothalamic nucleus preoptic area, supraoptic nucleus, dorsal nucleus, anterior hypothalamic nucleus
3. **Mesencephalon**
   superior colliculus, inferior colliculus, periaquaeductal gray
4. **Pons/Medulla**
   spinal trigeminal nucleus, dorsal motor nucleus, hypoglossal nucleus, dorsal parabranchial nucleus, pontine nucleus, central gray, tractus nucleus solitarii, facial nucleus, medial vestibular nucleus
5. **Cerebellum**
   molecular layer, granular layer
6. **Spinal cord**
   ventral horn, dorsal horn
7. **Retina**
8. **Testis**
9. **Gastrointestinal tract**
   gastric mucosa, Auerbach's and Meissner's nervous branch of the stomach, small intestine, colon

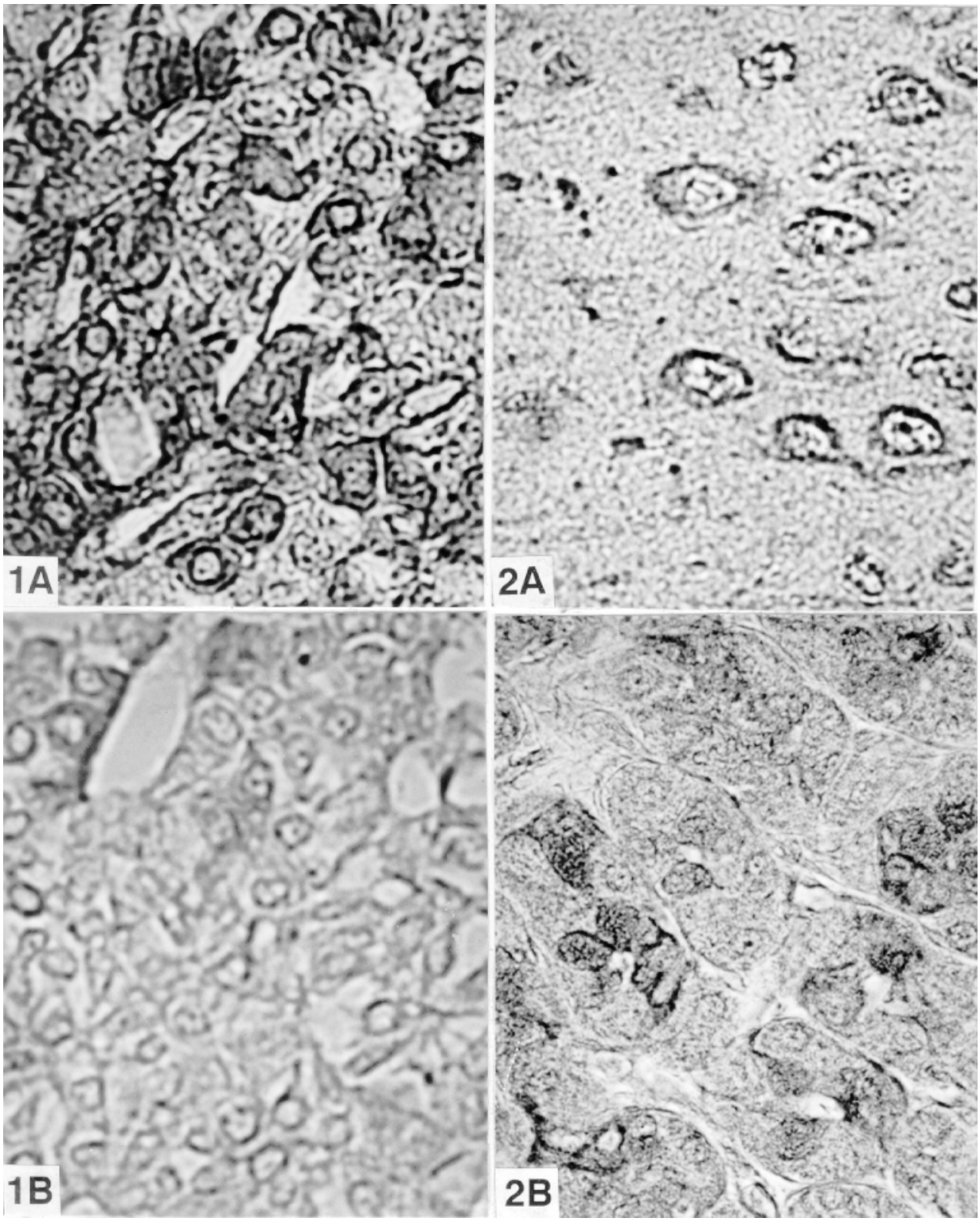

Fig. 1 TRH receptor type 2 immunoreactivity in the anterior pituitary:
A: positive staining in the anterior pituitary (x 200); B: positive staining disappeared when antibody was applied prior to incubation with an excessive amount of TRHR type 2 (x 200)

Fig. 2 Distribution of TRH receptor type 2 in: A. cerebrum; B. stomach (x 200)

2A), anterior pituitary (Fig. 1A,B), gastric mucosa (Fig. 2B), Auerbach's and Meissner's nervous complex of the stomach, small intestine and colon, testis and retina, but not in the heart, liver, lung, kidney, thyroid gland, parathyroid gland, adrenal gland and posterior pituitary. In the central nervous system, significant stain revealed neural perikrya, axons and dendrites.

Discussion

The distribution of TRHR type 2 immunoreactivity in the rat body was immunohistochemically investigated. Anti TRHR type 2 antiserum was raised in New Zealand white rabbits by repeated injections of the synthetic peptide (TRHR type 2 5-23) conjugate with BSA in complete Freund's adjuvant. This antiserum was characterized by the immunohistochemical method, e.g. TRHR immunoreactivity has been specifically eliminated by the preincubation of antibody with an excess amount of synthetic peptide (TRHR type 2 5-23) or anterior pituitary homogenates which contain TRHR type 2 (Fig. 1B). In addition, the immunoreactivity was reduced with the dilution of this antibody. these data indicate that this antibody is specific and that this method can be used to detect the distribution of TRHR type 2 in the rat body.

The present study clearly demonstrated that TRHR type 2 was widely distributed in the rat body. In the central nervous system it stained the perikarya, axons and dendrites of the hypothalamus, cerebrum, cerebellum, midbrain, pons, spinal cord, retina and also the testis and anterior pituitary, but not the heart, lung, kidney, thyroid gland, parathyroid gland, adrenal gland and posterior pituitary.

Itadani et al. (1998) reported than mRNA of TRHR type 2 was predominantly found in the rat brain. The present study partly confirmed this finding on the protein levels. We previously reported that also TRH receptor type 1 is widely distributed in the rats (Mitsuma et al. 1995). In this study we found that the central nervous system the distribution of TRHR type 2 was aproximately similar to the TRHR type 1. Nevertheless, the function of TRHR type 2 is currently still unknown. Considering this, it should be recalled that TRH show several actions upon the central nervous system (Reichlin 1986) and several analogues with weak TRH action and central nervous system effects have been demonstrated (Metcalf 1982). Thus, it may be speculated that TRHR type 2 may serve as another specific TRH receptor in the central nervous syetem and possibly also in several peripheral tissues.

These findings further suggest that TRHR type 2 is widely distributed and that the present method is a useful tool in studying the distribution of this specific receptor in the rat body.

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