A new type of antiserum to thyrotropin-releasing hormone; detection of new TRH-immunoreactive cell groups in rat hypothalamus

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Summary. We produced a new type of antiserum to thyrotropin-releasing hormone (TRH) in rabbits. The immunogen is TRH-BSA, the production of which is based on the formation of an amide bond using carbodiimide (EDC). The specificity of the antiserum was assessed by enzyme immunoassay (EIA) and immunohistochemistry. When using the anti-TRH serum for immunohistochemistry in rat hypothalamus, new magnocellular groups were detected in the ventrolateral parts of the posterior hypothalamus and the dorsal parts of the third ventricle. Colchicine treatment was found not to be necessary to visualize perikarya containing TRH.
Key words. TRH; new antibody; carbodiimide coupling method; enzyme immunoassay; immunohistochemistry; rat; hypothalamus; magnocellular groups.

The isolation and characterization of the thyrotropin-releasing hormone (TRH) as the tripeptide pGlu-His-Pro-NH_2 were carried out in 1969[1-3], but there are few immunohistochemical studies on the distribution of TRH in the central nervous system[4-8], compared with investigations using radioimmunoassay. Monospecific antibodies to TRH were produced at a high titer by Bassiri and Utiger, and since then most studies involving radioimmunoassay and immunohistochemistry have used anti-TRH sera which have been elaborated as reported by these authors. In the studies they report[9,10], synthetic TRH was coupled to bovine serum albumin (BSA) with bis-diazotized benzidine (BDB). As shown in figure 1, the diazonium of BDB is presumed to react both with lysyl, tyrosyl or histidyl groups of BSA and with a carbon atom at position 2 or 4 of the imidazole group of histidine in TRH.

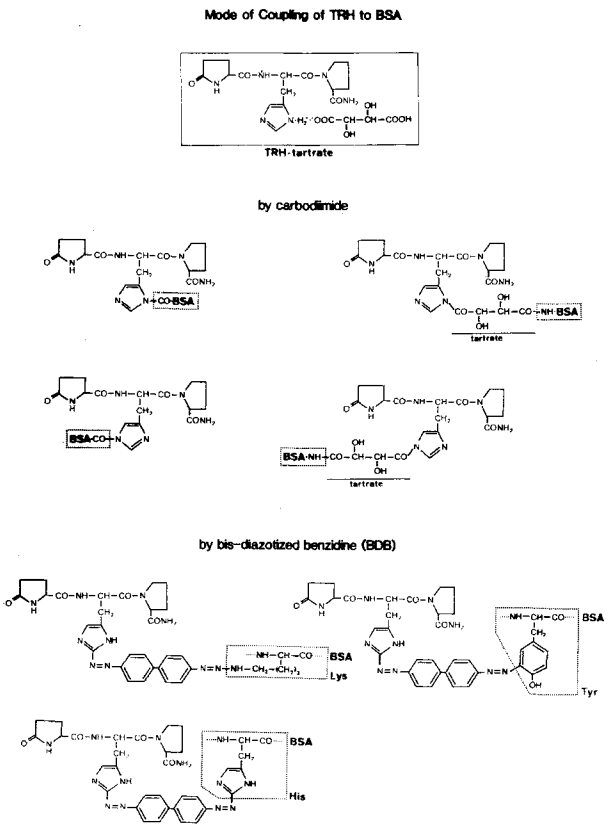


Figure 1. Two modes of coupling of TRH to BSA with carbodiimide (EDC) or bis-diazotized benzidine (BDB).

We considered that, since imidazole nitrogens in histidine residues are relatively active, it should be possible to conjugate them directly with BSA by the formation of amide bonds, using a carbodiimide (EDC) coupling method. Under these conditions, both carboxyl and amino residues, which are abundant in BSA, may react with the nitrogen atom at position 1 or 3 of the imidazole group of histidine in TRH, in the presence or in the absence of tartrate. These modes of coupling are illustrated in figure 1. Such structures suggest that TRH molecules can easily take on several three-dimensional conformations, including that of the native form of TRH. With this antigen, a highly specific anti-TRH serum was successfully produced in rabbits.

The specificity of the anti-TRH serum was assessed by both enzyme immunoassay (EIA) and immunohistochemistry. For EIA, TRH was conjugated to activated CH-sepharose 4B (Pharmacia, Sweden). The primary antisera were added to test tubes containing excess amounts of prepared TRH-sepharose beads. After washing, horseradish peroxidase (HRP)-labelled anti-rabbit IgG was added to the test tubes. Immunospecific binding of the antibodies was quantitated by estimating the bound HRP; measuring its activity by the 2,2′-azino-di(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) colorimetric method using a spectrophotometer to measure OD_{420}[11]. For the absorption test using EIA, antisera absorbed with various concentrations of TRH were used instead of primary antibodies for immunostaining. As the appropriate antiserum dilution for immunohistochemistry was 1/2000, we chose the same dilution for EIA. From these EIA and immunohistochemical studies, the anti-TRH serum was found to be adequately absorbed at quantities

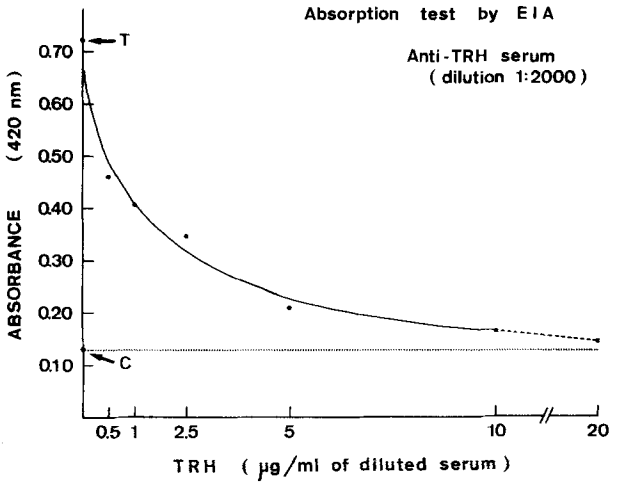
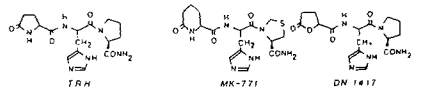


Figure 2. Absorption test by enzyme immunoassay (EIA). T, non-absorbed antiserum with TRH. C, non-immunized rabbit serum, instead of primary antibodies. Anti-TRH serum dilution was 1/2000.

Cross-reactivity of various compounds by EIA following preincubation of diluted antiserum (1:2000) at a concentration of 2×10^{-5} M.

Number	Compounds	% Cross-reactivity
1	TRH(pGlu-His-Pro-NH_2)	100.0
2	TRH-tartrate	100.0
3	TRH-BDB-BSA	35.0
4	DN-1417	<0.5
5	MK-771	<0.5
6	pGlu-His-Gly-NH_2	<0.5
7	Leu-enkephalin	<0.5
8	Oxytocin	<0.5
9	LH-RH	<0.5
10	Substance P	<0.5
11	Angiotensin I	<0.5
12	Angiotensin II	<0.5
13	Serotonin(5-HT)	<0.5
14	Histamine	<0.5
15	L-Pyroglutamic acid	<0.5
16	L-Histidine	<0.5
17	L-Prolinamide	<0.5
18	BSA*1	<0.5

*1 Bovine serum albumin ; 5×10^{-6}M



of 20 µg per milliliter of serum (fig. 2). The cross-reactivity of the TRH antiserum was also studied by EIA.

The diluted anti-TRH sera, incubated previously with various compounds, were used for immunostaining. Various TRH-analogs, neuropeptides, hypothalamic hormones, neurotransmitters and TRH constituents were tested, and found to have no effect on the staining. BSA, up to concentrations of 300 µg/ml, also showed no effect. The results of these experiments are shown in the table. TRH-BDB-BSA, an immunogen for generating anti-TRH serum used in previous reports, was prepared at a concentration of 2×10^{-5} M, like TRH alone. TRH-BDB-BSA caused some inhibition of staining, but was much less potent than TRH.

In the present study, the distribution of TRH-like immunoreactivity was observed in the hypothalamus of rats. The procedures for tissue fixation and immunohistochemical staining were essentially according to Kimura et al.[12]. Five Wistar rats (weighing 250–300 g) were used in this study. The animals were anesthetized with sodium pentobarbital, and perfused through the heart with ice-cold phosphate-buffered saline (PBS; 0.9% NaCl in 0.1 M phosphate buffer, pH 7.4) and later with a fixative composed of 2% paraformaldehyde, 0.3% glutaraldehyde and 0.2% picric acid in 0.1 M phosphate buffer. The volume of the fixative was 300 ml. After perfusion, the brain was removed and sliced into frontal blocks of 1.0 cm thickness. The tissue blocks were immersed for 2 days in a postfixative solution containing 2% paraformaldehyde and 0.2% picric acid in 0.1 M phosphate buffer at 4 °C. Vibratome sections (30 µm thickness) were cut, and were collected in PBS with 0.3% Triton X-100. The free-floating sections were initially incubated with anti-TRH antiserum for 2–4 days at 4 °C. This primary antiserum was used at a dilution of 1:2000. Thoroughly washed sections were then incubated overnight with goat anti-rabbit IgG (1:400) at 4 °C and finally with PAP (1:800) for 2 h at room temperature. Finally, the antigens were rendered visible by reaction with 50 mM Tris-HCl buffer (pH 7.6) containing 0.02% 3,3-diaminobenzidine and 0.003% H_2O_2 for 10 min at room temperature.

Many cell bodies, fibers and structures which were probably terminals throughout the hypothalamus showed TRH-immunoreactivity. TRH-positive neuronal somata were visible in normal animals, without colchicine treatment[13–15]. The distribution of TRH-immunoreactive (TRH-I) neuronal cell bodies in the hypothalamus is shown schematically in figure 3. TRH-I cells were found in the periventricular area, paraventricular nucleus, anterior hypothalamic nucleus, lateral hypothalamus, dorsomedial nucleus, perifornical area and arcuate nucleus. These results are similar to those reported previously, though with some variation. For instance, no TRH-I cells were found in the ventromedial nucleus as described by Johansson et al.[5]. We found TRH-I cell bodies and fibers in magnocellular rather than parvocellular parts of the paraventricular nucleus[8].

There were conspicuous differences between the results of the present study and previous reports[5,8] in the distribution of TRH-I cells in the hypothalamus. Figure 4a shows neuronal cell bodies in the ventrolateral hypothalamus of a rat which possess TRH-I products. These neurons were usually large (12–20 µm in diameter) and variously shaped. The regions of these cell groups included the caudal magnocellular nucleus of the hypothalamus. Although TRH-I cell bodies were distributed from the middle to the caudal hypothalamus, near the brain surface, the largest cluster was in the caudal hypothalamus where the posterior hypothalamic nucleus could be seen. Along a dorsal bank of the third ventricle in the rat hypothalamus, scattered cell bodies containing TRH-immunoreactivity were seen, together with some varicose commissural fibers. These cell groups were in the tuberal magnocellular hypothalamic nucleus (fig. 4b). In agree-

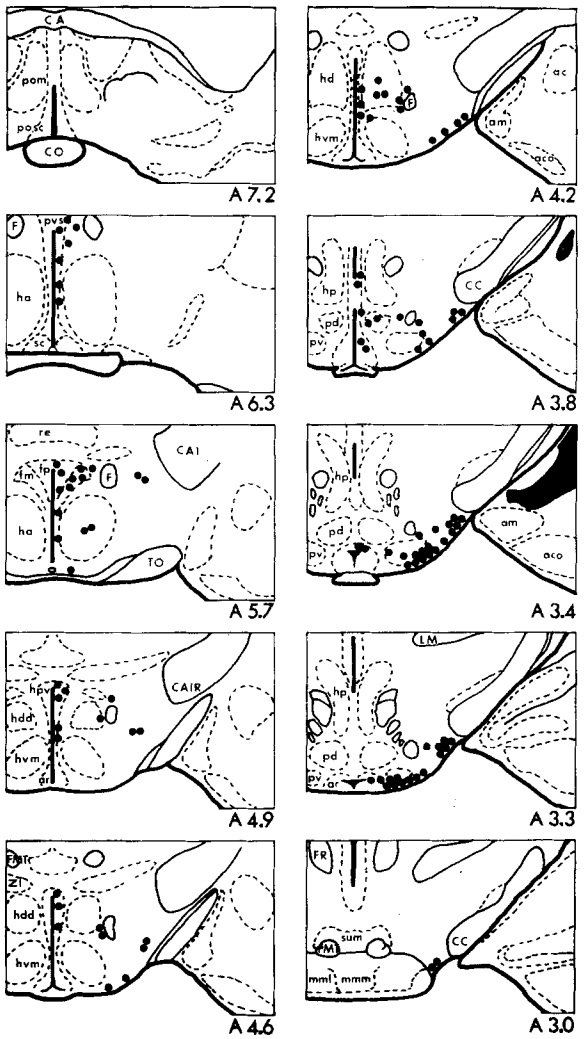


Figure 3. Schematic diagram of the distribution of TRH-immunoreactive cell bodies in the rat hypothalamus. Abbreviations[16]: am = Nucleus amygdaloideus medialis; ar = Nucleus arcuatus; CA = Commissura anterior; CAI = Capsula interna; CAIR = Capsula interna, pars retrolenticularis; CC = Crus cerebri; CO = Chiasma opticum; F = Columna fornicis; fm = Nucleus paraventricularis (filiformis), pars magnocellularis; FMT = Fasciculus mamillothalamicus; fp = Nucleus paraventricularis (filiformis), pars parvocellularis; FR = Fasciculus retroflexus; ha = Nucleus anterior (hypothalami); hd = Nucleus dorsomedialis (hypothalami); hdd = Nucleus dorsomedialis (hypothalami), pars dorsalis; hp = Nucleus posterior (hypothalami); hpv = Nucleus periventricularis (hypothalami); hvm = Nucleus ventromedialis (hypothalami); LM = Lemniscus medialis; mml = Nucleus mamillaris medialis, pars lateralis; mmm = Nucleus mamillaris medialis, pars medialis; pd = Nucleus premamillaris dorsalis; pom = Nucleus preopticus medialis; posc = Nucleus preopticus, pars suprachiasmatica; pv = Nucleus premamillaris ventralis; pvs = Nucleus periventricularis stellatocellularis; re = Nucleus reuniens; sc = Nucleus suprachiasmaticus; sum = Nucleus supramamillaris; TO = Tractus opticus; ZI = Zona incerta.

ment with a previous report[8], TRH-I cell bodies were also visible within the intraventricular space.

The structural differences in immunogens should give antibodies different characteristics. Both antisera are sufficiently neutralized by TRH; however, TRH-BDB-BSA failed to prevent adequately the immunoreactivity of our antibodies. Thus, the antigenic recognition sites of our

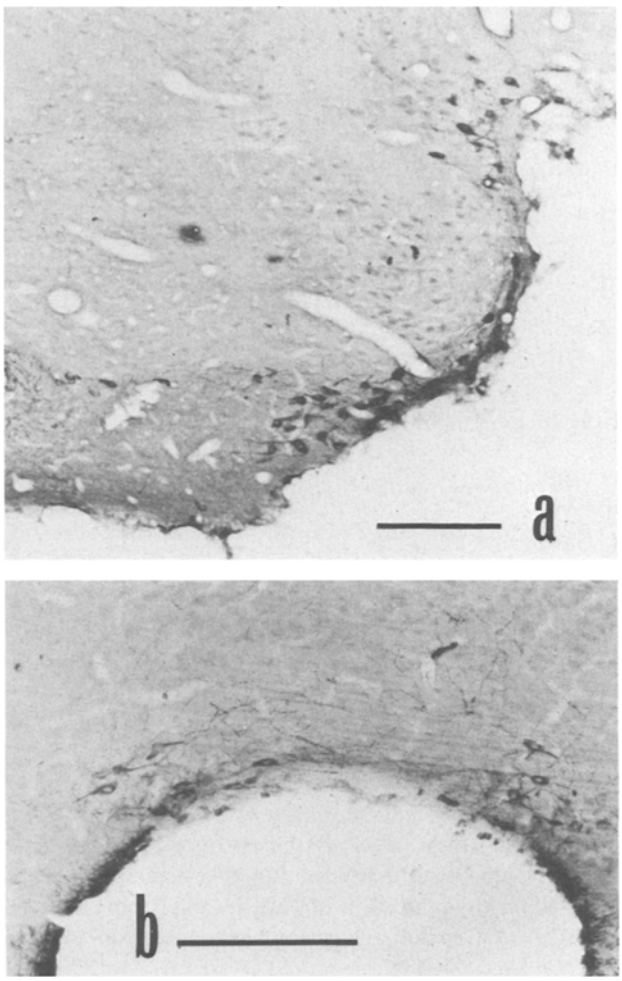


Figure 4. TRH-immunoreactive neuronal somata (*a*) in the ventrolateral parts of the posterior hypothalamus, (*b*) in dorsal parts of the third ventricle of the rat. Anti-TRH serum dilution was 1/2000. Bars: 100 µm.

antibodies are different from those of antibodies raised by a different immunogen, TRH-BDB-BSA. This is partly supported by immunohistochemical data in the present study which demonstrated new groups of neurons containing TRH. Furthermore, in contrast to previous reports[5–8], TRH-positive perikarya were easily observed without colchicine treatment. Therefore, the anti-TRH serum produced by this method should be useful for investigating the TRH system in the hypothalamus and in extrahypothalamic brain areas.

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Role of adenosine A_1 and A_2 receptors in the regulation of aldosterone production in rat adrenal glands

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Summary. To investigate the roles of adenosine A_1 and A_2 receptors in the regulation of aldosterone production, we examined the effects of adenosine and adenosine agonists (N^6-cyclohexyl adenosine; selective adenosine A_1 receptor agonist and 5'-N-ethylcarboxamine adenosine; selective adenosine A_2 receptor agonist) on aldosterone and cyclic AMP production in rat adrenal capsular cells. Neither adenosine nor 5'-N-ethylcarboxamine adenosine caused significant effects on basal aldosterone or cyclic AMP production. Also, adenosine (10^{-3} M) showed no consistent effects on aldosterone and cyclic AMP production induced by ACTH. On the other hand, N^6-cyclohexyl adenosine exhibited a significant inhibition of basal aldosterone and cyclic AMP production at doses of 10^{-4} M and 10^{-3} M; furthermore, 10^{-3} M N^6-cyclohexyl adenosine inhibited aldosterone and cyclic AMP production stimulated by ACTH. These results suggest that adenosine A_1 receptors are coupled to and inhibit adenylate cyclase and may be involved in the inhibition of aldosterone production.
Key words. Aldosterone; cyclic AMP; adenosine; adenosine A_1 receptor; adenosine A_2 receptor.

Adenosine has been shown to have a variety of biological activities including effects on hemodynamics, sympathetic nerve activity, renin and hormonal secretions[1-5]. However, the role of adenosine in the regulation of adrenal steroidogenesis is still controversial. Kowal and Fiedler[6], using monolayer cultures of adrenal cells and primary cultures of adrenal tumor cells, reported that adenosine stimulated adrenal steroidogenesis. Wolff and Cook[7] found that adenosine increased steroidogenesis and adenylate cyclase activity in adrenal tumor cells. Adenosine was also reported to cause a significant increase in cortiocosterone secretion in in situ isolated perfused rat adrenal preparations[8]. On the other hand, in other studies adenosine was shown to have no direct effect on adrenal steroidogenesis in vivo or in vitro[9,10]. Furthermore, Shima[11] observed that adenosine and its analog showed an inhibitory effect on ACTH-stimulated steroidogenesis and adenylate cyclase activity without affecting basal steroid production or adenylate cyclase activity in rat adrenal gland. With regard to the effect of adenosine on aldosterone production, Shima[11] reported that adenosine inhibited $ACTH^{1-24}$-stimulated aldosterone production without affecting basal aldosterone production in dispersed rat adrenal cells. Hinson et al.[8], however, indicated that adenosine did not exhibit a consistent effect on aldosterone secretion in perfused rat adrenal gland or aldosterone production in dispersed rat adrenal cells.

Recently, two functionally distinct adenosine receptors that stimulate or inhibit plasma membrane adenylate cyclase have been identified and reviewed[12]. These two subclasses of adenosine receptors are designated A_1 and A_2. A variety of adenosine analogues have been introduced in recent years. N^6-cyclohexyl adenosine (CHA) and 5'-N-ethylcarboxamine adenosine (NECA) are relatively selective agonists for A_1 and A_2 receptors, respectively. The present study was undertaken in an attempt to evaluate the effects of adenosine, and adenosine A_1 and A_2 receptor agonists (CHA and NECA), on aldosterone and cyclic AMP production in rat adrenal capsular cells. In addition, the effects of adenosine and CHA on ACTH-stimulated aldosterone production and ACTH-induced cyclic AMP production were also examined.

Materials and methods

Adenosine, CHA, NECA and bovine serum albumin (BSA) were purchased from Sigma Chemical (St. Louis, MO, USA). $ACTH^{1-24}$ was obtained from the Protein Research Foundation (Osaka, Japan). Medium 199 was from GIBCO Laboratories (Grand Island, NY, USA)