ALTERATIONS IN LEVELS OF THYROTOPHIN RELEASING HORMONE AND RATES OF HORMONE DEGRADATION IN HYPOTHALAMIC TISSUE OF THE DEVELOPING RAT

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SUMMARY

Developmental changes in levels of hypothalamic thyrotrophin releasing hormone (TRH) and the role that hypothalamic degrading enzymes may play in these alterations were investigated. Levels of TRH in male and female fetal rats and in male neonatal rats were measured by radioimmunoassay. The hormone content of the hypothalamus was shown to increase from less than 1 ng at 1–4 days of age to approximately 10 ng at 20 days of age. Thereafter, the content of TRH declined to the adult level of about 5 ng. The ability of fractionated hypothalamic homogenates to degrade TRH was measured over the same time. The 27 000 g supernatant fraction contained a degrading activity that yielded only radiolabelled deamido-TRH upon incubation with [L-proline 2,3-3H]TRH. The corresponding particulate fraction contained at least two distinct TRH degrading activities as determined by the number of metabolites present. Changes in rates of degradation were not large enough to account for the differences observed in levels of TRH.

INTRODUCTION

Enzymes which degrade thyrotrophin releasing hormone (TRH) have been found in homogenates of hypothalamic tissue and in other areas of the brain (Reichlin, Saperstein, Jackson, Boyd & Patel, 1976; Taylor & Dixon, 1976). The physiological role that these TRH degrading enzymes play is unclear. It has been suggested, however, that they could potentially serve to regulate concentrations of TRH (McKelvy & Epelbaum, 1978). It has also been pointed out that the TRH degrading enzymes in the brain can form metabolic products of TRH which possess distinct biological activities (Prasad, Matsui & Peterkofsky, 1977). To determine whether TRH degrading enzymes could alter levels of TRH during development we have measured the content of TRH and the TRH degrading activities in hypothalami of developing male rats.

MATERIALS AND METHODS

Reagents

[L-Proline 2,3-3H]TRH with a specific activity of 21.8 Ci/mmole was obtained from New England Nuclear Corporation, Boston, Massachusetts, U.S.A. Bovine serum albumin, thyroglobulin and Isatin spray reagent were from Sigma Chemical Company, St Louis, Missouri, U.S.A. Thyrotrophin releasing hormone (pyroGlu-His-Pro-NH₂), pyroGlu-His-Pro and luteinizing hormone releasing hormone (LH-RH) were purchased from Bachem Incorporated, Torrance, California, U.S.A. Vega–Fox Chemicals and its predecessor Fox
Chemical Company, Tucson, Arizona, U.S.A. supplied Glu-His and His-Pro respectively. Peninsula Laboratories Incorporated, San Carlos, California, U.S.A. produced Glu-His-Pro-NH₂ and pyroGlu-His-Pro-Gly-NH₂. Beckman Instruments Incorporated, Palo Alto, California, U.S.A. supplied pyroGlu-L-DOPA-Pro-NH₂. Iodoacetamide was supplied by Pierce Chemical Company, Rockford, Illinois, U.S.A. Finally N-ethylmaleimide was purchased from Aldrich Chemical Company, Milwaukee, Wisconsin, U.S.A.

Antibody to TRH

Antibody was prepared in a manner similar to that described by Bryce (1974) using the bis-diazobenzidine technique of Bassiri & Utiger (1972). Thyroglobulin was coupled to TRH instead of to albumin because the former protein appears to be more effective in eliciting an antibody-response to small molecules (Skowski & Fisher, 1972). All antiserum in this study was from a single rabbit. Blood was collected by puncture of an ear artery and allowed to clot at 4 °C for 1–2 h before the clot was separated by centrifugation. Solid ammonium sulphate was added to the serum to obtain 40% saturation and the solution was allowed to stand for 1 h. The precipitate was collected by centrifugation, redissolved in phosphate-buffered saline (PBS, 0.01 M-phosphate containing 0.15 M-NaCl, pH 7.4), dialysed overnight against the same buffer and stored frozen at -20 °C.

Radioimmunoassay of TRH

Levels of TRH were measured by radioimmunoassay according to the method of Bryce (1974) with the following exceptions. Incubation mixtures contained 10 µl diluted TRH antiserum (which bound 4000 counts/min [³H]TRH), 5 µl [L-proline 2,3-³H]TRH (12 500 counts/min, 0.13 µmol/l), 5–20 µl portions of the TRH solutions to be measured (buffer was substituted in control samples) and PBS to make a total volume of 35 µl. After incubation overnight at 4 °C, 10 µl non-specific immunoglobulin (16 mg/ml) were added before ammonium sulphate precipitation. A standard curve was generated for each group of assays by plotting the log TRH concentration in the assay versus radioactivity. This curve was normally linear between 0.1 and 2.5 ng TRH. The error among triplicate assays was less than 25%.

Specificity of antibody to TRH

The cross-reactivity of the antibody was measured by radioimmunoassay using the compound in question in place of TRH. The antibody did not cross-react with Glu-His, His-Pro, pyroGlu-His-Pro, Glu-His-Pro-NH₂ or the related peptide hormone, LH-RH. The only compounds tested that did cross-react with the TRH antibody were pyroGlu-His-Pro-Gly-NH₂ and pyroGlu-L-DOPA-Pro-NH₂. The latter peptide showed competition for the antibody that quantitatively paralleled that of authentic TRH.

Preparation of hypothalamic tissue

The hypothalamic fragments were obtained from male Wistar rats which were caged with lactating female rats. Animals which were 21 days of age had been weaned. The rats were decapitated and the brains quickly removed. The hypothalamus was excised by cutting vertically around its borders using the optic chiasma as the anterior border, the mamillary bodies as the posterior border and the hypothalamic fissures as the lateral borders. A horizontal cut under the hypothalamus at a depth of 3–4 mm allowed it to be lifted from the surrounding tissue. The hypothalamus of fetal rats was obtained in a similar manner after decapitation of the mother.

Measurement of TRH in the hypothalamus

Hypothalamic fragments were immediately placed in 400 µl absolute methanol, weighed and then disrupted with a Dounce tissue homogenizer. The homogenizer was washed three times
with 100 µl methanol and the combined washings and homogenate were sonicated for 25 s. The extract was centrifuged at 8000 g for 3 min and the insoluble material washed three times with 300 µl methanol. The pooled supernatant fluids were dried under a stream of nitrogen, redissolved in PBS and then radioimmunoassayed in triplicate.

Assay for hypothalamic TRH degrading activity
Hypothalami from male rats were dissected as described above and immediately placed into chilled beakers and weighed. The tissue was homogenized in 2 ml PBS for 30 s with a Brinkman polytron homogenizer. The homogenate was centrifuged at 27 000 g for 30 min at 4 °C. The supernatant fluid was decanted and used for determining soluble enzyme activity. The pellet was homogenized in 2 ml PBS for 15 s. This homogenate was used for determining particulate enzyme activity. The assay for enzyme activity was carried out in the following manner: 15 µl portion of the soluble enzyme fraction was mixed with 10 µl [L-proline 2,3-3H]TRH (0.46 µmol/l) and 25 µl PBS and incubated at 37 °C for the specified time. The reaction was stopped by immersing the reaction tube in boiling water for 3 min and then diluting with 250 µl PBS. The particulate fraction was assayed in a similar manner except that 20 µl homogenate and 20 µl PBS were used and the mixture was agitated during incubation. Triplicate 10 µl samples of the diluted reaction mixture were mixed with 20 µl rabbit anti-TRH and incubated overnight at 4 °C. Ten microlitres non-specific immunoglobulin (16 mg/ml) were then added, the mixture was brought to 50% saturation with (NH₄)₂SO₄ and allowed to stand on ice for 20 min. The precipitate was pelleted by centrifugation at 8000 g for 5 min, the supernatant fluid discarded and the pellet washed twice with 50% (NH₄)₂SO₄. The final pellet was dissolved in 100 µl 1 M NaOH and the radioactivity determined. Protein was measured by the method of Lowry, Rosebrough, Farr & Randall (1951) using bovine serum albumin as a standard.

Product analysis of the particulate and soluble degrading activities
Degradation products of [L-proline 2,3-3H]TRH were examined by thin-layer chromatography on silica gel G Plates (Analabs, North Haven, Connecticut, U.S.A.) using the methods described by Taylor & Dixon (1978). Radioactive products were located by scraping 0.5 cm sections of the plate into a vial and determining the radioactivity. The products were identified by a comparison of their relative mobilities with authentic samples of pyroGlu-His-Pro, TRH, proline and prolineamide. These compounds were visualized by either the Pauly or the Isatin reagents which detect the presence of histidine and proline respectively.

Inhibition of TRH degradation by sulphydryl reagents
The enzyme assay was performed as described above in the presence of either 4·0 mM iodoacetamide or N-ethylmaleimide.

RESULTS
Hypothalamic TRH
The content of TRH in the hypothalamus of fetal and neonatal male rats is shown in Fig. 1. The hypothalamus contained less than 1 ng TRH during late fetal and early neonatal life. After 4 days of age the level of TRH increased and reached a maximum of 10 ng/hypothalamus on day 20. Our results with male rats were similar to those reported by Ojeda, Kruích & Jameson (1976) in female neonatal and adult rats. However, the concentrations of TRH observed by the present radioimmunoassay in male animals were higher than those determined by in-vitro bioassay in female rats (Ojeda et al. 1976). Dussault & Labrie (1975) have also reported the hypothalamic content of TRH in young unsexed rats with similar results to those of the present study.
Fig. 1. Content of thyrotrophin releasing hormone (TRH) in the hypothalami of male rats measured by radioimmunoassay. The data represent the mean ± S.D. of the number of samples shown in parentheses.

TRH degrading activity in the hypothalamus

Fractionation of rat hypothalamic homogenates at 27 000 g yielded soluble and particulate TRH degrading activities. As shown in Fig. 2, incubation of either fraction with [L-proline 2,3-3H]TRH at 37 °C resulted in a loss of immunoprecipitable TRH. The decrease in concentration of TRH approximated to linearity for 15 min with either enzyme fraction.

Product analyses of the soluble and particulate TRH metabolites were examined in the various solvent systems shown in Table 1. The soluble fraction from either 3- or 20-day-old rats exhibited a single radioactive product which co-migrated with an authentic sample of pyroGlu-His-Pro. The enzyme(s) was also completely inactivated with sulphhydryl blocking reagents (i.e. N-ethylmaleimide). In contrast to the soluble TRH degrading enzyme the particulate fraction exhibited a minimum of three radioactive metabolites in two of the solvent systems examined. The relative mobilities \( R_F \) of the various compounds are listed in Table 1. It was possible to demonstrate that two of the radioactive compounds migrated with the same \( R_F \) values as pyroGlu-His-Pro and Pro. At least one additional major metabolite

Table 1. Relative mobilities \( R_F \) on thin-layer chromatograms of metabolites of [L-proline 2,3-3H]thyrotrophin releasing hormone formed upon incubation with male rat hypothalamic extracts in various solvent systems

<table>
<thead>
<tr>
<th>Solvent systems (by vol.)</th>
<th>Ammonia : methanol : n-Butanol : acetic acid : chloroform : ethylacetate : ( H_2O ) : Phenol : ( H_2O )</th>
</tr>
</thead>
<tbody>
<tr>
<td>Enzyme fraction</td>
<td>Soluble ( R_F )</td>
</tr>
<tr>
<td>Ammonia : methanol : n-Butanol : acetic acid : chloroform : ethylacetate : ( H_2O ) : Phenol : ( H_2O )</td>
<td></td>
</tr>
<tr>
<td>Soluble</td>
<td>0.54</td>
</tr>
<tr>
<td>Particulate</td>
<td>0.50</td>
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</tbody>
</table>
Changes in TRH levels and degradation

Fig. 2. [L-Proline 2,3-3H]thyrotrophin releasing hormone (TRH) degradation as a function of the time of incubation with male rat hypothalamic extract. The enzyme fractions were prepared from 12-day-old male rats. The soluble fraction (O) was from an homogenate of four hypothalami and the particulate fraction (●) was prepared from three hypothalami.

Fig. 3. [L-Proline 2,3-3H]thyrotrophin releasing hormone (TRH) degradation by soluble (O) and particulate (●) fractions. Fractions were prepared from homogenates of three hypothalami of rats of various ages. Each point represents the mean of determinations on two different preparations of fractions.
which did not exhibit an $R_t$ characteristic of pyroGlu-His-Pro, Pro, TRH or Pro-NH$_2$ was present. It is possible that this unidentified metabolite corresponded to histidylprolineamide (or the related diketopiperazine) which has been shown to result from removal of the pyroglutamic acid from TRH (Prasad & Peterkofsky, 1976).

In order to assess the role that TRH degrading enzymes may play in regulating hormone levels during development, the rates of degradation for both soluble and particulate enzymes were determined in male rats of varying ages. No corrections for endogenous content of TRH were made. The TRH degrading activities are shown in Fig. 3 as a function of the age of the rats. The variation in both soluble and particulate TRH degrading activities have $P$ values of $<0.01$. Although this value may be statistically significant, the levels of TRH degrading activity did not correlate with the changes in TRH levels.

**DISCUSSION**

The TRH content of young male rat hypothalami increased dramatically 5 days after birth, to reach a maximum at approximately 20 days, followed by a decline to the normal adult level. There are several possible explanations for this change.

The data could reflect hormone biosynthesis, the conversion of an inactive precursor (i.e. prohormone) into TRH, transport of the hormone (or prohormone) from another anatomical location to the hypothalamus, changes in the rates of hormone secretion or alteration in the TRH degrading activity of the hypothalamus. The latter explanation seems unlikely in view of the data presented here. Rates of degradation of TRH were only slightly altered during a time when the TRH content of the hypothalamus had increased significantly; the fluctuations in TRH degrading activity did not correspond to the changes in TRH levels. It seems unlikely that the changes in the rate of hypothalamic TRH degradation could account for the observed increase in TRH in the hypothalami.

Oliver, Parker & Porter (1977) have measured TRH degrading activity during development in the 900 g supernatant fraction of whole rat brain homogenates. Their results are similar to our data and also indicate relatively small changes from 4 to 20 days of age in the TRH degrading activity.

Although the levels of TRH degrading activity changed only slightly during maturation, concentration of TRH in the hypothalamus increased significantly, suggesting that the concentrations of the various TRH metabolites might also change. One of the metabolites of TRH degradation (histidyl-proline diketopiperazine) antagonizes ethanol narcosis and seems to have a characteristic physiological effect (Prasad et al. 1977). The hypothalamic TRH degrading enzymes have a potential role in the formation of such metabolites. It should also be pointed out that, although the hormone and degrading enzymes are both present in the hypothalamus, there is no information available about the cellular location of the TRH metabolizing enzymes. Localization of TRH and its metabolizing enzymes in the same cell population would help to establish the physiological significance of the TRH metabolizing enzymes.

It is interesting to compare the alterations in TRH levels during maturation with the appearance of thyroid-stimulating hormone (TSH) in the plasma. Levels of TSH in female rats increase from day 5 and peak at day 12 according to Ojeda et al. (1976) and Cons, Umez & Timiras (1975). However, there is some disagreement about when the TSH levels are maximal. Dussault & Labrie (1975) have reported that plasma TSH levels rise rapidly after birth and are maximal between day 4 and 22, after which they decline. In general, the rise in TSH levels correlates with the initial increase in hypothalamic TRH.

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REFERENCES


