Testosterone regulates the secretion of thyrotrophin-releasing hormone (TRH) and TRH precursor in the rat hypothalamic-pituitary axis

A. E. Pekary, M. Knoble, N. H. Garcia, S. Bhasin* and J. M. Hershman

Endocrinology Research Laboratory, Medical and Research Services, Veterans Administration Wadsworth Division Medical Center and *Harbor-UCLA Medical Center and Department of Medicine, University of California at Los Angeles, Los Angeles, California 90073, U.S.A.

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ABSTRACT

Orchidectomy has been reported to decrease concentrations of thyrotrophin (TSH) in the circulation of male rats without affecting serum levels of thyroid hormones. To understand the mechanism underlying this observation, we have measured the effect of gonadal status on the in-vitro release of TSH-releasing hormone (TRH) by male rat hypothalamic fragments. Because hormone release rates can be affected by changes in the post-translational processing of the hormonal precursors, we have also studied the corresponding changes in the concentrations of TRH and TRH-Gly, a TRH precursor peptide in hypothalamus and pituitary, by radioimmunoassay.

We observed a significant decline in the in-vitro release of TRH from incubated hypothalami 1 week after castration, which was quantitatively reversed by testosterone replacement. Concentrations of TRH and TRH-Gly in the posterior pituitary, on the other hand, which derive from neurones of hypothalamic origin, increased significantly with castration and were returned to the normal range by testosterone replacement.

We conclude that the primary effect of testosterone is the stimulation of hypothalamic TRH release, resulting in the depletion of TRH and TRH precursors from TRH-containing neurones which project into the median eminence and posterior pituitary. Journal of Endocrinology (1990) 125, 263–270

INTRODUCTION

Thyroid hormones regulate thyrotrophin (TSH) secretion by direct negative feedback upon the pituitary thyrotrophs (Hershman & Pekary, 1985). More recently, evidence for thyroid hormone inhibition of hypothalamic TSH-releasing hormone (TRH) release has become available (Rondeel, de Greef, van der Schoot et al. 1988). Less widely appreciated is the role of androgenic steroids in the regulation of TSH secretion in the male rat (Chen & Walfish, 1979; Watanobe & Takebe, 1987). We have reported previously that testosterone has profound effects on the conversion of TRH-Gly (pGlu-His-Pro-Gly) to TRH in the rat ventral prostate (Pekary, Knoble & Garcia, 1989). The existence of this regulatory relationship in an extrahypothalamic tissue coupled with our knowledge that the co-option of TRH as a hypothalamic releasing hormone for pituitary TRH and prolactin was a recent event in its evolution (Hershman & Pekary, 1985) suggested the possibility that a similar effect on TRH biosynthesis might be demonstrable in the hypothalamus. Because the α-amidation of TRH-Gly to form TRH is not rate-limited in the rat hypothalamus as it is in the rat prostate (Simard, Pekary, Smith & Hershman, 1989a,b), we were not able to measure TRH-Gly levels in individual rat hypothalami with our current TRH-Gly radioimmunoassay (RIA). We have, however, observed a significant decrease in TRH release by hypothalamic fragments in vitro following castration, and a concurrent increase in posterior pituitary TRH and TRH-Gly content which is of hypothalamic origin. These observations are consistent with previously reported reductions in serum TSH following castration of male rats (Chen & Walfish, 1979; Watanobe & Takebe, 1987) which may be mediated, in part, by a reduction in hypothalamic TRH release.

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MATERIALS AND METHODS

Animals
Male Sprague-Dawley rats (250-300 g) were obtained from Bantin-Kingman, Fremont, CA, U.S.A. and maintained in a temperature- and light-controlled environment (21-23 °C; lights on from 06.00 to 18.00 h). These animals were provided with water and Purina rodent chow ad libitum.

Surgical procedures
For experiment 1, animals were divided into two groups: castrated and intact control. In experiment 2, rats were divided into three groups: castrated, sham-castrated and control. In experiment 3, rats were divided into three groups: sham-castrated, castrated and castrated with testosterone replacement. Castrated rats had both testes removed through an abdominal incision, and sham-castrated rats had one testis briefly exteriorized 3 or 7 days before being killed. Control animals were left intact and untreated. Animals given testosterone replacement received a daily i.m. injection of 0.2 mg testosterone cypionate in 0.1 ml of 90% (v/v) sesame oil-10% ethanol (Depo-Testosterone; Upjohn, Kalamazoo, MI, U.S.A.). All surgical procedures were carried out under pentobarbital anaesthesia (5 mg/100 g body weight).

Extraction of blood and tissues for measurement of TRH and TRH-Gly
Following decapitation, trunk blood was collected in 16 × 150 mm glass test tubes in ice containing 1000 units heparin with continuous agitation by a vortex mixer. Whole blood (1 ml) was then rapidly transferred to another 16 × 150 mm test tube in ice which contained 10 ml methanol. This blood-methanol mixture was vortex-mixed to inactivate TRH-degrading enzymes rapidly and later centrifuged. The resulting supernatant was dried on a heater block at 60 °C with a fan blowing air into each tube. In experiment 1, the hypothalamus, pancreas, thyroid, ventral prostate and whole pituitary were removed from each rat, weighed, and placed in glass test tubes containing 1 mol acetic acid/l at 97 °C with a fan blowing air into each tube. The dried residues were rehomogenized in methanol, centrifuged and the supernatants redried as for the blood-methanol extract. In experiment 2, the hypothalamus, posterior pituitary, prostate, pancreas and thyroid from each animal were removed, weighed and extracted as described above.

Radioimmunoassays
The measurement of serum levels of luteinizing hormone (LH), follicle-stimulating hormone (FSH), TSH, thyroxine (T₄) and tri-iodothyronine (T₃), testosterone and concentrations of TRH immunoreactivity (TRH-IR) and TRH-Gly-IR in whole blood and tissue extracts has been described previously in detail (Roohk, Azukizawa, DiStefano et al. 1979; Bhasin, Fielder, Peacock et al. 1988; Simard et al. 1989a,b). Cross-reactivity curves for the TRH-Gly antisera used in the present study (898B2; Pekary et al. 1989) are given in Fig. 1. This antibody has greatest affinity for peptides such as TRH-Gly (pGlu-His-Pro-Gly) or Arg-TRH-Gly (Arg-Gln-His-Pro-Gly), but <0.01% cross-reactivity for TRH (pGlu-His-Pro-NH₂) and related peptides.

FIGURE 1. Cross-reactivity curves for rabbit antiserum to TRH-Gly (898B2) at a final dilution of 1:2500. Logit (B/B₀) = ln ((B/B₀)/(1–B/B₀)), where B₀/B₀ is the ratio of counts bound at finite dose divided by counts bound with zero dose of unlabelled antigen. Serial dilutions of each tissue extract studied displaced ¹²⁵I-labelled TRH-Gly in parallel with the TRH-Gly standard curve. The synthetic peptides tested for cross-reactivity in the TRH-Gly RIA included TRH-Gly and Arg-TRH-Gly (○); TRH-Gly-NH₂ (●); TRH-Gly-Lys (■); TRH-Gly-Lys-Arg (□); His-Pro-Gly, TRH, pGlu-His-Pro (TRH-OH) and pGlu-His-Gly-NH₂ (●).

High-pressure liquid chromatography (HPLC)
The individual methanol extracts were reconstituted with distilled water before RIA of TRH and TRH-Gly. Equal aliquots from the residual extracts within each experimental treatment group were pooled and defatted with ethyl ether. The aqueous phase was passed through a Sep-Pak C₁₈ reverse-phase cartridge (Waters Inc., Milford, MA, U.S.A.). The cartridge was washed with water and 50% (v/v) acetonitrile (Omnisolve grade; Matheson, Coleman and Bell, Cincinnati, OH, U.S.A.). The eluates were dried completely with the aid of a Speed-Vac centrifugal evaporator (Savant Instruments, Farmingdale, NY, U.S.A.). After removing particulate matter with a centrifugal filtration device (Bioanalytical Systems
Inc., West Lafayette, IN, U.S.A.), the samples were injected into a Model 421 programmable HPLC system (Altex Scientific, Berkeley, CA, U.S.A.) using a 4.6 × 25 cm Ultrasphere octadecylsiline reverse-phase column (Altex) previously equilibrated with 0.01 mol trifluoroacetic acid/l. At the time of injection, a linear 0-5% min gradient of acetonitrile at a flow rate of 1 ml/min was started. At 40 min the gradient was increased to 2%/min. The 1 ml fractions collected were dried completely and reconstituted with distilled water just before measurement of TRH-IR and TRH-Gly-IR by the corresponding RIA.

In-vitro incubation of hypothalamic fragments

Hypothalamic fragments, bounded by the posterior margin of the optic chiasm and the anterior margin of the mamillary bodies, and in a plane 1.5 mm lateral to the midline and 2 mm deep, were excised. Individual hypothalami were placed in 10 × 75 mm glass test tubes with 0.5 ml Hank’s balanced salt solution (Irvine Scientific, Santa Ana, CA, U.S.A.) containing 50 µg bacitracin/ml and 20 mmol Hepes/l, pH 8.0, and equilibrated with 95% oxygen and 5% carbon dioxide. The test tubes were sealed with Parafilm (American Can Co., Greenwich, CT, U.S.A.) and after incubating for 60 min at 37°C in a shaker bath, the medium was removed and replaced with 0.5 ml Hank’s medium equilibrated with 95% oxygen and 5% carbon dioxide containing 60 mmol KCl/l and 58 mmol NaCl/l to maintain osmolality (Shennan & Sheppard, 1983; Pekary, Mirell, Turner et al. 1987). After 30 min at 37°C the medium was removed. The media were extracted as described above. The hypothalami were homogenized in 1.0 ml methanol and the supernatants dried completely. The pellets were stored at −20°C for later measurement of protein by the bicinchoninic acid method (Pierce Chemical Co., Rockford, IL, U.S.A.). The TRH-IR content of the media and hypothalamic extracts was measured by RIA.

Peptides

TRH was obtained from Calbiochem, La Jolla, CA, U.S.A. and TRH-Gly was synthesized by Bachem Fine Chemicals, Torrance, CA, U.S.A.

Statistics

All results are reported as means ± s.d. Student’s unpaired t-test and one-way analysis of variance (ANOVA) were performed with the aid of StatView 512+, a statistical computer program for the Apple Macintosh computer (BrainPower Inc., Calabasas, CA, U.S.A.). The HPLC peak area for TRH divided by the total TRH-IR for pooled extracts was multiplied by the TRH-IR values for the corresponding individual tissue extracts to correct for the occurrence of immunoreactive peptides in pituitary tissues not identical to TRH. A similar correction for the TRH-Gly-IR was not made since the TRH-Gly antibody used can cross-react with TRH-Gly precursor peptides such as Arg-TRH-Gly (Fig. 1) which occur in rat posterior pituitary and are relevant to TRH biosynthesis.

RESULTS

Castration for 3 or 7 days led to a significant decrease in serum testosterone and significant increases in serum levels of LH and FSH in both experiments 1 and 2. This treatment did not affect significantly the serum concentrations of TSH, T4, T3 or prolactin (results not shown). After 3 (results not shown) or 7 days of castration, the whole pituitary concentration of TRH was significantly increased (Table 1). The levels of TRH-Gly-IR in whole pituitary were also significantly increased 7 days after sham-castration and castration (Table 2) but not after 3 days (results not shown). A significant effect of sham-castration on posterior pituitary levels of TRH-Gly-IR was not consistently observed. About 80% of the total TRH content of the whole pituitary resides in the posterior lobe, as seen in Fig. 2. For this reason, posterior lobes were dissected from the anterior lobe and extracted for TRH and TRH-Gly in experiment 2. As with the whole pituitary, a significant increase in posterior pituitary content of TRH and TRH-Gly-IR content was observed 7 days after castration (Tables 1 and 2).

The effect of castration on the HPLC chromatographic profile of TRH-Gly-IR in the rat anterior and posterior pituitary is given in the two bottom panels of Fig. 2. The anterior pituitary profiles consist of two major and several minor peaks. The effect of castration was to reduce significantly the levels of these peaks of TRH-Gly immunoreactivity. The profiles for posterior pituitary consisted of a large number of peaks of TRH-Gly-IR. The relative areas of these peaks appear to be affected by castration, particularly in the region of fraction 30.

The HPLC profiles for TRH-IR for pooled extracts of posterior pituitaries for the six experimental treatment groups of experiment 2 are displayed in Fig. 3. The significant increase in the TRH peak area following 7 days of castration is apparent. Because the TRH-Gly RIA is less sensitive than that for TRH, the corresponding profiles for TRH-Gly-IR were not reliably measurable. The HPLC profile of TRH-Gly obtained in a separate experiment from a larger pool.
TABLE 1. Tissue levels of TRH and prostate weights in rats 7 days after removal of testes (castrated), manipulation of testes without removal (sham-castrated) or no treatment (control). Values are means ± s.d.; numbers of animals are given in parentheses.

<table>
<thead>
<tr>
<th>TRH (pmol/g)</th>
<th>Whole pituitary</th>
<th>Posterior pituitary</th>
<th>Hypothalamus</th>
<th>Prostate weight (mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Experiment 1</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control (9)</td>
<td>127 ± 72*</td>
<td>—</td>
<td>1395 ± 199</td>
<td>454 ± 80*</td>
</tr>
<tr>
<td>Castrate (10)</td>
<td>204 ± 130</td>
<td>—</td>
<td>1508 ± 218</td>
<td>81 ± 25</td>
</tr>
<tr>
<td><strong>Experiment 2</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control (5)</td>
<td>—</td>
<td>326 ± 149*</td>
<td>718 ± 99</td>
<td>326 ± 35*</td>
</tr>
<tr>
<td>Sham-castrated (4)</td>
<td>—</td>
<td>489 ± 436</td>
<td>867 ± 232</td>
<td>306 ± 64*</td>
</tr>
<tr>
<td>Castrated (6)</td>
<td>—</td>
<td>1011 ± 517</td>
<td>818 ± 122</td>
<td>77 ± 17</td>
</tr>
</tbody>
</table>

*P < 0.05 compared with castrated group (unpaired t-test), †P < 0.05 compared with castrated group (one-way analysis of variance).

Experiments 1 and 2 were the same except for the inclusion of a sham-castrated group and dissection and extraction of the posterior pituitary in experiment 2.

TABLE 2. Tissue levels of TRH-Gly immunoreactivity (IR) and prostate weights in rats 7 days after surgery. Values are means ± s.d.; numbers of animals are given in parentheses.

<table>
<thead>
<tr>
<th>TRH-Gly-IR (pmol/g)</th>
<th>Whole pituitary</th>
<th>Posterior pituitary</th>
<th>Hypothalamus</th>
<th>Prostate weight (mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Experiment 1</strong></td>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Control (9)</td>
<td>343 ± 88††</td>
<td>—</td>
<td>98 ± 36</td>
<td>454 ± 80††</td>
</tr>
<tr>
<td>Castrate (10)</td>
<td>581 ± 257</td>
<td>—</td>
<td>124 ± 83</td>
<td>81 ± 25</td>
</tr>
<tr>
<td><strong>Experiment 2</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control (5)</td>
<td>—</td>
<td>421 ± 148</td>
<td>202 ± 129</td>
<td>326 ± 35*</td>
</tr>
<tr>
<td>Sham-castrated (4)</td>
<td>—</td>
<td>1426 ± 569*</td>
<td>312 ± 212</td>
<td>306 ± 54*</td>
</tr>
<tr>
<td>Castrated (6)</td>
<td>—</td>
<td>1493 ± 443*</td>
<td>329 ± 107</td>
<td>77 ± 17</td>
</tr>
</tbody>
</table>

*P < 0.05 compared with control group (one-way analysis of variance), ††P < 0.02 compared with castrated group (unpaired t-test).

Experiments 1 and 2 were the same except for the inclusion of a sham-castrated group and dissection and extraction of the posterior pituitary in experiment 2.

of control rats (Fig. 2), revealed a pattern with a major peak corresponding to TRH-Gly. This observation would suggest that the total TRH-Gly-IR and the tetrapeptide TRH-Gly levels should be reasonably proportional.

In experiment 3, the TRH levels in whole pituitaries of the castrated rats (serum testosterone 0.45 ± 0.14 nmol/l, n = 7) increased significantly above those for sham-castrated animals (testosterone 15.3 ± 5.6 nmol/l, n = 5), while testosterone replacement (testosterone 8·1 ± 2·6 nmol/l, n = 6) in this experiment returned serum testosterone to the control range and significantly decreased the pituitary TRH concentration as seen in Fig. 4. In a preliminary experiment, testosterone replacement to 2·6 times the mean sham-castrated serum value maintained the whole pituitary TRH concentration at almost exactly the mean value for the sham-castrated group (results not shown).

In-vitro incubation of rat hypothalami for 60 min at 37°C in Hank's medium revealed a significant (P < 0.05, one-way ANOVA) decrease in hypotalamic TRH secretion in rat castrated for 7 days compared with sham-castrated rats and castrated rats receiving testosterone replacement, as seen in Fig. 5. This decrease was still observable during a subsequent 30 min incubation with isotonic medium containing 60 mmol K⁺/l. The TRH release rate in all hypothalamic fragments was significantly (P < 0.05, paired t-test) increased by incubation with 60 mmol K⁺/l suggesting that these tissues remained viable during the full 90-min incubation period. This experiment was repeated with qualitatively similar results. A significant decrease in hypothalamic TRH release in the castrated group compared with the control group was again observed, while the K⁺ stimulation led to an average four-fold increase in the TRH secretion rate. The basal rate of TRH secretion by the castrated
3-20 ¬ 30 ¬ 40 ¬ 50 ¬

\( \text{Fraction no.} \)

Figure 2. High-pressure liquid chromatography (HPLC) for extracts of (a) rat anterior (broken lines) and posterior pituitary (solid lines); and (b) whole, (c) anterior and (d) posterior pituitary of castrated (solid lines) and control rats (broken lines). Ten rats were used for each chromatogram. The TRH-Gly immunoreactivity peaks at fractions 22 and 30 in the HPLC profile for rat posterior pituitary (d) coeluted with added synthetic TRH-Gly and Arg-TRH-Gly, a TRH-Gly precursor, respectively.

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group receiving testosterone replacement, however, had a high variance, making the comparison of the castration group and the control group by one-way ANOVA statistically insignificant. The concentrations of TRH in the fragments after incubation (sham-castrated 11.1 ± 1.0, castrated 10.0 ± 2.3, castrated + testosterone 9.1 ± 1.1 nmol/g protein) did not differ significantly by one-way ANOVA. The hypothalamic content of TRH-Gly in hypothalamic fragments was not detectable by the present TRH-Gly RIA because of its insensitivity relative to the TRH RIA and the low TRH-Gly:TRH ratio (1:26) in adult rat hypothalamus (Glembotski, Manaker, Winokur & Gibson, 1986; Simard et al. 1989a). Blood levels of TRH-Gly-IR were not significantly affected by castration, while the blood TRH-IR levels were very low and not consistently measurable (results not shown). Levels of TRH- and TRH-Gly-IR in pancreas and thyroid were not significantly affected by castration compared with sham-castrated and control groups (results not shown).

![Graph](image)

**FIGURE 5.** Effect of sham-castration (S-C; n = 7), castration (C; n = 5) or castration and daily injections of 0.2 mg testosterone (C + T; n = 8) on in-vitro TRH release by rat hypothalamic fragments incubated for 60 min in Hanks’ medium (open bars) or for 60 min in Hanks’ medium plus 30 min in isotonic medium containing 60 mmol K+/l (hatched bars). Values are means ± s.d. *P < 0.05 compared with castrated rats (one-way analysis of variance).

**DISCUSSION**

In the posterior pituitary, the effect of castration was to increase consistently the concentrations of both TRH-Gly and TRH (Tables 1 and 2). The TRH content of the posterior pituitary was returned to the normal range by testosterone replacement (Fig. 4). In-vitro release of TRH by hypothalamic fragments was decreased at 7 days after castration compared with sham-castrated and castrated rats receiving testosterone replacement (Fig. 5). The TRH content of the hypothalamic fragments did not differ between treatment groups (Tables 1 and 2) because most of the TRH-containing neurones of the hypothalamus synapse with neurones in other areas of the brain rather than the portal vessels, and presumably mediate autonomic rather than neuroendocrine processes (Griffiths, 1985; Reichlin, 1986). Since posterior pituitary TRH and TRH-Gly occur in neurones originating in the hypothalamus, the proportional increase in TRH and TRH-Gly in the posterior pituitary (Tables 1 and 2) concomitant with an inhibition of in-vitro hypothalamic secretion following castration (Fig. 5) is consistent with a decrease in TRH release and a resulting accumulation of TRH and its precursors. A similar relationship has previously been noted for TRH content and secretion by pancreatic islets in response to glucose addition to the incubation medium (Dolva, Nielsen, Welinder & Hanssen, 1983). The up-regulation of TRH receptors in the anterior pituitary of neonatally castrated male rats (Watanobi & Takebe, 1987) is also consistent with a castration-induced inhibition of hypothalamic TRH release. A simultaneous stimulation of preproTRH biosynthesis and/or decrease in its metabolism however, cannot be excluded.

The highest concentrations of TRH in the rat occur in the posterior pituitary, hypothalamus, ventral prostate and β cells of the pancreas (Jackson, 1982; Pekary, Sharp, Briggs et al. 1983; Bhasin, Pekary, Hershman & Swerdloff, 1984; Simard et al. 1989a,b). TRH within the paraventricular nucleus of the hypothalamus is secreted into the portal circulation of the median eminence, which then stimulates release of thyrotrophin and prolactin (Griffiths, 1985; Hershman & Pekary, 1985). Evidence for a regulatory role for TRH on insulin, glucagon and somatostatin secretion by the β, α and δ cells of the islets of Langerhans respectively, has recently been reported (Vara & Tamararit-Rodrigues, 1988). The function of TRH in the posterior pituitary and the ventral prostate, on the other hand, is not known definitively. Indirect evidence for its regulation of anterior pituitary hormone secretion is accumulating, as discussed below.

The existence of a short portal vessel drainage system which carries blood from the posterior to the anterior lobe of the pituitary (Halasz, 1985) suggests the possibility that TRH release by the posterior lobe affects anterior pituitary function. The stimulation of prolactin release by i.v. TRH, but the inhibition of prolactin release by intraventricular TRH due to stimulation of dopamine secretion (Ohta, Kato, Matsushita et al. 1985), and the absence of a prolactin rise in suckling rats after posterior pituitary removal
(Murai & Ben-Jonathan, 1987) suggest a physiological role for TRH secretion from the posterior pituitary. Recent studies showing a significant increase in LH but not FSH following i.v. TRH in the follicular and luteal phases of the menstrual cycle (Colon, Lessing, Yavetz et al. 1988) and the results of Fig. 5 demonstrating a significant increase in hypothalamic TRH release in castrated rats receiving testosterone replacement compared with hypogonadal animals are also consistent with this possibility. Chen & Walfish (1979) have reported that castration and testosterone replacement have significant effects on the pituitary-thyroid system of the rat.

The TRH-Gly-IR peaks observed in the HPLC profiles of the anterior pituitary (Fig. 2c) decrease while the total TRH-Gly-IR in the posterior pituitary (Table 2) and many of the TRH-Gly-IR peaks in the HPLC profiles of Fig. 2d increase during the 7 days following castration. TRH biosynthesis has recently been demonstrated to occur in the anterior pituitary within a minor, non-thyrotropic, multihormone-containing cell population (May, Wilber, U'Prichard & Childs, 1987). The TRH-Gly-IR in the rat anterior pituitary is most likely to be of endogenous origin because it consists of TRH precursor peptides for anterior pituitary TRH biosynthesis; the TRH-Gly-IR content of the hypothalamus and therefore the probable hypothalamic secretion rate for this peptide should be insignificant compared with TRH (Glembotski et al. 1986; Simard et al. 1989a). The processing of preproTRH to form peptides of intermediate length is very different for tissues of hypothalamic and extrahypothalamic origin (Cockle, Morrell & Smyth, 1989). The divergent effects of testosterone on preproTRH processing in the anterior and posterior pituitary, evident in Fig. 2, suggest the influence of steroid hormones on TRH biosynthesis may be complex and highly variable among the known TRH-biosynthesizing tissues (Pekary et al. 1989).

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**REFERENCES**


