Thyrotropin-releasing hormone regulates the diurnal variation of pyroglutamyl aminopeptidase II activity in the male rat adenohypophysis

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Abstract

Objective: Thyrotropin-releasing hormone (TRH) is inactivated in the extracellular compartment by pyroglutamyl aminopeptidase II (PPII), a narrow specificity ectopeptidase present in the brain and in the lactotrophs of the adenohypophysis. TRH and various hypothalamic/paracrine agents regulate the activity of PPII on the surface of adenohypophyseal cells in primary culture. The activity of the hypothalamic–pituitary–thyroid axis presents circadian variations including an increase of serum thyrotropin levels in the early hours of the day. The purpose of this study was to determine whether adenohypophyseal PPII activity fluctuates during the daytime in the male rat and the role of TRH in these regulatory events in vivo.

Results: Adenohypophyseal PPII specific activity and mRNA levels presented diurnal variations. A decrease in specific activity occurred with a minimum between 0930 and 1130 h, associated with increased serum thyrotropin levels. PPII mRNA levels were lowest at 0800 h. Intraperitoneal injection at 0800 or 1000 h of [3-Me-His2]-TRH, a potent agonist of the TRH receptor, reduced PPII specific activity at 30 min post-injection which was followed by a return to basal levels at 2 h. A second phase of decrease occurred between 4 and 8 h post-injection. Intravenous injection of a TRH-immune serum induced, at 2 h post-injection, an increase in adenohypophyseal PPII specific activity, which lasted up to 6 h.

Conclusions: Adenohypophyseal PPII activity and mRNA levels fluctuate during the day; TRH down-regulates PPII activity in vivo, contributing to some of these variations. These new findings, and previous data, suggest that adenohypophyseal PPII activity varies in distinct physiological events, in response to endocrine and hypothalamic/paracrine factors, potentially modulating responses to TRH.
Apart from these relatively slow effects, PPII activity can be altered in minutes by various treatments, in primary cultures of female adenohypophyseal cells. Combining dopamine withdrawal with the addition of TRH causes a transient enhancement of PPII activity (16) while arachidonic acid mobilization leads to a reversible down-regulation (17). TRH alone produces a rapid decrease (authors, unpublished observations). The mechanisms underlying these rapid responses have not been investigated; they are likely a consequence of the activity of transduction pathways but not a direct effect of extracellular messengers on the enzyme.

Indirect evidence suggests that the secretory activity of the TRH neurons of the paraventricular nucleus of hypothalamus (PVN) is circadian. In male rats, one peak of TRH mRNA levels occurs in the morning, another at night (18) and TRH levels in the median eminence exhibit 24-h variations (19). Whether these variations are associated with phasic secretion of TRH in portal blood is unknown; however, circadian fluctuations of TRH and TRH mRNA levels are associated with circadian fluctuations of TRH release in hypothalamic slices (20), suggesting that the variations in levels of PVN TRH mRNA and median eminence TRH indicate fluctuations in TRH release in portal blood. It is reasonable to assume that a circadian rhythm of secretion occurs, contributing to that of serum thyrotropin (TSH) levels observed in male rats (peak at the beginning of the day) (21, 22).

Our purpose was to determine: (a) whether the diurnal variability of the hypothalamic–pituitary–thyroid axis is paralleled by a rhythm of adenohypophyseal PPII activity and (b) TRH involvement in the control of PPII activity in vivo. We focused on the diurnal part of the circadian cycle since we expected that hypothalamic events contributing to the morning rise in serum TSH levels could regulate PPII activity.

Materials and methods

Reagents

[3-Me-His²]-TRH (3MeTRH) was purchased from Peninsula Laboratories (Belmont, CA, USA); M-MLV reverse transcriptase was from Gibco BRL (Grand Island, NY, USA); Taq DNA polymerase from Biotechnology Universities (DF, Mexico); oligo-dT15 from Boehringer Mannheim; agarose from Bio-Rad Laboratories (Hercules, CA, USA); TRH-β-naphthylamide (TRH-βNA) from Bachem Bioscience Inc. (King of Prussia, PA, USA); and solvents were from J T Baker (DF, Mexico). All other reagents (including deoxy-nucleotides) were from Sigma (St Louis, MO, USA). Dipeptidyl aminopeptidase IV (DAPIV) was purified from goat cortical kidney according to Yoshimoto & Walter (23). TRH-immune serum (serum code r1) was prepared and characterized as described (24). Its specificity has been reported previously (25); it did not cross-react with pglu-his-pro-OH, pglu-his, pglu-met-pro-NH₂, his-pro-NH₂, pro, his, glu, gln, luteinizing hormone-releasing hormone and α-melanocyte-stimulating hormone, each tested up to 5 ng/tube.

Animals

Male Wistar rats (80 – 100 days old, 260 – 300 g) were used in all experiments. Rats kept (4 – 5 animals/cage per group) under controlled lighting conditions (lights on from 0700 to 1900 h) and fed ad libitum were killed by decapitation. The Society for Neuroscience (USA) ‘Guidelines for the Use of Animals in Neuroscience Research’ were followed.

Experimental procedures

Diurnal variations

Animals were killed between 0500 and 2330 h. Adenohypophyses and serum were stored at −80 °C until adenohypophyseal PPII mRNA levels or specific activity and serum TSH concentration were determined.

3MeTRH treatments

3MeTRH (50 μg/kg) in 300 μl 0.9% NaCl or vehicle was injected i.p. at 0800 or 1030 or 1500 h. In some experiments, an absolute control (intact animals) was also used. Animals were killed at the times indicated. Adenohypophyseal PPII specific activity and serum TSH levels were determined.

Normal or TRH-immune serum injection

Rats were anesthetized with an s.c. injection of ketamine (100 mg/kg), acepromazine (4 mg/kg) and xylazine (10 mg/kg) and a catheter was aseptically inserted in the right jugular vein 3 days before the experiment. Each animal was housed in an individual cage until the experiment. TRH-immune or normal serum (100 μl) was administered through the catheter at 0800 h and animals were killed after 2, 4 or 6 h. A third group of animals (intact control) did not undergo anesthesia or catheter insertion. Adenohypophyseal PPII specific activity and serum TSH levels were determined.

TSH radioimmunoassay (RIA)

Serum TSH was analyzed by RIA using reagents and protocol provided by the National Hormone and Pituitary Program (NIDDK, Bethesda, MD, USA).

PPII activity

PPII activity was measured in membranes obtained from each adenohypophysis. Adenohypophyses were sonicated twice for 15 s in 0.5 ml NaPO₄ (50 mM) buffer, pH 7.5 (buffer A) and the homogenate was centrifugated at 1000g for 15 min. The pellet was
resuspended in 0.25 ml buffer A and centrifuged as above. Both supernatants were pooled and centrifuged for 15 min at 14,000 g; the pellet was resuspended once with buffer A–1 M NaCl, centrifuged again, resuspended with buffer A and centrifuged. The pellet was finally resuspended in buffer A. Membrane PPII activity was determined with the substrate TRH-BNA in a coupled assay in the presence of excess DAPIV as previously described (13). Activity was linear with time and refers to membrane protein content.

**PPII RNA quantification**

Each adenohypophysis was homogenized in 400 µl lysis buffer; total RNA was purified according to Chomczynski & Sacchi (26). RT-PCR was performed as previously described (13) (using glyceraldehyde-3-phosphate dehydrogenase (G3PDH) cDNA as an internal control which was co-amplified for 30 cycles with PPII cDNA) except that reverse transcription was for 2 h and cDNA bands stained with ethidium bromide were quantified with a Fluor-S-Imager (Bio-Rad). These amplification conditions generate semi-quantitative results consistent with Northern analyses (13). PPII cDNA/G3PDH cDNA ratio is an estimate of PPII mRNA levels relative to those of G3PDH. This ratio was taken as an estimate of TRH mRNA levels since G3PDH cDNA signal per µg total RNA did not change significantly during the diurnal phase of the circadian cycle (not shown).

**Statistical analyses**

Data generally represent the mean±S.E.M. values for each experiment (n = 4 or 5), except for data in Fig. 3 (injection at 0800 h) in which data from three

![Figure 1](image_url) Adenohypophyseal PPII specific activity and serum TSH levels fluctuated during the day. Animals were killed between 0800 and 2330 h. Each panel corresponds to an independent experiment. In (B), rats were injected with vehicle at 0800 h; vehicle injection had no significant effect on serum TSH levels and adenohypophyseal PPII specific activity (see text). Data are the mean PPII specific activity±S.E.M. or the mean serum TSH levels±S.E.M. Some S.E.M. values were smaller than the size of the symbol. Data were analyzed by one-way ANOVA followed by Duncan’s multiple range test. ANOVA analyses showed significant differences (P < 0.05) for PPII specific activity (PPII-S.A.) in (A), P < 0.001 for PPII-S.A. in (B) and for serum TSH levels in (A) and (B). Duncan’s multiple range test results: *P < 0.05, ***P < 0.01. (A) PPII-S.A. data compared with the 0930 h time-point; serum TSH levels data compared with the 1100 h time-point; (B) PPII-S.A.: 0830 h data compared with all time-points; 1000, 1130 and 1930 h compared with 1530 h time-point; serum TSH level: 1000 h data compared with all time-points.
independent experiments were pooled. Data in Figs 1 and 2 were analyzed by one-way ANOVA followed by Duncan’s multiple range test to determine statistical significance between individual means. For the other data, individual means of controls and treatment groups were compared using Student’s t-test. Differences were considered to be significant at $P < 0.05$ or less.

## Results

In a first series of experiments we analyzed adenohypophysial PPII specific activity and mRNA levels, and serum TSH levels, during the diurnal phase of the circadian cycle. Serum TSH levels increased in the morning with a peak between 0930 and 1300 h (Fig. 1); a peak of serum TSH levels was also observed in two other independent experiments either at 0800 or 1000 h (not shown). PPII specific activity fluctuated during the day. While the patterns of activity varied somewhat between experiments, reproducible trends were observed: a decrease during the morning hours with a minimum between 0930 h and 1130 h and maxima at 0630–0800 h and 1300–1530 h (Figs 1 and 2B). In another independent experiment, PPII specific activity fluctuated with a minimum at 0930 h and maximum at 0800 h (data not shown). The morning drop in PPII specific activity was concomitant with, or preceded by, the increase in serum TSH (Fig. 1). Relative PPII mRNA levels had a minimum at 0800 h and maximum at 0630 h and at/or after 1730 h (Figs 1 and 2B). This pattern was similar, in part, to that observed for PPII specific activity. Minimum PPII mRNA levels preceded that of PPII specific activity by 0130–0300 h; although a discrepancy was observed in the late afternoon when specific activity was stable while mRNA levels were increasing (Fig. 2).
Since the main stimulator of TSH secretion is TRH, a regulator of PPII activity in vitro, the correlation between serum TSH levels and PPII activity suggested that release of endogenous TRH may contribute to down-regulation of PPII specific activity in the morning. To test this hypothesis, we analyzed the effect of an injection of 3MeTRH, a high-affinity agonist of the TRH receptor. Injected at 0800 h, the vehicle had no significant effect on PPII specific activity or serum TSH levels 30 min or 3 h after its injection (PPII: 30 min, 4.19 ± 2.83 (vehicle) vs 39.69 ± 8.71 (vehicle) pmol βNA/min per mg protein (n = 4); 3 h, not shown; TSH: 30 min, 0.93 ± 0.16 (intact) vs 1.74 ± 1.06 (vehicle) ng TSH/ml serum (n = 4); 3 h, not shown). 3MeTRH induced a significant increase in serum TSH levels at 30 min (vehicle: 2.46 ± 0.47; 3MeTRH: 19.9 ± 4.84 ng/ml; n = 4; P < 0.02); values returned to vehicle levels from 2 h (vehicle: 5.87 ± 0.44; 3MeTRH: 5.74 ± 2.09 ng/ml). 3MeTRH rapidly decreased PPII specific activity (30 min after injection), with values recovering to control at 2 h, further decreasing between 4 and 8 h and normalizing at latter times. This pattern was observed in three independent experiments. The effect of 3MeTRH was observed if injected at 0800 or 1000 h but not at 1500 h (Fig. 3).

To determine the role of endogenous TRH on PPII activity, TRH-immune or normal serum was injected. TRH immuno-neutralization decreased serum TSH values (13–43% decrease compared with control vehicle values; not shown) and increased PPII specific activity by 90% 2 h after injection, with the effect still significant at 6 h post-injection (Fig. 4).

**Discussion**

TRH has short- and long-term inhibitory effects on PPII activity in primary cultures of adenohypophyseal cells; the long-term effect probably due to regulation of PPII mRNA levels (12, 13) (authors, unpublished observations). In this study, we have shown that PPII specific activity and mRNA levels fluctuated during the day and that the injection of 3MeTRH transiently reduced PPII activity but the sensitivity to 3MeTRH was lost in the afternoon. Finally, immuno-neutralization of endogenous TRH led to an increase in PPII specific activity.

Adenohypophyseal PPII activity varies in vivo during the estrous cycle and in response to modifications of estrogen or TH levels (5–7). Our results show that PPII activity changed during the day although the pattern of serum TSH levels, adenohypophyseal PPII activity or PPII mRNA levels differed between experiments probably due to the variation in pituitary–thyroid function that occurs even within and among animals living in a common controlled environment (27). Reproducible trends were however detected: maximum serum TSH levels occurred prior to, or concomitant with, minimum PPII specific activity (0930–1130 h); the nadir in activity was preceded by that of PPII mRNA levels. To our knowledge, this is the first evidence that a diurnal variation in the physiologically relevant mechanism of peptide inactivation occurs in one of its target organs.

Many adenohypophyseal (including TSH and prolactin) and peripheral hormone serum levels present a circadian cycle. Reports are inconclusive with regard to serum TH levels in male rats, suggesting either none or low-amplitude fluctuations with lowest levels at night (22, 28). Since THs positively regulate PPII activity by a direct effect on the adenohypophysis (6, 8), TH fluctuations could contribute to the diurnal variations in PPII activity.

Our data demonstrate that TRH, acting directly on the adenohypophysis, is a major regulator of PPII activity during diurnal variation. This conclusion stems from (a) the correlation between increased serum TSH levels and drop of PPII activity, (b) the rapid effect of exogenous 3MeTRH injected either at 0800 or 1000 h, (c) the effect of the TRH immuno-neutralization on PPII activity and (d) the effect of TRH in primary cultures of adenohypophyses. This is the first demonstration that a hypothalamic/paracrine factor regulates the activity of its inactivating peptidase in vivo in the adenohypophysis. These effects are likely due to transduction through the TRH receptor, as demonstrated for the long-term effects in vitro (12,
14), most probably by TRH released from the hypothalamus although local release from the adenohypophysis cannot be excluded (29).

3MeTRH was not effective if injected in the afternoon. To our knowledge, a diurnal variation of sensitivity to TRH has not been reported in the rat; however, a peak of serum TSH levels occurs at night in humans (30) and the TRH response in the healthy male is more efficient in the morning than in the afternoon (31). This could be due to a transitory down-regulation of TRH receptor.

The kinetics of the initial drop of PPII specific activity in response to 3MeTRH was similar to that observed in vitro (authors, unpublished observations). The molecular mechanisms leading to this rapid decrease are unknown: a down-regulation of surface PPII initiated by enzyme phosphorylation (32), followed by internalization and degradation, as described for endopeptidase 24.11 (33) may occur. The second decrease could be due to TRH-induced down-regulation of PPII mRNA, as observed in vitro (13).

During the morning, the minimum specific activity was preceded by a minimum in PPII mRNA levels, suggesting that changes in mRNA levels drive the activity changes. This is consistent with TRH control of PPII mRNA levels as a major contributor to the long-term (hours) drop in PPII activity observed in vitro (13). Since this correlation was lost during the afternoon, translational or post-translational regulation of PPII production, or increased degradation, may compensate for the enhanced levels of PPII mRNA.

In conclusion, we have shown diurnal variations of adenohypophyseal PPII activity and PPII mRNA levels. Furthermore, the correlation between the nadir of adenohypophyseal PPII specific activity and the peak of serum TSH levels, the effects of exogenous 3MeTRH and of TRH immuno-neutralization demonstrate that endogenous TRH participates in adenohypophyseal PPII activity control during the day. Various physiological circumstances, in addition to the estrous cycle and the time of day, might influence PPII activity on the surface of lactotrophs in vivo, through the action of hormones and/or hypothalamic/paracrine factors. These activity fluctuations should have a local effect, i.e. on TRH potency in the adenohypophysis, which in turn may contribute to adjust the time-course or intensity of adenohypophyseal hormone secretion in response to TRH according to previous hypothalamic/paracrine and endocrine activities.

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References

TRH degradation in adenohypophysis


9 Schomburg I & Bauer K. Thyroid hormones rapidly and stringently regulate the messenger RNA levels of the thyrotropin-releasing hormone (TRH) receptor and the TRH-degrading ectoenzyme. Endocrinology 1995 136 3480–3485.


18 Zoeller RT, Kabeer N & Alberts HE. Cold exposure elevates cellular levels of messenger ribonucleic acid encoding thyrotropin-releasing hormone in paraventricular nucleus despite elevated levels of thyroid hormones. Endocrinology 1990 127 2955–2962.


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