Fasting enhances pyroglutamyl peptidase II activity in tanycytes of the mediobasal hypothalamus of male adult rats

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Fasting down-regulates the hypothalamus-pituitary-thyroid (HPT) axis activity through a reduction of thyrotropin releasing hormone (TRH) synthesis in neurons of the parvocellular paraventricular nucleus of the hypothalamus (PVN). These TRH neurons project to the median eminence (ME), where TRH terminals are close to the cytoplasmic extensions of β2 tanycytes. Tanycytes express pyroglutamyl peptidase II (PPII), the TRH-degrading ectoenzyme that controls the amount of TRH that reaches the anterior pituitary. We tested the hypothesis that regulation of ME PPII activity is another mechanism by which fasting affects the activity of the HPT axis. Semi-quantitative *in situ* hybridization histochemistry data indicated that PPII and deiodinase 2 mRNA levels increased in tanycytes after 48 h of fasting. This increase was transitory, followed by an increase of PPII activity in the ME, and a partial reversion of the reduction in PVN pro-TRH mRNA levels and the number of TRH neurons detected by immunohistochemistry. In fed animals, adrenalectomy and corticosterone treatment did not change ME PPII activity 72 h hour later. Methimazole-induced hypothyroidism produced a profound drop in tanycytes PPII mRNA levels, which was reverted by 3 days of treatment with T4. The activity of thyroliberinase, the serum isoform of PPII, was increased at most fasting time points studied. We conclude that delayed increases in both the ME PPII as well as the thyroliberinase activities in fasted male rats may facilitate the maintenance of the deep down-regulation of the HPT axis function, in spite of a partial reactivation of TRH expression in the PVN.

The brain of vertebrates detects adverse environmental changes, as food deficit. Homeostatic responses to food restriction or fasting lead to adjustments in the activity of the neuroendocrine axes, which control growth and energy metabolism. During energy deficits, the hypothalamic-pituitary-thyroid (HPT) axis is regulated through multiple tissue-specific changes (1). In the paraventricular nucleus of the hypothalamus (PVN), food restriction or fasting decrease the expression of prothyrotropin releasing hormone (pro-TRH) and of PC1/3 and PC2, convertases that contribute to pro-TRH processing into mature peptide (2–7). The concentration of TRH in both the median eminence (ME) and the hypothalamic portal blood, as well as the level of β-TSH mRNA in anterior pituitary, and the concentration of TSH in serum decrease during fasting (2, 5, 8, 9, 10). A negative energy balance is associated with decreased liver deiodinase 1 (D1) mRNA levels and activity (11, 12, 13), and increased catabolism of T4 (14). These events lead to a dramatic reduction of serum triiodothyronine (T3) and T4 concen-

Abbreviations:

trations, which contribute to reducing energy expenditure when food reserves are low (8, 9).

Down regulation of both TRH biosynthesis and release during fasting likely contributes to the decrease of TRH concentration in the hypothalamic portal blood, but TRH hydrolysis in the extracellular space likely adds another level of control. TRH is hydrolyzed in vitro by pyroglutamyl peptidase II (PPII; EC 3.4.19.6; TRH-degrading ectoenzyme), a narrow specificity ectoenzyme (15). PPII is found in various brain regions, including the mediobasal hypothalamus (MBH) (16, 17). In the MBH, PPII is expressed in all tanycyte subtypes (17), a specialized ependymal cell type that forms the central and ventral parts of the wall of the third ventricle. While $\alpha1$ and $\alpha2$ tanycyte subtypes send basal processes into the dorsomedial and ventromedial nuclei, the $\beta1$ and $\beta2$ tanycyte subtypes are localized ventrally, and the $\beta2$ tanycytes project basal processes to the external layer of the ME (18). Functional evidence suggests PPII regulates TRH concentration in the ME extracellular space, before TRH reaches the anterior pituitary, and thus thyrotropin levels in serum (17). The expression and activity of PPII in the ME are up-regulated by thyroid hormones; these changes are faster than those operating on TRH synthesis in the PVN, and may contribute to the negative feedback that adjusts HPT axis activity (17, 19).

PPII is also expressed in the anterior pituitary, where its activity is regulated by multiple hypothalamic and peripheral factors, including thyroid hormones that up-regulate it (15, 20). In this tissue, PPII is not localized to thyrotrophs, and functional studies in cell culture indicate that it controls TRH effect on prolactin secretion, but not TRH effect on TSH secretion (21, 22). Additionally, a soluble PPII isoform, called thyroliberinase, is produced by the liver (23), and may hydrolyze circulating TRH. Thyroliberinase activity is up-regulated by thyroid hormones (24, 25), but the impact of negative energy balance conditions on the activity of this enzyme is unknown.

The current study aimed to test the hypothesis that PPII expression and activity in the ME of adult male rats is adjusted during fasting, as an additional mechanism to regulate HPT axis activity. The sensitivity of ME PPII expression and activity to thyroid and adrenal status was analyzed in fed animals. Additionally, we compared the dynamics of ME PPII expression and activity with pro-TRH synthesis in the PVN of fasted animals, and explored the effect of fasting on the activity of PPII in the anterior pituitary, serum and the parietal cortex.

Materials and Methods

Animals

Adult male Wistar rats were obtained from the animal care facility of Instituto de Biotecnología, UNAM, México, except that for experiment 5 Wistar rats were purchased from Harlan Laboratories (Mexico), and for experiment 7 we used Sprague-Dawley rats generated at the animal care facility of the Multidisciplinary Institute of Cell Biology (IMBICE). Before experiments, animals were acclimatized for at least 5–10 days to a 12-hour light/12-hour dark cycle (lights between 07:00 and 19:00 hours) in a room with controlled temperature (22 ± 1°C) and humidity. Rat chow (Harlan 2018SX) and tap water were provided ad libitum.

Experimental protocols

Experiment 1. Rats (400–450 g, age 120–130 days, n = 5 in each group) were isolated in individual cages for either 48 or 72 hours and allowed free access to food and water (control animals), or isolated and deprived of food for either 48 or 72 hours with water ad libitum (experimental animals). Between 9–11 AM, animals were overdosed with pentobarbital (50 mg/kg ip), blood withdrawn from aorta, and perfused transcardially with 20 ml 0.01 M PBS (pH 7.4) containing 15 000 U/liter heparin sulfate, followed by 150 ml 4% paraformaldehyde in PBS. The brains were rapidly removed and postfixed by immersion in the same fixative for 2 hours at room temperature. Brain tissue was cryo-protected in 25% sucrose in PBS at 4°C overnight, snap frozen on dry ice, and stored at –70°C. Since data were identical in control animals isolated for either 48 or 72 hours, data from both groups were pooled.

Experiment 2. Rats, raised 2 per cage since weaning, were isolated 5 days before initiation of fasting. Animals (300–350 g, age 78 days, n = 4 in each group) were separated in 2 experimental groups, either maintained with free access to food and water, or deprived of food for 48 hours with water ad libitum. Between 9–12 AM, animals were overdosed with pentobarbital, blood withdrawn, and brain tissue prepared as described for experiment 1.

Experiments 3 and 4. Rat weight: 250–300 g, ages 65 or 75 days for experiments 3 or 4 respectively; n = 5 in each group. Control animals were isolated for either 36, 48, 60 or 72 hours and allowed free access to food and water, whereas experimental animals were isolated and deprived of food for either 36, 48, 60 or 72h with water ad libitum. Finally, animals were euthanized by decapitation between 9–12 AM, brain, anterior pituitary and liver removed, and tissues frozen on dry ice; trunk blood was collected for hormone and metabolite dosage. A brain coronal slice (bregma –2.12 to –3.6 mm) (26) was used to dissect the ME with a sample corer (0.5 mm internal diameter; Fine Science Tools, Foster City, CA) centered between the base of the brain as the ventral limit and the floor of third ventricle as a dorsal limit. The same slice and sample corer were used to obtain parietal cortex (area 1) samples from the right and left hemispheres.

Experiment 5. Between 9–12 AM rats (270–330 g, age 70 days, n = 6 in sham and 40% corticosterone / 60% cholesterol group, and n = 5 for the adrenalectomized and 25% corticosterone / 75% cholesterol groups) were either sham operated, or bilaterally adrenalectomized under ketamine 100 mg/Kg and xylazine 10 mg/Kg anesthesia and implanted with a subcutaneous corticosterone pellet (27, 28). The pellets were made of either cholesterol, or 25% corticosterone/75% cholesterol, or 40% corticosterone/ 60% cholesterol (percentages of w/w). Animals were maintained 2 per cage with free access to food, and either water (sham) or 0.5% saline solution (adrenalectomized rats).

Animals were sacrificed 3 days later, and tissue and blood collected as described for experiments 3 and 4.

Experiment 6. Rats (200–300 g, age 60–75 days) were separated in five groups: control (intact, n = 3, vehicle (200 μl sterile saline/ 0.01 N NaOH per 3 days beginning on day 19, n = 1, hypothyroid (0.1% methimazole + 1% sodium perchlorate in their drinking water per 21 days, n = 2), hypothyroid + vehicle (200 μl per 3 days beginning on day 19, n = 3) and hypothyroid + L-T4 (10 μg in 200 μl vehicle per 3 days beginning on day 19, n = 3). Rats were perfused transcardially 6 hours after the last injection and brain tissue prepared as described for experiment 1. Blood was collected from the aorta prior to infusion. Because

of the small number of animals, data from control and vehicle groups were pooled; the same applied for hypothyroid and hypothyroid plus vehicle groups.

Experiment 7. Rats (250–300 g, age 60–80 days, n = 3 in each group) were isolated and separated in three experimental groups. Rats were either maintained with free access to food and water or deprived of food for either 48 or 72h with water ad libitum. On the morning of the experimental day, animals were perfused with formalin as previously described (29). Brains were removed, postfixed, immersed in 20% sucrose.
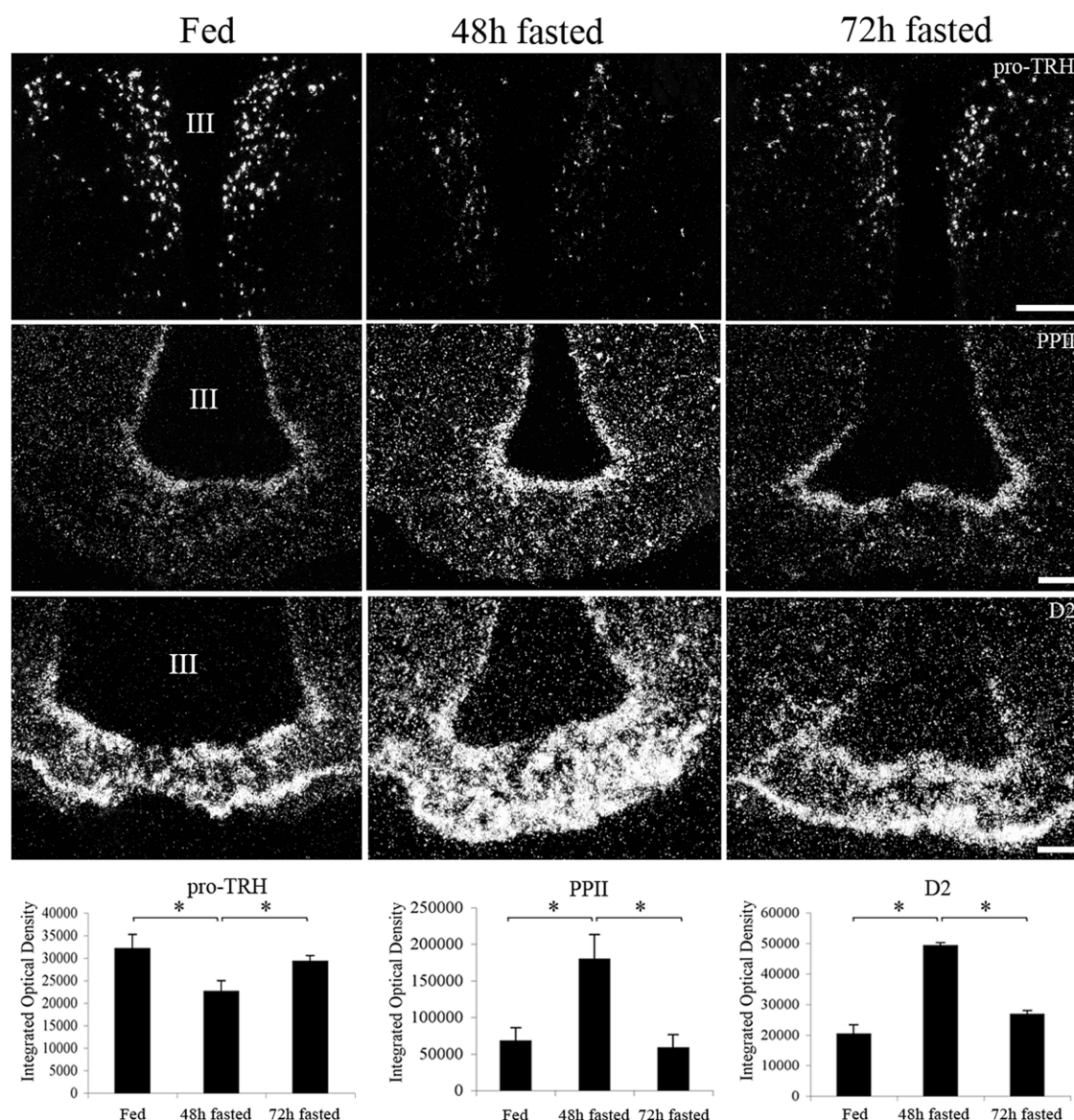

Figure 1. Effect of fasting on pro-TRH, D2 and PPII mRNA expression in mediobasal hypothalamus; experiment 1. mRNAs were detected by in situ hybridization with [^{35}S]-labeled cRNA probes; sections viewed at 5X under dark-field illumination. Upper row: photomicrographs of coronal slices through the medial PVN hybridized with a pro-TRH mRNA probe; left: control; middle: rats submitted to 48 hours of fasting; right: rats submitted to 72 hours of fasting. Second row: Photomicrographs of coronal slices through the mediobasal hypothalamus hybridized with a PPII mRNA probe. Third row: Photomicrographs of coronal slices through the mediobasal hypothalamus hybridized with a D2 mRNA probe. Lower panels: the graphs show the densitometric analyses of the in situ hybridization autoradiograms. Data were analyzed by one way ANOVA and post hoc Student-Newman-Keuls multiple comparisons test. Data are mean ± S.E.M. Pro-TRH: n = 3–5; D2: n = 3–5; PPII: n = 4–5. *: $P < .05$, **: $P < .01$. III = third ventricle, scale bar = 100 μm.

In situ hybridization (ISH) and image analysis

Serial 18-μm coronal sections through the rostrocaudal extent of the ME were cut on a cryostat (OTF model, BRIGHT Instruments Company Ltd, Huntingdon, UK), adhered to Superfrost/Plus slides (Fisher Scientific, Pittsburgh, PA), desiccated overnight at 42°C, and stored at -80°C until prepared for in situ hybridization. Every fourth section of the ME (bregma -2.56 to -3.14 mm) was hybridized with either a 644-bp single stranded [^{35}S]-UTP labeled RNA probe complementary to the coding region of the rat PPII gene (nucleotides $129-773$) (17), or a 800-bp single stranded [^{35}S] UTP labeled cRNA probe complementary to the entire coding region of the rat deiodinase 2 (D2) gene (30). Sections at PVN level (mid and caudal, bregma -1.56 to -2.04) were hybridized with a 1241-bp single stranded [^{35}S] UTP labeled cRNA probe complementary to the rat pro-TRH (31). Hybridization was performed as previously described (17). Slides were dipped into emulsion, and the autoradiograms developed after 4 (pro-TRH), 11 (D2), 33 or 38 days (PPII) of exposure at 4°C.

Silver grains were visualized under dark field illumination with a 5X objective (Zeiss Axioscop microscope) and a CCD Sony video camera. Positive areas were delineated, and integrated density values (density x area) were measured in each section using Mercator Explora Nova imaging software (La Rochelle, France). For each ISH trial, the sum of the integrated density values of up to six slices/animal was calculated and taken as one determination.

The anatomical distribution of hybridization data indicated that results were specific for the target mRNAs (Figures 1 and 4). Pro-TRH mRNA signal was high in the PVN, in a distribution pattern consistent with published studies (32). In the mediobasal hypothalamus PPII mRNA was detected along the medial and ventral parts of the border of the third ventricle wall, within the ME and, with much lower concentration in the surrounding tissue, consistent with previous results (16, 17). D2 mRNA coincided with the distribution of tanycytes and was also detected in the substance and external layer of the median eminence (33).

Immunocytochemistry and image analysis

Twenty-five μm coronal sections were used to perform pro-TRH staining (29). Briefly, brain sections were incubated overnight with an anti-pro-TRH antibody (1:3,000). Rabbit anti-pro-TRH antibody was generated against C-terminal prepro-TRH$_{239-255}$ sequence (KQSPQVEPWDKEPLEE) plus a tyrosine added at the N-terminal end (34). Sections were treated with a biotinylated donkey antirabbit antibody and with Vectastain Elite ABC kit. Visible signal was developed with a 3–3′-diaminobenzidine/nickel solution. Immunostaining of negative control, which did not show any antiserum immunolabeling, included sequential elimination of either the primary or secondary antibody from the staining procedure. Bright-field images were acquired with an Eclipse 50i microscope and a DS-Ri1 digital camera. The software program Adobe PhotoShop 7.0 was used to combine the photomicrographs into plates and adjust levels, contrast and brightness in the images. To determine the total number of pro-TRH-immunoreactive (IR) cells in the medial PVN, cells containing distinct black/purple precipitate were quantified in one out of three complete series of coronal brain sections. For analysis, the pro-TRH-IR cells of the PVN were subdivided into parvo and magnocellular subregions (35) and estimated in the parvocellular subregion of the mid level of the PVN ranging between bregma -1.60 and -1.88 mm. Data were corrected for double counting, according to the method of Abercrombie (36), and expressed as the number of pro-TRH-IR cells per coronal section. Blind quantitative analysis was performed independently by two observers.

Measurement of PPII, and thyroliberinase activities

PPII activity was determined as described (37). Membranes were collected by centrifugation. Protein concentration was determined by the Bradford assay. PPII and thyroliberinase activities were determined using TRH-βNA as substrate in a coupled assay with excess dipeptidyl aminopeptidase IV (EC 3.4.14.5) and inhibitors of pyroglutamyl peptidase I (EC 3.4.19.3), and of prolylendopeptidase (EC 3.4.21.26). Enzymatic assays were performed at 37°C under initial velocity conditions. In agreement with previous data (17), in control animals PPII specific activity was higher in the ME than in the anterior pituitary (experiment 3; control ME = 48.8 ± 2 pmoles βNA/min/mg protein; control anterior pituitary = 10.9 ± 0.5; control parietal cortex = 80.4 ± 5.8. Differences between groups were significant (n = 19; $P < .0001$; ANOVA, followed by Student-Newman-Keuls multiple comparisons test). Since expression and activity drastically diminish from the third ventricle wall to the brain parenchyma (17), the activity of ME PPII was not standardized per mg protein, but reported by structure, to minimize variation due to dissection.
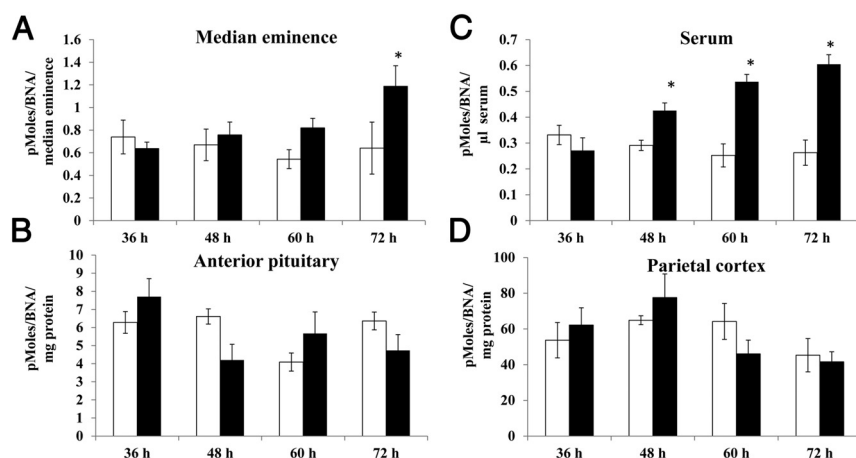

Figure 2. Effect of fasting on activity of PPII in ME, parietal cortex, anterior pituitary and serum; experiment 3. The graphs show activity or specific activity of PPII in control animals and fasted animals between 36–72 hours. Fasting increased PPII activity in the ME (A). Fasting did not change specific activity of anterior pituitary or parietal cortex PPII (B, D). Fasting enhanced thyroliberinase activity (C). White bars (control); black bars (fasted). Values are pmoles product per min per tissue (A), or per mg protein (B, D), or per μL of serum (C). Data were analyzed by one way ANOVA and post hoc Student-Newman-Keuls multiple comparisons test. Data are mean ± S.E.M. Panels A and B: n = 5; Panel C: n = 4–5; Panel D: n = 4–5. *: $P < .05$.

Liver deiodinase 1 activity

D1 activity was determined in liver microsomes (10 μg of protein) with ^{125}I-

rT3 (Perkin Elmer, NEX109) as a substrate (38); the labeled hormone was purified before use by chromatography on Sephadex LH 20. Released ^{125}I was separated by chromatography on Dowex 50WX2, 100–200 mesh, and quantified with a gamma counter. Blank values (without homogenate) were subtracted; proteins were determined with the Bradford assay. Values were linear with time (between 5 and 15 minutes) and protein concentration (between 5 and 10 μg protein). Specific activity values, and the effect of fasting on activity, were consistent with previous studies (11, 12, 13), suggesting that the assay primarily detected D1.

Measurements of hormone concentrations in serum

Serum TSH levels were analyzed by RIA using rNIDDK reagents (Bethesda, MD). Serum corticosterone levels were analyzed by RIA with reagents from Merck-Millipore, Perkin Elmer and Sigma. In experiments 2, 3 and 5, total serum T3 and T4 concentrations were analyzed by ELISA (Diagnóstica Internacional, Méx). In experiment 4, total T3 and T4 concentrations were analyzed by RIA with RK-6CT1 and RK-11CT1 kits (Siemens Diagnostic Products Corporation; Los Angeles, CA) respectively. Serum leptin concentrations were measured with an ELISA kit (Crystal Chem Inc., Downers Grove, IL).

Detection limits: TSH: 25 ng/ml; T4-RIA: 30 nMol/L; T4-ELISA: 16 nMol/L; T3-RIA or -ELISA: 0.3 nMol/L; corticosterone: 25 ng/mL; leptin: 0.2 ng/mL. The intra-assay precision was ≤ 5%, ≤ 10%, and ≤ 4.3%, and the interassay precision was ≤ 9%, ≤ 10%, and ≤ 4.5% for TSH, corticosterone, and leptin respectively. The intra assay precision was < 6.8% for T4-RIA and < 4.3% for T4-ELISA, < 6.0% for T3-RIA and < 9.6% for T3-ELISA. The inter assay precision was < 6.0% for T4-RIA and < 4.5% for T4-ELISA, < 10.6% for T3-RIA and < 10.3% for T3-ELISA. Proteins were determined with the Bradford assay.

Statistical analysis

Results are presented as means ± S.E.M. Statistical significance between control and experimental groups was determined by unpaired t-Test (experiment 2) or one- or two-way ANOVA, followed by Student-Newman-Keuls multiple comparisons test. When data did not pass the normality test, they were reanalyzed after transformation to root square or logarithm base 10. Differences were considered significant at $P < .05$. ANOVA data are shown in Supplemental Table 1. Results of post hoc tests are shown in figures, tables or text. For experiment 1, linear correlation analyses were performed with Sigma Plot software and considered significant when $P \leq .05$ (Supplemental Figure 3).

Results

Fasting increases PPII mRNA expression in the mediobasal hypothalamus, PPII activity in the ME and thyroliberinase activity in serum

To determine whether PPII expression in tanycytes is regulated by fasting, we subjected male rats to either 48 or 72 hours of fasting (experiment 1). Serum TSH levels tended to be lower in fasted than in control animals (control: 2.2 ± 0.9, fasting 48 hours: 1.24 ± 0.14, fasting 72 hours: 0.9 ± 0.2 ng/mL, n = 5). PPII mRNA levels in the ME were increased 2.5 fold at 48 hours of fasting. This increase was transitory since PPII mRNA levels were similar to control values at 72 hours of fasting (Figure 1). The increase in PPII mRNA levels was even more intense, but equally reversed with time, if PPII mRNA expression was quantified in the ME together with the lateral ventricular walls (ie, the region where both β and α tanycytes are found) (not shown). These data suggest that PPII expression was regulated by fasting both in α and β tanycytes.

Since animals were isolated concomitantly with fasting initiation, the results may have been dependent on the stress produced by isolation (39). Thus, male rats accustomed to isolation for 5 days were subjected to 48 hours of fasting in an independent experiment (experiment 2). Body weight gain dropped in fasted animals, and serum TSH, T4 and T3 concentrations were lower in fasted than in control animals (Supplemental Table 2). PPII mRNA levels in the ME were increased 1.4 fold by 48 hours fasting (Supplemental Figure 1); this result suggests that fasting regulates ME PPII mRNA levels independently of isolation. Therefore, the following experiments were done according to experiment 1 protocol.

Two other experiments were performed to determine the dynamics of fasting effects on PPII activity. In experiment 3, fasting decreased body weight gain as well as serum TSH, total T3 and T4 concentrations, and increased serum corticosterone levels; these effects were detected at 36 hours and/or at later time points (Table 1). In fed animals, the ME PPII activity tended to decrease when the time of isolation increased. Fasting enhanced ME PPII ac-

Table 1. Body weight gain and serum hormone concentrations in normally fed male animals and 36–72 h of fasting; experiment 3.

	Control 36 h	Fasting 36 h	Control 48 h	Fasting 48 h	Control 60 h	Fasting 60 h	Control 72 h	Fasting 72 h
Body weight gain[a]	13.2 ± 0.3 (5)	−26.4 ± 0.9 (5) ***	6.6 ± 2.1 (5)	−32.2 ± 1.5 (5) ***	7 ± 0.5 (5)	−25.2 ± 1.7 (5) ***	8.8 ± 1.4 (5)	−20.6 ± 1 (5) ***
TSH[b]	1.60 ± 0.1 (5)	1.16 ± 0.1 (4)	2.15 ± 0.2 (5)	1.49 ± 0.1 (5)	2.87 ± 0.5 (5)	1.32 ± 0.1 (5) *	3.31 ± 0.9 (5)	1.07 ± 0.7 (5) ***
T3 (total)[c]	1.39 ± 0.16 (5)	0.90 ± 0.05 (4) *	1.45 ± 0.13 (5)	1.25 ± 0.11 (5)	1.40 ± 0.10 (5)	1.14 ± 0.15 (5)	1.34 ± 0.11 (5)	0.79 ± 0.09 (3) *
T4 (total)[c]	108 ± 9 (5)	62.6 ± 4.5 (5) ***	106 ± 7.1 (5)	37 ± 4.7 (5) ***	89.2 ± 11.5 (5)	44.4 ± 2.5 (5) ***	77.9 ± 5.1 (5)	48.2 ± 11.6 (5)
Cortico-sterone[b]	101 ± 14.4 (5)	570 ± 124 (4) *	121 ± 20.9 (5)	253 ± 65 (5)	26.8 ± 4.2 (5)	587 ± 116 (5) ***	34.2 ± 12.1 (5)	215 ± 75 (5) *
Serum proteins[d]	74.3 ± 6.5 (5)	68.8 ± 5.1 (5)	77.4 ± 2.3 (5)	66.1 ± 3.9 (5)	71.7 ± 3.4 (5)	74.4 ± 2.5 (5)	72.7 ± 3.0 (5)	69.7 ± 8.3 (5)

Data are mean ± SEM [a]: g; [b]: ng/mL; [c]: nMol/liter; [d]: μg/μl. Post hoc Student-Newman-Keuls multiple comparisons test: *: $P < 0.05$, ***: $P < 0.005$, compared to control.

tivity, with a significant increase at 72 hours of fasting (Figure 2A). Notably, parietal cortex or anterior pituitary PPII specific activities were not affected by fasting (Figure 2B, D). Finally, serum thyroliberinase activity in fasted animals was increased after 48 hours of fasting, and at later time points (Figure 2C).

In experiment 4, fasting decreased body weight gain, serum concentrations of leptin, TSH, and total T4, as well as liver D1 activity, while it increased serum corticosterone concentration; these effects were detected at 36 hours of fasting and/or at later time points (Supplemental Table 3). In fed animals, the ME PPII activity tended to decrease while the time of isolation increased. Fasting increased ME PPII activity between 48 and 72h, with significant effects at 48 and 72 hours (Supplemental Figure 2A). Fasting decreased parietal cortex PPII specific activity after 72 hours of fasting (Supplemental Figure 2D). In the anterior pituitary, PPII specific activity was not affected by fasting (Supplemental Figure 2B). Finally, serum thyroliberinase specific activity was increased at 36 or more hours of fasting (Supplemental Figure 2C).

Effect of adrenalectomy and corticosterone treatment on PPII activity in the ME and thyroliberinase activity in serum

Based on the relevance of adrenal status for HPT axis activity (40) and the differential changes in serum corticosterone concentration during isolation and fasting, we evaluated the effect of adrenalectomy and corticosterone

treatment on PPII activity in ME and serum (experiment 5). In adrenalectomized animals, corticosterone levels were undetectable; implantation of pellets with 25 or 40% corticosterone led to serum corticosterone values 3 or 4 fold higher than in sham animals (Figure 3A). Adrenalectomy decreased body weight gain and serum protein concentration and failed to affect the relative concentration of serum TSH (amount/mg protein). Corticosterone treatment did not reverse the decrease in body weight gain, but did reverse the serum protein concentration change, and tended to decrease the relative concentration of serum TSH (moles/mg protein). The relative serum concentrations of total T3 and T4 were not affected by adrenalectomy and corticosterone treatments. The relative concentration of serum leptin tended to decrease with adrenalectomy; this effect was partially reverted with corticosterone treatment (Table 2). Adrenalectomy and corticosterone treatments did not change ME PPII activity, or the specific activity of thyroliberinase (Figure 3B,C).

Thyroid status regulates PPII mRNA expression in the mediobasal hypothalamus

In situ hybridization histochemistry data indicated that ME D2 mRNA expression is enhanced after 48 hours of fasting; however, it returned to fed values at 72 hours of fasting (Figure 1). The ME PPII mRNA levels correlated positively with ME D2 mRNA levels (Supplemental Figure 3B). In experiment 6, we evaluated the sensitivity of PPII mRNA expression in the rat ME to thyroid status. Methimazole-induced hypothyroidism increased serum TSH level at day 21 after treatment; treatment with T4 for 3 consecutive days beginning at day 19 after methimazole treatment reverted this serum TSH increase (control: 2.14 ± 0.5 ng/mL (2), methimazole: 18.4 ± 5.2 (4)*, methimazole + T4: 3.8 ± 0.3 (3), *: $P < .05$ compared to control). ISH data show that hypothyroidism produced a drastic decrease in ME PPII mRNA levels; this drop was reversed if methimazole-treated animals were treated with T4 for 3 consecutive days and sacrificed 6 hours after the last injection (Figure 4A-D).

Prolonged fasting partially reinitiates pro-TRH expression in the PVN

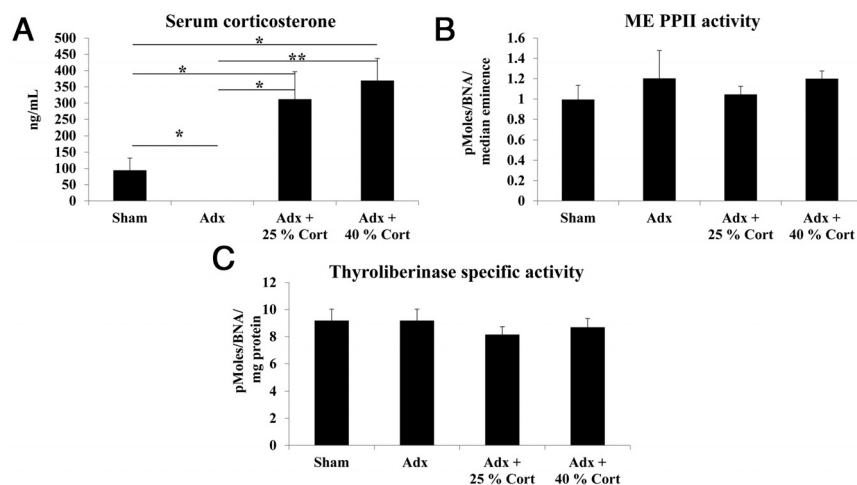Data from experiment 1 showed that 48 hours of fasting reduced pro-

Figure 3. Effect of adrenalectomy and corticosterone replacement on ME PPII activity and thyroliberinase activity; experiment 5. The graphs show serum corticosterone concentrations, and activity or specific activity of PPII in sham, adrenalectomized, and adrenalectomized animals implanted with 25 or 40% corticosterone pellets at 72 hours. Adrenalectomy decreased serum corticosterone concentration (A). Adrenalectomy and corticosterone implants did not change ME PPII specific activity (B) or specific activity of thyroliberinase (C). Values are in ng/mL (A), pmoles product per min per tissue (B), or per mg protein (C). Data were analyzed by one way ANOVA and post hoc Student-Newman-Keuls multiple comparisons test. Data are mean ± S.E.M. n = 6, except for adrenalectomy and adrenalectomy plus 25% corticosterone groups, n = 5. *: $P < .05$, **: $P < .01$, ***: $P < .001$.

Table 2. Serum concentrations of TSH, thyroid hormones, and leptin in normally fed male animals adrenalectomized (Adx) and treated with corticosterone (Cort); experiment 5.

	Sham	*Adx*	*Adx + 25% Cort*	*Adx + 40% Cort*
***Body weight gain**[a]*	−4.33 ± 3.09 (6)	−17.8 ± 4.23 (5) *	−21.4 ± 3.85 (5) *	−16.0 ± 3.52 (6) *
***Serum protein**[b]*	47.9 ± 2.67 (6)	34.0 ± 3.92 (5) **	44.7 ± 3.3 (5) [&]	49.7 ± 2.1 (6) [&]
***TSH**[c]*	35.2 ± 6.9 (6)	40.4 ± 9.1 (5)	22.6 ± 2.1 (5)	23.3 ± 2.9 (6)
***Leptin**[c]*	141 ± 37.9 (6)	62.4 ± 21.7 (5)	119 ± 24.9 (5)	115 ± 28.1 (6)
***T3 (total)**[d]*	12.3 ± 1.8 (6)	15.0 ± 1.46 (5)	11.8 ± 4.51 (5)	9.66 ± 0.95 (6)
***T4 (total)**[d]*	1620 ± 400 (6)	2000 ± 400 (5)	1390 ± 164 (5)	1270 ± 52.1 (6)

Data are mean ± SEM [a]: g; [b]: $\mu g/\mu l$; [c]: ng/mg protein; [d]: nMol/mg protein. Post hoc Student-Newman-Keuls multiple comparisons test: *: $P < 0.05$, **: $P < 0.01$ compared to sham; &: $P < 0.05$ compared to adrenalectomy.

TRH mRNA levels in the medial and caudal parts of the PVN; noticeably, pro-TRH mRNA levels at 72 hours of fasting were intermediary between those in fed and 48 hours fasted animals (Figure 1). Pro-TRH mRNA values correlated negatively with ME D2 mRNA levels (Supplemental Figure 3A). To confirm that PVN pro-TRH expression spontaneously reverts if fasting occurs for more than 2 days, we quantified the number of pro-TRH cells
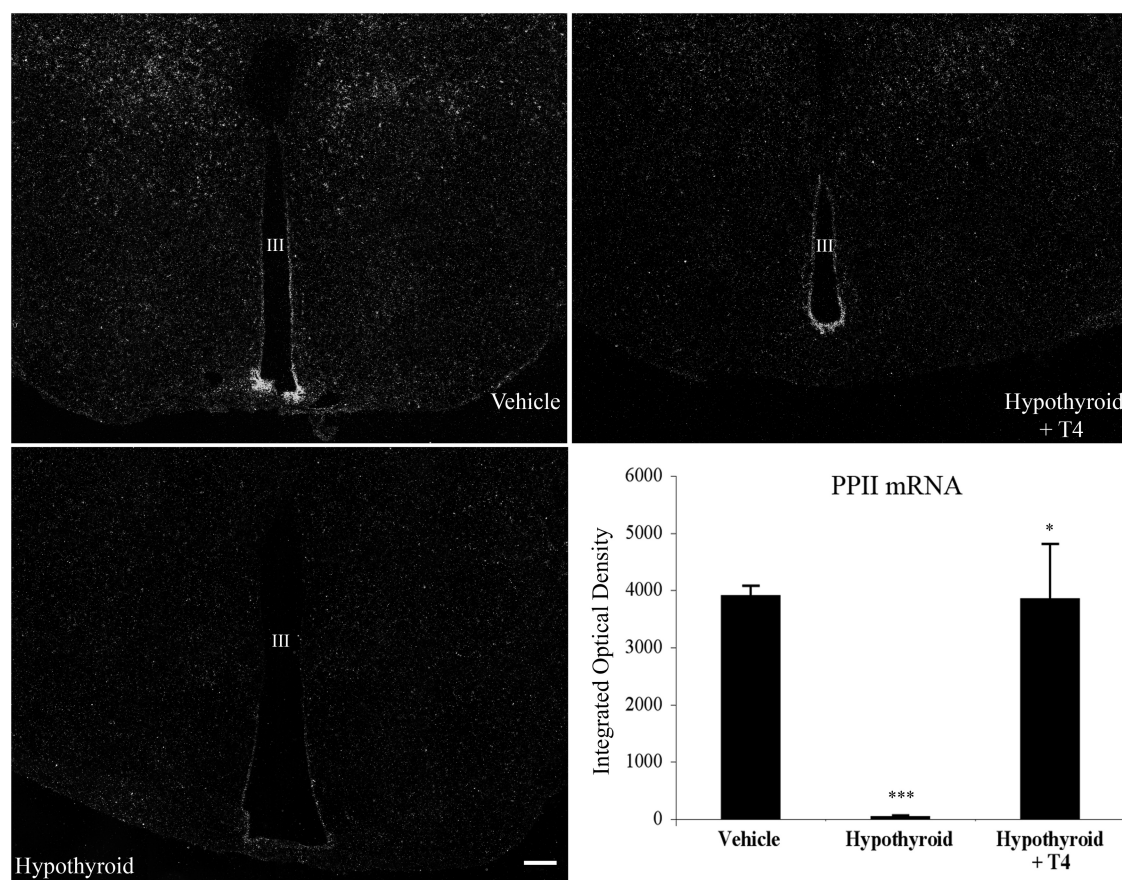

Figure 4. Effect of hypothyroidism and T4 restitution on expression of PPII in ME; experiment 6. PPII mRNA was detected by in situ hybridization with a [^{35}S]-labeled cRNA probe. A-C) dark-field photomicrographs of coronal sections through the medio basal hypothalamus (exposure time: 38 days); images are made by a collage of 16 images taken at 5X. A) rat injected with vehicle per 3 days beginning on day 19; B) rat made hypothyroid with 0.1% methimazole + 1% sodium perchlorate in drinking water per 21 days; C) hypothyroid rat treated with L-T4 (10 μg in 200 μl vehicle per 3 days beginning on day 19). D) The graph shows the densitometric analyses of in situ hybridization microradiograms (exposure time: 33 days); data in vehicle group include intact (n = 3) and vehicle (n = 2) (200 μl sterile saline/ 0.01 N NaOH per 3 days beginning on day 19) treated animals; data in hypothyroid group include data from hypothyroid (0.1% methimazole + 1% sodium perchlorate in their drinking water per 21 days, n = 2), and hypothyroid + vehicle (200 μl per 3 days beginning on day 19; n = 3) rats; hypothyroid + L-T4 (10 μg in 200 μl vehicle per 3 days beginning on day 19, n = 3. Data were analyzed by one way ANOVA and post hoc Student-Newman-Keuls multiple comparisons test. Data are mean ± S.E.M. *: $P < .05$. III= third ventricle, scale bar = 100 μm.

positive in the parvocellular subregion of the medial level of the PVN in experiment 7. The number of pro-TRH positive cells in the medial parvocellular region of the PVN decreased at 48 hours of fasting and increased towards values found in fed animals at 72 hours of fasting (Figure 5).

Discussion

Multiple molecular changes in specific organs orchestrate an inhibition of HPT axis in response to fasting (1, 8). The data obtained in this study indicate that regulation of HPT axis activity in response to fasting includes an adjustment in ME PPII activity, the ectopeptidase that hydrolyzes TRH, in male rats. This change in activity is probably localized in tanycytes, including β2 tanycytes, since PPII mRNA expression increased in cells that correspond to tanycytes localization. PPII is likely present on the surface of β2 tanycytes and expected to affect HPT axis activity since in vitro PPII inhibition enhances the recovery of TRH released from the ME, and in vivo PPII inhibition augments serum TSH concentration in cold-stressed animals (17). Thus, we propose that a delayed increase of ME PPII activity during fasting contributes to reduce TRH concentration in the portal blood (2), and therefore to down-regulate HPT axis activity. The enhancement of the extracellular hydrolysis of TRH may limit the possibility that

a partial reactivation of TRH neurons reinitiates thyroid axis function when fasting is prolonged.

Prolonged fasting promotes a partial reactivation of pro-TRH expression in the parvocellular PVN and a reversion of D2 mRNA induction in the mediobasal hypothalamus

Responses to fasting are likely dependent on its severity (41), but the effect of fasting on the dynamics of D2 expression is poorly described. In male rats, D2 mRNA expression is enhanced after 72 hours of fasting in α and β tanycytes (42). D2 activity increases in the MBH after 48 hours of fasting (43). In our experimental conditions, ME D2 expression increased after 48 hours of fasting, but normalized to values found in fed animals after 72 hours of fasting. Known enhancers of D2 activity in MBH are the inverse changes in circulating levels of corticosterone and leptin (43), which may have led to the peak of expression we detected after 48h of fasting. The drop in D2 expression in tanycytes after 72 hours of fasting may be due to a reduction of serum corticosterone concentration, as animals habituated to the stress.

As for D2 expression, the effect of fasting on the dynamics of PVN pro-TRH mRNA expression and TRH release in male rats is poorly defined; PVN pro-TRH mRNA levels are lower after 48 to 65 hours of fasting as compared to values detected in fed animals (2, 3, 4, 44). We noticed a late and partial reversal of the fasting-induced reduction in PVN pro-TRH mRNA expression. This partial inversion was confirmed in an independent experiment in which we measured the number of cells immuno-reactive for pro-TRH in the parvocellular part of the medial PVN. Immunohistochemical data were consistent with ISH results, showing a reactivation of pro-TRH translation during late fasting. ISH and immunohistochemical data were consistent in two different strains (Wistar in exp. 1 and Sprague-Dawley in exp. 7), in spite of strain differences in serum thyroid hormones and corticosterone concentrations (45).

During fasting, the decrease of serum leptin concentration reduces PVN pro-TRH mRNA levels and TRH release (3, 6). An increase of T3 production in tanycytes may be a necessary link for down regulation of PVN pro-TRH mRNA levels (4), but data in D2 or thyroid hormone receptor β2 KO mice do not support this hypothesis (10, 46–48). As for D2 expression, a reduction in serum corticosterone concentration may drive the reactivation of pro-TRH synthesis since, on a time scale of days glucocorticoids inhibit TRH mRNA levels in the PVN (40).
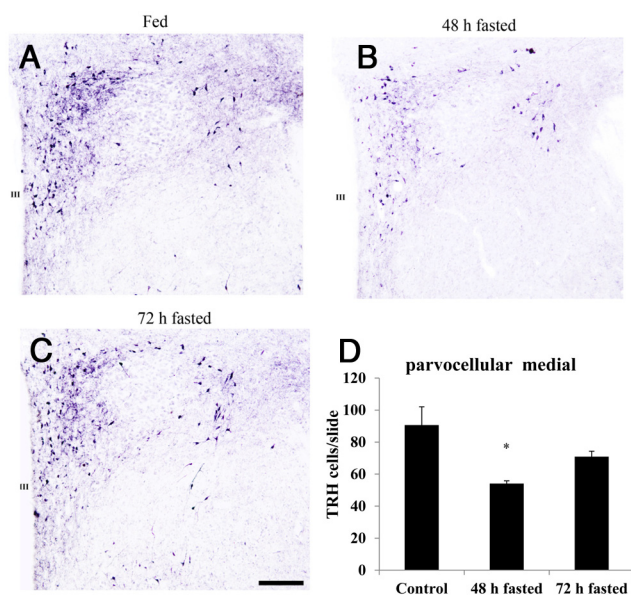

Figure 5. Effect of fasting on the number of TRH immunopositive neurons in the medial PVN in male rats; experiment 7. Coronal slices of the medial PVN immunolabeled with an antibody against pro-TRH. A: control; B: rats submitted to 48 hours of fasting; C: rats submitted to 72 hours of fasting; D: quantification of pro-TRH immunoreactivity in the medial PVN. Data are mean ± S.E.M., n = 3 *: $P < .05$. III= third ventricle, scale bar = 100 μm.

The partial reactivation of pro-TRH expression in the PVN during late fasting coincides with an up-regulation of PPII activity in the ME

An increase in PPII mRNA levels in the ME as well as in the medial and ventral parts of the ventricle walls, presumably in tanycytes, was detected at 48 hours of fasting, the earliest time point analyzed by ISH. This increase in PPII expression was apparently independent of tanycyte subtypes and reproduced in an independent experiment with younger animals previously habituated to isolation. Thus, enhanced expression of MBH PPII at 48 hours of fasting is a consistent response, independent of adult animal age (in the 2–4.5 month range) or housing conditions. The increase in tanycyte PPII expression was transient, since it was not detected after 72 hours of fasting. ME PPII activity was not affected by 36 hours of fasting but increased either after 48 hours of fasting in one experiment, or after 72 hours of fasting in another experiment. Thus, an increase in ME PPII expression in response to fasting produces a concomitant or delayed increase in ME PPII activity.

TRH hydrolysis by PPII produces His-Pro-NH2, that may be either hydrolyzed by dipeptidyl peptidase IV (49), an enzyme abundant on the surface of many cell types (50), or spontaneously cyclize to cyclo His-Pro (49). Cyclo His-Pro is detected in the brain, including the hypothalamus; it may not be uniquely derived from TRH (51, 52). However, it is relevant to note that cyclo His-Pro concentration increases in the hypothalamus of fasted animals; the mechanism leading to its accumulation has not been clarified (51); it may be related to increased PPII activity in the ME.

Together, these results suggest that during prolonged fasting the catabolism of TRH by PPII is increased in the ME extracellular fluid, concomitant with positive adjustments in TRH expression in PVN neurons which, if unbalanced, might partially reactivate TRH secretion in the portal capillaries. The control of ME PPII activity may thus contribute to maintain the reduction in the portal concentration of TRH that is detected in rats subjected to fasting (2, 53).

Putative drivers of up-regulation of PPII activity in the ME during fasting

Up-regulation of PPII activity in ME during fasting suggests the activity is coordinated with other aspects of HPT axis function. The hypothesis that adrenal status, which is relevant for HPT axis activity (40), regulates ME PPII activity in fed animals was not supported by our data. Thus, it seems unlikely that the effect of fasting on ME PPII activity is mediated by changes in serum corticosterone concentration per se. It is however possible that the early inverse changes in serum corticosterone and leptin con-

centrations that increase D2 activity in the MBH during fasting (43) led together to an increase in MBH PPII expression. An effect of thyroid hormones on ME PPII expression and activity during fasting is consistent with available data. Fasting increases not only D2 expression and activity in ME (42, 43, this study), but also hypothalamic T3 levels (4). Thyroid hormones up-regulate PPII expression in all subtypes of tanycytes in the context of thyrotoxicosis (17), or in hypothyroid mice, with changes faster than those operating on pro-TRH synthesis in the PVN (19); finally, tanycyte PPII expression is exquisitely sensitive to thyroid status (this study). Thyroid hormones positively regulated PPII expression in tanycytes and MBH regions from bregma –2.56 to –3.14 mm (Figure 4), while brain PPII activity sensitivity to thyroid status is poor (20, 25). Thus, the opposite effects of fasting on PPII activity in the cortex and median eminence coincide with region specific effects of thyroid hormones on PPII activity.

Fasting promotes an increase in the activity of thyroliberinase in serum

Thyroliberinase was the first TRH hydrolyzing activity biochemically characterized. Thyroliberinase activity increased during fasting, with significant changes detected relatively early (after 36 or 48 hours fasting) and maintained at least up to 72 hours of fasting. Since thyroid hormones up-regulate thyroliberinase activity (24, 25), the fasting-induced increase of thyroliberinase was unexpected. Additionally, thyroliberinase activity was insensitive to manipulation of serum corticosterone levels in fed animals. The mechanisms leading to enhanced thyroliberinase activity during fasting remain unknown. The increase in thyroliberinase activity detected in peripheral blood presumably coincides with an increase of the activity of this enzyme in the hypothalamus-pituitary portal blood vessels, and may contribute to reduce the concentration of TRH reaching the thyrotrophs during fasting.

Relevance of anterior pituitary PPII activity

Since anterior pituitary PPII activity is sensitive to thyroid status and rapidly up-regulated by thyroid hormones in vivo, it was initially proposed that PPII contributes to the negative feedback that thyroid hormones exert on the HPT axis (20, 25). However, PPII is not expressed by thyrotrophs and does not control thyrotropin secretion in response to TRH in cell culture (21, 22) suggesting that PPII unlikely controls TSH secretion in this locus. We observed that fasting does not modify PPII activity in the anterior pituitary. Likewise, 7 days of food restriction in adult female rats does not change the expression of PPII in the anterior pituitary (54). It should be however noted that 48 hours of fasting decreases anterior pituitary PPII ac-

tivity in 28 days old but not in three-month-old male rats (55); the reason for this age-dependent response is unknown. Thus, in adult animals anterior pituitary PPII activity is not regulated by fasting and may not contribute to the adjustments of the HPT axis activity.

In conclusion, the initial drop of PVN pro-TRH expression in adult male rats that is induced by fasting is partially reversed between 48 and 72 hours postfasting initiation, when ME PPII as well as thyroliberinase activities are up-regulated. The adjustments of ME PPII and thyroliberinase activities may oppose the partial reactivation of TRH secretion and contribute to the reduced HPT axis activity that characterizes prolonged fasting. These data reveal unsuspected fluctuations in the dynamics of HPT axis during fasting, and are consistent with the evidence that tanycytes respond to changes in energy balance (10).

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