Deficiencies in Pro-thyrotropin-releasing Hormone Processing and Abnormalities in Thermoregulation in Cpefatfat Mice*

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Cpefatfat mice are obese, diabetic, and infertile. They have a mutation in carboxypeptidase E (CPE), an enzyme that converts prohormone intermediates to bioactive peptides. The Cpefat mutation leads to rapid degradation of the enzyme. To test whether pro-thyrotropin-releasing hormone (TRH) conversion to TRH involves CPE, processing was examined in the Cpefatfat mouse. Hypothalamic TRH is depressed by at least 75% compared with wild-type controls. Concentrations of pro-TRH forms are increased in homozygotes. TRH-[Gly4-Lys5-Arg6] and TRH-[Gly4-Lys5-Arg6] represent approximately 45% of the total TRH-like immunoreactivity in Cpefatfat mice; they constitute ~1% in controls. Levels of TRH-[Gly4] were depressed in homozygotes. Because the hypothalamus contains some TRH, another carboxypeptidase must be responsible for processing. Immunocytochemical studies indicate that TRH neurons contain CPE- and carboxypeptidase D-like immunoreactivity. Recombinant CPE or carboxypeptidase D can convert synthetic TRH-[Gly4-Lys5-Arg6] and TRH-[Gly4-Lys5-Arg6] to TRH-[Gly4]. When Cpefatfat mice are exposed to cold, they cannot maintain their body temperatures, and this loss is associated with hypothalamic TRH depletion and reduction in thyroid hormone. These findings demonstrate that the Cpefat mutation can affect not only carboxypeptidase activity but also endoproteolysis. Because Cpefatfat mice cannot sustain a cold challenge, and because alterations in the hypothalamic-pituitary-thyroid axis can affect metabolism, deficits in pro-TRH processing may contribute to the obese and diabetic phenotype in these mice.

Neuropeptides and peptide hormones are first biosynthesized as precursors that must undergo a series of conversions to become biologically active (1, 2). Typically, maturation of these precursors begins with limited proteolysis. Excision of the pro-peptide usually occurs at monobasic, dibasic, or dibasic residues where subtilisin-like processing enzymes cleave the precursor on the C-terminal side of these amino acids. The endoproteolysis is usually followed by the sequential removal of the basic amino acid(s) residues by a carboxypeptidase-like enzyme (3). In some situations, additional modifications can occur in the form of N-terminal acetylation or pyroglutamate formation, sulfation, and C-terminal amidation (4). These alterations usually serve to yield a peptide that is both biologically active and resistant to degradation.

Further evidence that peptide processing is physiologically relevant has come from a mouse identified at the Jackson Laboratories. This animal was reported to be obese, diabetic, and infertile because of a spontaneous mutation in the fat gene (5). Subsequent studies have shown that the fat/fat mouse has a single point mutation in the carboxypeptidase E (CPE)1 gene, and hence, this mutation has been termed Cpefat (6). A Ser202 to Pro202 transition renders the enzyme catalytically inactive and subject to rapid degradation soon after synthesis. With respect to the diabetes in the Cpefatfat mouse, the mechanism underlying this dysfunction has been partially attributed to a deficiency in converting pro-insulin intermediates to insulin.

To date, the mechanisms underlying the obesity in the Cpefatfat mouse are not well understood. The reason for these circumstances is probably because of the multifactorial nature of the regulation of food intake, absorption, and caloric utilization (7, 8). In the latter case, the control of metabolism may be especially important. One hormonal system that plays an important role in the regulation of metabolism is the hypothalamic-pituitary-thyroid axis that is controlled by thyrotropin-releasing hormone (TRH).

Hyphysiostrophic pro-TRH is synthesized in the hypothalamic paraventricular nucleus, and it must undergo a number of different processing steps to yield mature TRH (9). Upon stimulation, TRH is released from median eminence nerve terminals into the hypophyseal blood where it is transported to the pituitary to stimulate the biosynthesis and secretion of thyroid-stimulating hormone (TSH; see Ref. 10). TSH, in turn, is carried in blood to the thyroid where it stimulates thyroid hormone biosynthesis and release. Besides this role, TRH can also exert some control over the release of prolactin, growth hormone, vasopressin, and insulin, as well as the classic neurotransmitters, norepinephrine and epinephrine. Furthermore, TRH is present in brain regions outside of the hypothalamus where it may serve as a neurotransmitter or neuromodulator.

In previous reports, it has been shown that the pro-TRH is cleaved endoproteolytically by two different members of the

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‡ The abbreviations used are: CPE, carboxypeptidase E; CPD, carboxypeptidase D; FITC, fluorescein isothiocyanate; HPLC, high pressure liquid chromatography; IR, immunoreactivity; PC, prohormone convertase; RIA, radioimmunoassay; T₄, triiodothyronine; T₃, thyroxine; TRH, thyrotropin-releasing hormone; TSH, thyroid-stimulating hormone; WT, wild-type; Tricine, N-[2-hydroxy-1,1-bis(hydroxymethyl)-ethyl]glycine; dansyl, 5-dimethylaminonaphthalene-1-sulfonylethylglycine; dansyl, 5-dimethylaminonaphthalene-1-sulfonylethylglycine; dansyl, 5-dimethylaminonaphthalene-1-sulfonylethylglycine;

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prohormone convertase (PC) family of enzymes, PC1/3 and PC2 (11). In this scheme, PC1/3 appears to be primarily responsible for most of the major cleavage events. By contrast, although PC2 can perform many of these same conversions, this enzyme is required for the formation of the peptide, and it is specifically involved in the processing of prepro-TRH (268-387) to generate the pP28, and pSE14 peptides (11, 12). Because PC1/3 and PC2 seem to cleave the pro-TRH on the C-terminal side of dibasic residues, these basic amino acids must be removed from the N-terminal TRH sequences before the TRH to be acted upon is generated. An enzyme that has been hypothesized to produce these conversions is CPE. Because the Cpefat/fat mouse has a mutation in this enzyme, this animal should allow us to examine this hypothesis and determine whether this mutation exerts any effects on hypothalamic-pituitary-thyroid function.

**EXPERIMENTAL PROCEDURES**

**Animals**—The BKS.HRS-Cpefl/fl mice were received as a gift from Dr. Edward H. Leiter at Jackson Laboratories. The mouse colony was maintained at the Duke University Medical Center by heterozygous matings. Initially, mice were genotyped using the microsatellite markers D8Mit68 and D8Mit131. In these PCR, the primers were denatured at 94 °C for 3 min, and the reaction was run at 94 °C for 45 s, 50 °C for 45 s, and 72 °C for 2 min in 35 cycles. Reaction products were separated on a 3.5% 1:1 agarose gel (FMC Corp., Rockland, ME) where DNA from C57BLKS/J and B6.A-T18b HRS-hr mice were run as controls. Subsequently, most mice were genotyped by the procedure of Mدادatu and Naggett (13). Here, DNA was denatured at 94 °C for 2 min and then the reaction was run at 94 °C for 30 s, 50 °C for 45 s, and 72 °C for 45 s in 40 cycles at a Stratagene Rotocycler. The PCR products were separated on a 3% MetaPhor agarose gel (FMC Corp.) where wild-type (WT) H11006 samples were identified by a 113-bp product, and rated on a 3% MetaPhor agarose gel (FMC Corp.) where wild-type (WT) H11006 samples were identified by a 101-bp product.

Liquid chromatography (HPLC) studies, samples were boiled for 10 min in a buffer containing 25% acetic acid, 25 mM EDTA, 25 mM EGTA, and various enzyme inhibitors (0.1% concentrations each for phenylmethylsulfonyl fluoride, aprotinin, bacitracin, bestatin, and pepstatin) (12). Samples were sonicated, homogenized further using a Dounce homogenizer, and vortexed for 1 min and briefly centrifuged at 900 × g for 5 min. The supernatant was removed, and a small aliquot was taken from the pellet for protein analysis.

**Electrophoresis**—Supernatants were lyophilized, resuspended in 0.0625 M Tris (pH 6.8), 1% SDS, 15% glycerol, and 15 mM dithiothreitol buffer, boiled for 5 min, and loaded onto a 1.5-mm discontinuous polyacrylamide Tricine-SDS gel (11). Gels were run in a Protein 16 cm cell system (Bio-Rad). After electrophoresis, gels were cut into 2-mm slices with a gel slicer (Biorad Scientific Instruments, San Francisco, CA). Each slice was placed into 25 mM acetic acid for protein extraction prior to radioimmunoassay (RIA) and incubated for 4 days, and the gel slices were removed. Recovery of proteins/peptides from the gel slices was approximately 90%. The molecular masses of the sample proteins/peptides were determined using pre-stained molecular mass markers as follows: 106, 80, 49.5, 32.5, 27.5, and 18.5 kDa (Bio-Rad) and 29, 20.4, 14.4, 6.5, and 2.1 kDa (Bio-Rad, Newton, MA). A 250-μmol column run at 1 ml/min. In preparation for HPLC, peptide samples were filtered through Sep-Pak C18 cartridges (Waters Associates, Milford, MA). The lyophilized samples were reconstituted in 0.5% trifluoroacetic acid and injected onto the HPLC. Initial conditions (1% methanol + 0.05% trifluoroacetic acid) were held for the first 5 min, and a gradient was developed over 20 min to 3% methanol + 0.05% trifluoroacetic acid. These changes in mobile phase permitted baseline separation of synthetic TRH, TRH-(Gly4-Lys5)-Arg3, TRH-(Gly4-Lys5)-Arg3 (Peninsula), Lys-Arg-Gln-His-Pro-Gly (Peninsula), Lys-Arg-Gln-His-Pro-Gly-Lys5-Arg (Peninsula), TRH-(Gly4-Lys5)-Arg3, and TRH-(Gly4-Lys5)-Arg3 (Phoenix Pharmaceuticals). The RIA kit used to measure mouse TSH was developed and provided by Dr. A. F. Parlow (Harbor-UCLA Medical Center, Torrance, CA). Briefly, standards (0.195 to 50 ng of mouse TSH) and serum samples were added in a volume of 0.2 ml and were incubated with 0.1 ml of guinea pig anti-mouse TSH serum (1:8,000 dilution) for 24 h at 4 °C. The next day 2% normal guinea pig serum (Antibodies Incorporated, Davis, CA) and ~20,000 cpm of rat [125I]TSH (COVANCE, Vienna, VA) were added in a 0.1-ml aliquot and incubated for 24 h at 4 °C. On the third day, anti-guinea pig sera (1:25 dilution) were used and incubated for 4 h at 4 °C. The antibodies were separated by HPLC, and fractions surrounding and including the elution of the CPD was pooled. Reversal from the column was greater than 95%. The RIA kit was reconstituted with RIA buffer and screened with the TRH and TRH-(Gly4-Lys5) Arg3 assays. Fractions that comprised and were in the vicinity of the elution positions of synthetic TRH-(Gly4-Lys5)-Arg3 and TRH-(Gly4-Lys5)-Arg3 were pooled with recombinant carboxypeptidase D (CPD) for 4 h (see below) and submitted to the TRH-(Gly4-Lys5) Arg3 RIA. The N-terminal antisera (anti-CPD1) that recognizes prepro-TRH25-36 (26-kDa pro-TRH, prepro-TRH25-115 (15-kDa intermediate), prepro-TRH25-112 (9.5-kDa intermediate), and prepro-TRH25-129 (9-kDa intermediate) of the rat were used to study prepro-TRH processing (16). The sensitivity of the TRH and TRH-(Gly4)-Arg3 assays were 0.7 and 6.8 pg, respectively, and the intra- and interassay variabilities were 5–6 and 9–12%, respectively.

**In Vitro Processing of TRH Intermediates by CPD and CPE—Baculoviral recombinants of duck CPD, rat CPD, and dasyly-Phe-Ala-Arg were generously supplied by Dr. Lloyd D. Fricker (Albert Einstein College of Medicine, Bronx, NY). For the CPD assay, 63 units (specific activity, 42 fluorescent units/μg protein) of CPD were added to 100 μl Tris acetate buffer (pH 6.4) containing 2.5 μs cobalt chloride, 0.01% Triton X-100 (see Ref. 19). The CPD assay consisted of 63 units (specific activity, 49 fluorescent units/μg protein) of CPD in 125 mM sodium acetate (pH 5.5), 2.5 μs cobalt chloride buffer (see Ref. 20). The solutions were incubated on ice for 15 min to activate the enzymes, and 15 μg of synthetic TRH-(Gly4-Lys5)-Gly3 or TRH-(Gly4-Lys5)-Arg3 were added. Incubations proceeded for 0.5, 4, 24, or 72 h at 37 °C. At the end of this time, the samples were boiled for 5 min and fractions were separated by HPLC (see above), and processing was quantified by UV absorbance at 214 nm.

To determine the levels of TRH-(Gly4-Lys5)-Gly3 and TRH-(Gly4-Lys5)-Arg3 in hypothalami from WT and Cpefat/fat mice, samples were separated by HPLC, and fractions surrounding and including the elution of the CPD was pooled. Reversal from the column was greater than 95%. The RIA kit was reconstituted with RIA buffer and screened with the TRH and TRH-(Gly4-Lys5)-Arg3 assays. Fractions that comprised and were in the vicinity of the elution positions of synthetic TRH-(Gly4-Lys5)-Arg3 and TRH-(Gly4-Lys5)-Arg3 were pooled with recombinant carboxypeptidase D (CPD) for 4 h at 37 °C, boiled for 5 min, and submitted to the TRH-(Gly4)-Arg3 RIA.

**Immunocytochemistry**—Experiments designed to determine whether CPE and/or CPD were expressed in TRH neurons were instituted with hypothalamic neuronal cultures. Briefly, hypothalamic neurons (3 × 104/cm2) were plated onto the synthetic poly-L-lysine-coated four-chamber Lab Tek slides (Nunc, Naperville, IL). Twelve-day-old cultures were fixed with 4% paraformaldehyde in phosphate-buffered saline and submitted to immunocytochemistry as described (11, 12). To identify CPE-containing neurons, cells were first incubated (1:500 dilution) with a monoclonal antibody.
antiserum (1:500; Research Diagnostics, Flanders, NJ) for 24 h. The

antiserum was incubated with the neurons for 2 h at room temperature. For visualization of pro-TRH, slides were incubated first with anti-pAV37 antiserum (1:1,000 dilution) at 4°C for 24 h. The following day, slides were incubated for 2 h at room temperature with goat anti-rabbit immunoglobulin (1:500 dilution) that had been conjugated with fluoroscein isothiocyanate (FITC; Vector Laboratories, Burlingame, CA). The CPD antiserum was kindly donated by Dr. Lloyd Fricker at Albert Einstein College of Medicine in Bronx, NY, and its specificity has been described (21). The CPD immunostaining was achieved by incubating (1:500 dilution) the hypothalamic neurons with the anti-CPD antiserum at 4°C for 24 h. The following day, slides were incubated for 2 h at room temperature with FITC conjugated secondary anti-rabbit sera (236/11006). Co-localization studies were performed with sequential immunostaining for CPE and pro-TRH or CPD and pro-TRH as described above. Control experiments included the incubation of cells with pre-immune sera or in the absence of the primary antibody/antiserum. Thirty-mm slides were digitized with a video camera using BioVisionframe grabber software (Perceptice Corporation, Knoxville, TN). Images of red and green panes were combined using Adobe Systems software (Mountain View, CA) to show areas of co-localization. The objective was ×40.

Statistics—The data are presented as means ± S.E. The data were analyzed by t tests and one- or two-way analysis of variance tests with genotype or genotype and time as the main dependent variables. A posteriori analyses were performed by Duncan and Newman-Keuls tests.

RESULTS

Hypothalamic Pro-TRH Processing—In an initial examination of pro-TRH processing, we analyzed the levels of TRH in the hypothalamus by RIA. The concentrations of immunoreactive TRH were depressed by at least 75% in the Cpefat/fat mice (52 ± 9 pg/mg protein, n = 10) relative to those in the heterozygous (298 ± 12 pg/mg protein, n = 9) and WT controls (249 ± 14 pg/mg protein, n = 10). Hypothalamic protein contents did not differ among the three genotypes (data not shown). These data indicate clearly that whereas the amounts of TRH are substantially reduced in homozygote hypothalamus, some small amount of pro-TRH may be fully converted to TRH in these animals.

The reduced levels of TRH could be because of depressed biosynthesis of pro-TRH, or they could be attributed to some deficiency in processing the pro-hormone. In these studies we used antisera that were raised against the rat pro-TRH and TRH sequences, because no mouse-specific reagents were available. It should be emphasized that the amino acid sequence homology between the two species is ~88% with 100% conservation around the processing sites for the five cryptic TRH sequences (see Refs. 22 and 23, and see Fig. 1). In these experiments, we used anti-TRH, anti-TRH-[Gly4], and anti-pCC10 serum. The latter antiserum was generated against the synthetic decapptide, Cys-Lys-Arg-Gln-His-Pro-Gly-Lys-Arg-Cys (where the underlined amino acids represents the TRH sequence), and it binds to the pro-TRH (26 kDa) and 15-, 9.5-, and 6-kDa intermediate peptides (11). Separation of pro-TRH and its high molecular mass intermediates by SDS-PAGE revealed that the precursor was processed into a number of different intermediates (Fig. 2). Indeed, six peptides unidentified previously were detected in the hypothalami from WT and Cpefat/fat mice using the anti-pCC10 serum. Interestingly, five of these peptides (15, 11, 7.5, 5, and 4 kDa) were increased substantially in the homozygotes over those of the controls (Table I). In both genotypes, the majority of the immunoreactivity was found in the 1.6-kDa species. Collectively, these data indicate that processing of the pro-TRH is aberrant in hypothalami from Cpefat/fat animals. Moreover, they also demon-

![Image](317x140 to 563x482)

![Image](566x566 to 563x738)

**FIG. 1. Comparison of the amino acid sequences of the rat and mouse pro-TRH.** Differences between the rodent sequences are shown in bold. The TRH sequences are underlined in italics. Both the rat (M36317) and mouse sequences (X59387) were obtained from GenBank™ (22, 23). The rat sequence is 255 amino acids in length, whereas the mouse sequence is 256. When the sequences are aligned, the mouse sequence is found to have an insertion at amino acid 194. There is ~88% homology between the sequences.

![Image](123x123)

**FIG. 2. Electrophoretic separation of hypothalamic high molecular mass pro-TRH-derived peptides from male WT and Cpefat/fat mice.** Proteins were separated by SDS-PAGE, eluted from the gel, and submitted to RIA using the anti-pCC10 serum. Top, separation from WT hypothalamus. Bottom, separation from mutant hypothalamus. Recovery from the gel was ~90%, and each data point was replicated twice. The sizes of the pro-TRH species were estimated by molecular mass standards.
samples, the concentrations of this peptide were reduced by
and
these neurons (Fig. 4
seen (11, 12). CPE-like IR can be
colocalization studies were employed in our well established
whether this carboxypeptidase is expressed in TRH neurons,
abley, these amino acids are removed by CPE. To determine
confined almost exclusively to the perikarya (Fig. 4
C
pro-TRH are present within the same neurons (Fig. 4
C
immunoreactivities (Fig. 3). TRH concentrations were reduced
4-fold in hypothalami from the Cpe
fat/fat
animals compared with their WT littermates (Table II). Although some TRH-[Gly 4]
was present in WT samples, the concentrations of this peptide were reduced by
10-fold in the mutants.
To quantitate the concentrations of the TRH-[Gly4-Lys5]
and TRH-[Gly4-Lys5-Arg6], fractions corresponding to the elution positions of the synthetic standards were incubated with recombinant CPD to convert the endogenous peptides to TRH-
Gly4
antiserum. The
Gly4
is noted when TRH-
Gly4-Lys5
is the substrate
A
displays the elution positions of the synthetic peptide standards. Top,
chromatogram of materials from WT animals separated by HPLC and
screened with the TRH and TRH-[Gly4]
antiseria. Bottom, a chromato-
graphic profile of materials from homozygous mice. Recoveries were
95%, and each data point was replicated three times. Although each
original chromatogram was run with at least 10 mice, the values in the chromatogram are presented in terms of those for an individual animal.
used the primary hypothalamic neuronal cultures. CDPh-like IR
(green) can be visualized to be distributed almost exclusively in the perikarya of the neurons (Fig. 4D). Similarly, TRH-like IR
(red) is also present in the cell body (Fig. 4E). Co-localization of the immunostaining demonstrates that CDPh-like and TRH-like immunoreactivities (orange-yellow) reside within the same neurons (Fig. 4F). The pro-TRH, CPE, and CPD immunostain-
ings are specific, because no immunoreactivity is detected for any of these antigens when pre-immune sera (for pro-TRH) is
used or when the secondary antisera/antibody (for CPE and
CPD) is omitted from the reaction (Fig. 4, G–I). These data support the idea that a carboxypeptidase besides CPE is present in TRH neurons.
In Vitro Processing of TRH Intermediates—To evaluate whether CPE or CPD can process the low molecular mass TRH intermediates, synthetic TRH-[Gly4-Lys5] or TRH-[Gly4-Lys5-Arg6] were incubated with recombinant CPE or CPD using identical units of enzyme. During the first 30 min of incubation with CPE, only ~10% of the TRH-[Gly4-Lys5-Arg6] is converted to TRH-[Gly4-Lys5] (Fig. 5A). Complete conversion to TRH-
Gly4
takes ~72 h. A similar time course of conversion to TRH-[Gly4]
is noted when TRH-[Gly4-Lys5] is the substrate

| Pro-TRH products | Wild types | Homozygotes | Mol %<sup>a</sup> | WT/fat MR<sup>b</sup> |
|------------------|------------|------------|-----------------|---------------------|
| 15-kDa peptide   | 3.0        | 0.70       | 5.3             | 1.17                |
| 11-kDa peptide   | 2.7        | 0.63       | 10.0            | 2.21                |
| 7.5-kDa peptide  | 4.2        | 0.98       | 31.4            | 6.94                |
| 5-kDa peptide    | 12.0       | 2.79       | 28.0            | 6.19                |
| 4-kDa peptide    | 32.5       | 7.57       | 52.5            | 11.62               |
| 1.6-kDa peptide  | 375.0      | 87.33      | 325.0           | 71.87               |
| Total            | 429.4      | 100.00     | 452.2           | 100.00              |

<sup>a</sup> Percent refers to the mole percent of total pro-TRH products that is contributed for a given pro-TRH product.

<sup>b</sup> Mol % refers to the femtomol ratio of a given peptide product for Cpe
fat/fat
mice compared to that same product for WT animals times 100.

WT/fat MR refers to the femtomol ratio of peptide product for WT and Cpe
fat/fat
mice.

Because conversion of pro-TRH to its constituent high molecular mass intermediates is perturbed in hypothalamus of Cpe
fat/fat
mice, we next examined whether processing of the low molecular mass peptides is also abnormal. When the HPLC fractions were screened with either the TRH or the TRH-[Gly4]
antiseria, each of the antisera only detected a single immunoreactive peak, and these materials were found to elute in the same positions as the respective synthetic peptide standards (Fig. 3). TRH concentrations were reduced ~4-fold in hypothalami from the Cpe
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animals compared with their WT littermates (Table II). Although some TRH-[Gly4]
was present in WT samples, the concentrations of this peptide were reduced by ~10-fold in the mutants.

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Gly4
antisera. Materials eluting in the positions of the synthetic TRH-[Gly4-Lys5] (TRH-GK) and TRH-[Gly4-Lys5-Arg6] (TRH-GKR) standards were incubated with recombinant CPD and assayed with the TRH-[Gly4]
antiseria. The top of each representative chromatogram displays the elution positions of the synthetic peptide standards. Top,
chromatogram of materials from WT animals separated by HPLC and
screened with the TRH and TRH-[Gly4]
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is noted when TRH-[Gly4-Lys5] is the substrate
These data suggest that removal of the Lys\(^5\) residue by the CPE may be more protracted than excision of the Arg\(^6\) from the intermediate. By comparison, CPD processing of TRH-[Gly\(^4\)-Lys\(^5\)-Arg\(^6\)] to TRH-[Gly\(^4\)-Lys\(^5\)] and TRH-[Gly\(^4\)] occurs very rapidly and is essentially complete within the first 30 min of incubation (Fig. 5, C and D). Collectively, these data indicate that both CPE and CPD can process TRH intermediates in vitro. 

**Status of the Hypothalamic-Pituitary-Thyroid Axis in the Cpefat/fat Mice**—Because processing of the pro-TRH is deficient, and TRH levels are reduced substantially in hypothalami from male Cpefat/fat mice, concentrations of TSH, T\(_4\), and T\(_3\) were measured in serum. Baseline levels of serum TSH (Fig. 6A) and T\(_3\) (Fig. 6B) were not distinguished by genotype. By contrast, circulating T\(_4\) values were significantly higher in homozygote blood (Fig. 6C). These data demonstrate that, for the most part, mutation of the Cpe gene does not substantially affect pituitary or thyroid function under basal conditions.

To study the dynamics of hypothalamic-pituitary-thyroid interactions, mice were placed into a cold room (4–7 °C) for 0, 30, 60, or 120 min. At the outset of the experiment (0 min), the core body temperatures in the mutants were somewhat lower (\(\pm 0.6°\) C) than those in the WT animals. Controls were able to maintain their core temperatures, with little fluctuation (increase of 0.2 °C) over the 2-h period (Fig. 7A). By contrast, within the first 30 min of cold exposure, core body temperatures in mutants declined significantly, and by the end of 2 h, their body temperatures were reduced by at least 3 °C (two mice showed a reduction by 6 °C). Because the hypothalamic-pituitary-thyroid axis is involved intimately in thermoregulation (26), these data suggest that this axis in the Cpefat/fat mice is deficient.

Assessments of hormonal function were also performed during the 2-h exposure to cold. Hypothalamic TRH values in homozygous mice were already depressed at the zero time point by \(-79%\) compared with their WT littermates (Fig. 7B). Con-

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**Fig. 4. Co-localization of CPE- and CPD-like IR in hypothalamic TRH neurons.** Primary hypothalamic cultures were cultured for up to 14 days, fixed with 4% paraformaldehyde, and immunostained for CPE, CPD, and TRH. A, CPE immunoreactive perikarya (arrows) and processes (arrowheads) revealed with an anti-serum conjugated to Texas Red. B, pro-TRH immunoreactive cell bodies (arrow) as visualized with FITC-conjugated anti-serum. C, co-localization of CPE- and pro-TRH-like IR (arrowheads) within the same neurons. D, CPD immunoreactive neurons (arrow) detected with FITC-conjugated antisera. E, pro-TRH immunoreactive neurons (arrow) revealed with Texas Red antisera. F, co-localization of CPD- and pro-TRH-like immunoreactivity (arrowheads). G, pro-TRH immunoreactivity with pre-immune serum. H, CPE immunostaining in the absence of the primary antiserum. I, CPD immunoreactivity in the absence of primary antiserum. Objective, \(\times 40\).
tinal exposure to cold over the next 2 h depressed hypothalamic TRH levels significantly in the mutants by ∼38%, whereas those in the WT animals were only attenuated by ∼13%. An examination of TRH-[Gly 4] levels revealed that these values did not change over the 2-h period for either genotype (data not shown). Because processing of the Arg6- and Lys5-extended intermediates is blocked in the mutant, these findings suggest that the TRH-Gly4 is not readily available for secretion. Together, these data indicate that cold exposure reduces hypothalamic TRH contents in both genotypes and that the stores of this peptide are reduced more dramatically in the homozygotes than in the WT mice.

Exposure to cold increased serum TSH levels in both genotypes; however, the response in the Cpefat/fat animals was initially more robust than that in the WT controls (Fig. 7C). This significantly increased response could not be maintained in the mutants, and serum TSH values declined significantly to a value that was 83% from baseline. By contrast, in WT animals there was a tendency for serum TSH contents to be increased by cold, but by the end of the 2-h period these values were reduced by less than 20%. These data suggest that the Cpefat/fast mice may be able to defend their temperatures during brief exposure to cold, but more prolonged conditions compromise the ability of the hypothalamic-pituitary axis to mount a sustained response.

An examination of serum T4 concentrations revealed that these values effectively mirror those for TSH (Fig. 7D). Hence, serum T4 levels in homozygotes were increased during the first 30 min in the cold environment; however, they declined over time. By comparison, the WT mice showed a more protracted response to cold, and the levels of this hormone were increased and maintained over the 2-h period of exposure. Hence, at the end of this time, the concentration of T4 was significantly higher in sera from the WT animals compared with that for the Cpefat/fast mice.

As previously noted, basal serum T3 contents are already elevated in the Cpefat/fast animals relative to their WT controls. Upon cold exposure, the mutants showed no further increase in serum T3 levels; instead, values declined significantly by ∼30% over the 2-h period (Fig. 7E). By comparison, WT animals responded with significant increase in circulating T3 levels to the cold during the first 30 min of cold exposure, with a decline to baseline values by 120 min. Collectively, these data indicate that although both genotypes of mice show endocrine responses to the cold, those from the mutants are transient and are not sufficient to maintain their core body temperatures.

**DISCUSSION**

CPE is an exopeptidase that is responsible for removing basic amino acids from the C-terminal of proteins and peptides (3). A point mutation in the CPE gene (e.g. Ser202 to Pro202 transition) is sufficient to reduce the efficiency of endoproteolytic cleavage of the pro-TRH and its high molecular mass products in Cpefat/fat hypothalamus. These data indicate that mutation of this gene can exert effects upstream of its normal processing activity. In rats, pro-TRH is processed by PC 1/3 and 2 (11, 12). Both endoproteases are synthesized as pro-enzymes and must be processed to attain full activity (1, 2). It has been proposed that CPE may participate in the full activation of these convertases through removal of C-terminal basic amino acids. The data presented here suggest that the mutation of CPE influences the efficiency of pro-TRH cleavage, which in turn affects the endocrine responses to cold. This could have implications for the regulation of body temperature and energy expenditure, as altered hypothalamic TRH levels and TSH and T4 responses may contribute to the thermoregulatory dysfunction observed in Cpefat/fast mice.
acids or by inactivation of endogenous inhibitors (27–29). Recently, it has been reported that protein levels and enzymatic activities of both enzymes are altered in the brains of the Cpefat/fat mice (30). As a consequence, levels of pro-dynorphin and its high molecular mass intermediate (e.g., dynorphin A-17) are increased (30, 31). Additionally, concentrations of pro-insulin in pancreas (6) and pro-opiomelanocortin in pituitary are enhanced (29, 31, 32). Despite these findings, impaired endoproteolysis of pro-peptides is not necessarily a common feature of the CPE mutation, because the levels of pro-cholecystokinin in brain are unchanged (33, 34), whereas those in intestine are reported to be either enhanced (34) or unaltered (33) from the WT controls.

Besides alterations in TRH levels, concentrations of some high and low molecular mass pro-TRH intermediates were also changed in the Cpefat/fat hypothalamus. Although the function of TRH has been studied for many years, only recently have physiological roles been ascribed to some of the other pro-TRH products (9). For instance, although 100-fold less potent than TRH, TRH-Gly_4 stimulates gastric acid secretion in a dose-dependent manner (35). Additional peptides that have biological activity include the prepro-TRH_160–169 and the prepro-TRH_178–199 (36, 37). The prepro-TRH_160–169 augments TRH-stimulated TSH secretion and potentiates TRH-induced gastric acid secretion when microinjected into the dorsal motor nucleus of the vagus nerve. The prepro-TRH_178–199 can serve as a corticotropin-releasing hormone inhibiting factor (37, 38), and it can stimulate prolactin release from the pituitary (12).

Inasmuch as these pro-TRH-derived peptides possess biological activity, and because processing of the pro-TRH intermediates are affected by the CPE mutation in mice, it may be expected that some of these functions will be abnormal in the Cpefat/fat mouse.

In the present study, hypothalami from Cpefat/fat mice were found to contain high levels of the low molecular mass intermediates, TRH-[Gly_4-Lys_5-Arg_6], and TRH-[Gly_4-Lys_5]. These data indicate that carboxypeptidase activity is deficient in vivo and that CPE is primarily responsible for this activity in TRH neurons. A role for CPE in TRH processing is strengthened further by the in vitro conversion of TRH-[Gly_4-Lys_5-Arg_6] and TRH-[Gly_4-Lys_5] to TRH-[Gly_4] and by the co-localization of CPE-like IR in TRH neurons. It should be noted that besides TRH intermediates, an increase in additional C-terminal basic amino acid-extended peptides has been observed for insulin (6), cholecystokinin (33, 34), gastrin (39, 40), and neurotensin in the Cpefat mutant (41).

In murine hypothalami, the Cpefat mutation depresses the levels of fully processed TRH. Additional peptides in brain that are also reduced include dynorphin (30, 31), cholecystokinin (33), neurotensin (41), and substance P (42). Besides removal of basic amino acids, amidation may also be affected in the Cpefat/fat mouse. For instance, the molar ratio of TRH-[Gly_4] to TRH and the percent of this glycine-extended peptide relative...
to the other TRH-like low molecular mass peptides were depressed in mutant hypothalami. A similar relationship has also been reported for glycine-extended cholecystokinin (34) but not for gastrin (40) in gut. Together, these data suggest that ameliorative activity may be influenced in the Cpefat/fat mouse.

Despite perturbations in pro-TRH processing, Cpefat/fat hypothalamic contains low quantities of TRH. This result suggests either that some residual CPE is present or that an additional carboxypeptidase resides in TRH neurons. One possible candidate is CPD. This enzyme is expressed in many brain regions including the hypothalamic paraventricular nucleus where the hypothalamic TRH neurons are located (25). Moreover, CPD is co-localized with CPE in many different brain regions. In the present report, we show that hypothalamic TRH neurons contain CPE- and CPD-like IR. Moreover, recombinant CPD can process synthetic TRH-[Gly4-Lys5-Arg6] or TRH-[Gly4-Lys5] in vitro. These findings clearly show that CPD can mimic the actions of CPE. Despite this fact, hypothalamic levels of TRH-[Gly4-Lys5-Arg6] and TRH-[Gly4-Lys5] are augmented in Cpefat/fat mice. One reason for the discrepancy between the in vitro and in vivo results may be the differential locations of the enzymes. Our immunocytochemistry results show that CPE-like IR is located in the perikarya and processes, whereas the CPD-like IR is confined to the cell body region of TRH neurons. These findings complement other studies where CPE has been localized primarily to mature secretory granules (43), whereas CPD resides in the pre-Golgi network and immature secretory granules (44). Because pro-TRH processing begins in the Golgi and continues in the secretory granules (45), CPD could begin conversion of the pro-TRH intermediates in vivo; however, these effects would be transient as the intermediates passed to the mature granule and a build-up of intermediates could occur.

Cold exposure stimulates the hypothalamic-pituitary-thyroid axis strongly (26). In rats, cold temperatures increase TRH mRNA levels in the hypothalamic paraventricular nucleus (46), augment TRH release from the median eminence (47), stimulate TSH secretion from pituitary, and potentiate T4 and T3 contents in blood (48). In our studies, basal levels of serum TSH and T3 are similar among WT, heterozygous, and Cpefat/fat mice. A similar relationship has been reported for serum T3 concentrations in lean controls (7). Although Cpefat/fat animals respond to cold, they cannot maintain their core body temperatures or sustain their endocrine responses. The already depressed hypothalamic TRH levels in the Cpefat/fat mice are reduced further by an additional 38% with cold exposure. Although TRH neurons reside in several different hypothalamic areas, only the paraventricular nucleus contains CPE- and CPD-like IR. Moreover, CPD is co-localized with CPE in many different regions of the central nervous system (10), a deficiency in many regions of the central nervous system (10), a deficiency in the hypothalamic-pituitary-thyroid axis is impaired in its hypothalamic levels of TRH-[Gly4-Lys5-Arg6] and TRH-[Gly4-Lys5] are augmented in Cpefat/fat mice. One reason for the discrepancy between the in vitro and in vivo results may be the differential locations of the enzymes. Our immunocytochemistry results show that CPE-like IR is located in the perikarya and processes, whereas the CPD-like IR is confined to the cell body region of TRH neurons. These findings complement other studies where CPE has been localized primarily to mature secretory granules (43), whereas CPD resides in the pre-Golgi network and immature secretory granules (44). Because pro-TRH processing begins in the Golgi and continues in the secretory granules (45), CPD could begin conversion of the pro-TRH intermediates in vivo; however, these effects would be transient as the intermediates passed to the mature granule and a build-up of intermediates could occur.

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36. Roussel, J.-P., Hollande, F., Bulant, M., and Astier, H. (1991) Neuroendocrinology 54, 559–565
37. Redei, E., Hilderbrand, H., and Aird, F. (1995) Endocrinology 136, 3557–3563
38. McGivern, R. F., Rittenhouse, P., Aird, F., Van de Kar, L. D., and Redei, E. (1997) J. Neurosci. 17, 4886–4894
39. Udupi, V., Gomez, P., Song, L., Varlamov, O., Reed, J. T., Leiter, E. H., Fricker, L. D., and Greeley, G. H., Jr. (1997) Endocrinology 138, 1959–1963
40. Hansen, L. F., and Rehfeld, J. F. (2000) Biochem. Biophys. Res. Commun. 267, 638–642
41. Rovere, C., Viale, A., Nahon, J.-L., and Kitabgi, P. (1996) Endocrinology 137, 2954–2958
42. Perloff, M. D., Kream, R. M., and Beinfeld, M. C. (1998) Peptides 19, 1115–1117
43. Fricker, L. D., and Snyder, S. H. (1982) Proc. Natl. Acad. Sci. U. S. A. 79, 3886–3890
44. Varlamov, O., Eng, F. J., Novikova, E. G., and Fricker, L. D. (1999) J. Biol. Chem. 274, 14759–14767
45. Perez de la Cruz, J., and Nillni, E. A. (1996) J. Biol. Chem. 271, 22736–22745
46. Zoeller, R. T., Kabeer, N., and Albers, H. E. (1999) Endocrinology 127, 2955–2962
47. Arancibia, S., Tapia-Arancibia, L., Assenmacher, I., and Astier, H. (1983) Neuroendocrinology 37, 225–228
48. Rondeel, J. M. M., de Greef, W. J., Hep, C. J., Rowland, D. L., and Visser, T. J. (1991) Neuroendocrinology 54, 477–481
49. Donders, S. H., Pieters, G. F., Heevel, J. G., Ross, H. A., Smals, A. G., and Kloppenborg, P. W. (1985) J. Clin. Endocrinol. Metab. 61, 56–59
50. Lechan, R. M., and Jackson, I. M. D. (1982) Endocrinology 111, 55–65
51. Yamada, M., Saga, Y., Shibusawa, N., Hirata, J., Murakami, M., Iwasaki, T., Hashimoto, K., Satoh, T., Wakahayashi, K., Taketo, M. M., and Mori, M. (1997) Proc. Natl. Acad. Sci. U. S. A. 94, 10862–10867
52. Ahima, R. S., Prabakaran, D., Mantzoros, C., Qu, D., Lowell, B., Maratos-Flier, E., and Flier, J. S. (1996) Nature 382, 250–252
53. Nillni, E. A., Vaslet, C., Harris, M., Hollenberg, A., Bjorbak, C., and Flier, J. S. (2000) J. Biol. Chem. 275, 36124–36133