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Review

Neuroregulation of ProTRH Biosynthesis and Processing

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This review presents an overview of the current knowledge on proTRH biosynthesis, its processing, its tissue distribution, and the role of known processing enzymes in proTRH maturation. The neuroendocrine regulation of TRH biosynthesis, the biological actions of its products, and the signal transduction and catabolic pathways used by those products are also reviewed. The widespread expression of proTRH, PC1, and PC2 rnRNAs in hypophysiotropic and extrahypophysiotropic areas of the brain, with their overlapping distribution in many areas, indicates the striking versatility provided by tissue-specific processing in generating quantitative and qualitative differences in nonTRH peptide products as well as TRH. Evidence is presented suggesting that differential processing for proTRH at the intracellular level is physiologically relevant. It is clear that control over the diverse range of proTRH-derived peptides within a specific cell is accomplished most from the regulation at the posttranslational level rather than the translational or transcriptional levels. Several examples supporting this hypothesis are presented in this review. A better understanding of proTRH-derived peptides role represents an exciting new frontier in proTRH research. These connecting sequences in between TRH molecules to form the precursor protein may function as structural or targeting elements that guide the folding and sorting of proTRH and its larger intermediates so that subsequent processing and secretion are properly regulated. The particular anatomical distribution of the proTRH end products, as well as regulation of their levels by neuroendocrine or pharmacological manipulations, supports a unique potential biologic role for these peptides.

Key words: proTRH; TRH; neuroendocrine, peptides; HPT axis; prohormone processing; TRH degradation; proTRH-derived peptides; central nervous system; hypothalamic cells; catecholamines; prohormone convertases; AtT20 cells; glucocorticoids; leptin; suckling; TRH-degrading enzymes; TRH receptors.

Introduction

In recent years considerable research has focused on the expression of neuropeptide genes and their tissue-specific regulation. However, it has become clear that the peptides derived from these genes play significant neuromodulatory roles in the control of the central nervous system (CNS) neurotransmitters and neuroendocrine peptides. The discovery that multiple neuropeptides with distinct physiological functions arise from the processing of single polypeptide precursors *(1–5)* established the landmark of a new era of hormone biology. Thus, to fully understand the biology of a neuropeptide, one must understand the biosynthesis and processing of its preprohormone gene product, as well as the regulation of the gene's transcription. The different levels of one peptide with respect to another when both derive from the same precursor protein sequence is achieved through differential post-translational processing followed by its regulated degradation by the action of specific enzymes acting in specific cellular and extracellular compartments. This mechanism is critical to increase biological and functional diversity. Thus, identification of the molecular events involved in the action of neuronal inputs (stimulation of the target cell, signal transduction)–biosynthesis (processing and secretion)–enzymatic inactivation will provide us with a better understanding of the life cycle of each neuropeptide. The parvocellular paraventricular nucleus of the hypothalamus (PVN) producing prothyrotropin releasing hormone (proTRH) is a prime example were different neuroendocrine systems integrate signals from different areas of the brain to stimulate differentially one common pathway, the hypothalamic-pituitary-thyroid axis (HPT). In addition, immunoreactive TRH in the brain is also found outside of the traditional "thyrotrophic zone" of the hypothalamus *(6,7)*. This extrahypophysiotropic TRH is believed to function as a neuromodulator of known neurotransmitters *(5,8,9)*.

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Regulation of proTRH Neurons and Production of Biologic Peptides

The PVN is composed of two major components, the magnocellular and the parvocellular divisions. The parvocellular division contains most of the TRH neurons that project to the median eminence (ME) *(10)*. However, not all TRH containing neurons in the PVN project to the ME *(11)*. In addition to the PVN, TRH neurons are present in many other regions of the hypothalamus *(10)*. As these populations of neurons have no known projections to the ME, and are not regulated in conjunction with the thyrotropic neurons of the PVN, it is presumed that they do not subserve a direct hypophysiotropic function.

The TRH molecule synthesized in the PVN is responsible for the biosynthesis and secretion of thyroid-stimulating hormone (TSH) from the anterior pituitary *(12,13)*. TSH in turn stimulates thyroid hormone biosynthesis and release *(14,15)*. TRH also influences the release of other hormones, including prolactin, growth hormone, vasopressin and insulin *(16–18)*, and the classic neurotransmitters noradrenaline and adrenaline *(19)*. In addition to playing a central role in regulating the HPT axis, TRH is also present in many brain loci outside of the hypothalamus, supporting a potential role as a neuromodulator or neurotransmitter outside of traditional HPT axis function *(20,21)*. For example, TRH is implicated as a modulator of seizure activity *(22)* and gastrointestinal function *(23)*. TRH has been also found outside the central nervous system (CNS) in the gastrointestinal tract, pancreas, reproductive tissues including placenta, ovary, testis, seminal vesicles and prostate, retina, and blood elements *(24)*.

TSH is synthesized and secreted by the thyrotrophic cells in the anterior pituitary and is the major regulator of the thyroid gland. TSH secretion is primarily regulated by negative feedback from circulating thyroid hormone. TSH secretion is also regulated by stimulatory inputs from the hypothalamus through TRH action on thyrotrophs. Feedback effects of thyroid hormone occurs at the hypothalamus and pituitary level. Thyroidectomy increases preproTRH mRNA and hyperthyroidism produces the opposite in the medial parvocellular neurons of the PVN *(25,26)*. However, preproTRH mRNA levels do not change under the influence of thyroid hormone in other regions of the brain containing proTRH such as nucleus reticularis and olfactory bulb (OB) *(27)*. This is due probably to the tissue-specific thyroid hormone receptor distribution, which is not present in the OB *(28)*. There are other regulatory factors known to control TSH secretion, including glucocorticoids in the systemic circulation, and somatostatin (SRIF) and dopamine (DA) from the hypothalamus. There is evidence supporting the view that glucocorticoids in man and rat suppress endogenous hypothalamic TRH secretion *(29,30)*. The action of glucocorticoids on PVN preproTRH mRNA-expressing neurons may involve the hippocampus and amygdala, which convey negative feedback information to the CRF-expressing neurons in the PVN *(31)*. However, in vitro glucocorticoids have opposite effects on TRH expression. *(32)*. Glucocorticoids are known to affect the gene expression of many eukaryotic cells, also regulate the posttranslational maturation of many proteins *(32–35)*. Glucocorticoids enhance TRH gene expression in several in vitro cell systems, including hypothalamic neurons, anterior pituitary cells, and thyroid C cells, an effect that occurs, at least in part, through transcriptional activation *(32,36)*. Dexamethasone substantially elevates biosynthesis of the 26 kDa TRH prohormone and its intermediate products in cultured anterior pituitary cells, consistent with an overall upregulation of both the biosynthesis and processing of the TRH precursor *(32)*. It was reasoned that glucocorticoids act not only at the transcriptional level, but also at the translational/posttranslational level. This question was addressed in experiments with AtT_{20} cells transfected with preproTRH cDNA driven by a CMV-IE promoter not responsive to physiological signals. Dexamethasone causes a 75% increase in newly synthesized 26 kDa proTRH without altering preproTRH mRNA levels, suggesting that glucocorticoids raise translation rates and/or slow processing of proTRH. In fact, dexamethasone treatment accelerates TRH precursor processing. Processing of the N- versus the C-terminal intermediates is influenced differentially by glucocorticoids. Levels of the N-terminally derived peptide $preproTRH_{25-50}$ are enhanced while levels of the 5.4 kDa C-terminally derived peptide are reduced. TRH content is increased *(32)*. Thus, glucocorticoids induce changes in the biosynthesis and processing of proTRH by affecting both transcription and translation rates, and by differentially influencing the processing of N- versus C-terminal intermediates of proTRH. At the translational and posttranslational level these effects result in an increase in TRH production, with more complicated differential effects on the accumulation of other N- and C-terminal proTRH-derived peptides.

Even though TRH is the major regulator of the synthesis and secretion of TSH, and thus plays a pivotal role in the HPT axis, in a recent study, homozygous TRH gene knockout mice were shown to be viable, fertile, and exhibit normal development *(37)*. Whereas the TRH–/– mice showed normal serum prolactin and growth hormone levels, thyroid hormone levels were significantly reduced as compared with the wild-type heterozygous mice. The targeted disruption of the preproTRH gene caused a characteristic tertiary hypothyroidism, and a substantial decrease in insulin secretion resulting in a profound hyperglycemia. These authors suggested that in addition to abnormalities of the thyroid function, TRH may be involved in the pathogenesis of diabetes mellitus *(37)*. TRH outside the traditional HPT axis was shown to be involved in many neurological functions such as ergotrophic effects, arousal and sleep, TRH in cognition and locomotor activation, antidepressant effects, autonomic nervous system function, gastrointestinal function, pancreas and liver, cardiovascular function, respira-

tion seizure modulation, and electroconvulsive seizures. A full review of the extrahypophysiotropic role of TRH is described in detail elsewhere *(5)*.

TRH plays a prominent role in integrating a number of thermogenic responses to cold [Arancibia, 1996 #2067]. A principal site of TRH thermoregulation is the anterior hypothalamic preoptic area (POA) *(38)*. TRH into the POA inhibits warm-sensitive neurons, and activates cold-sensitive neurons *(39)*, which results in increased body temperature through peripheral vasoconstriction, increased metabolic heat production, and shivering *(40)*. Cold-exposure also elevates preproTRH mRNA levels *(41)* and TRH secretion *(42)* in the PVN. These changes elevate thyroid hormones and increase heat generation in brown adipose tissue *(43)*. Cold-induced increases in preproTRH mRNA also are seen in the dorsal motor nucleus of the vagus (DMN) *(44)* and caudal raphe nuclei *(45)*. Excitation of spinal cord preganglionic sympathetic neurons results in postganglionic NE release, and increased facultative thermogenesis via beta- and alpha 1-adrenoreceptors on brown adipocytes *(46)*. Therefore, the leading pathways to heat production involve stimulation of the HPT axis and extra hypophysiotropic proTRH neurons.

In comparison to the known roles of TRH there is little information about the biological activities of the other proTRH-derived peptides. Both TRH-Gly and preproTRH$_{178-199}$ are regulated by dexamethasone in the hypothalamus, but not in cerebellum, brainstem, retina, and stomach *(47)*. Thus, clues to the roles of proTRH-derived peptides other than TRH must come from an examination of their regional distribution, or evidence of regulation under specific physiological or pathological conditions.

PreproTRH$_{160-169}$ (also known as Ps4 and TRH-potentiating peptide) enhances TRH-stimulated TSH release from the anterior pituitary, and stimulates TSHβ gene promoter activity *(48)*. Thus, it acts in an opposite manner to feedback by T3, which decreases TSH secretion *(15)*, and inhibits TSH subunit gene expression *(49)*. This peptide also potentiates TRH-induced-gastric acid secretion when microinjected into the dorsal motor nucleus of the vagus *(50)*. The peptide has been isolated from bovine hypothalamus and its amino acid sequence confirmed by Edman degradation *(51)*. PreproTRH$_{160-169}$ is also unique in that a receptor for this peptide has been characterized *(52)*. PreproTRH$_{160-169}$ receptor binding is developmentally regulated, with an increase from birth to weaning, then a gradual decline to adult levels at postnatal day 60 *(53)*. Within the CNS, preproTRH$_{160-169}$ is most enriched in hypothalamus, with lesser amounts in the spinal cord and olfactory bulb. The pituitary and striatum contain moderate levels. Its receptor binding is highest in the pituitary, hypothalamus, spinal cord, olfactory bulb, and hippocampus *(54)*. Finally, preproTRH$_{160-169}$ function is not restricted to a hypophysiotropic one. A review of the distribution and postulated functions of this peptide recently has been presented *(55)*.

PreproTRH$_{178-199}$ is also released from rat hypothalamic slices and the ME *(56,57)*, and is localized in dense-core granules of PVN neurons that project down to the ME. PreproTRH$_{178-199}$ has been reported to be a corticotropin-inhibiting factor, acting to reduce POMC mRNA and inhibit ACTH release *(58,59)*. Despite this logic, the relationship between the HPA and HPT axes remains incompletely understood, and other investigators have been unable to reproduce corticotropin inhibition by preproTRH$_{178-199}$ *(60)*. Suckling was shown to increase preproTRH mRNA in PVN and markedly increases TRH release during the first period of lactation *(61)*. In recent studies done in this laboratory, it was shown that preproTRH$_{178-199}$ acts as a prolactin secretagogue in primary pituitary cultures, and its levels rise during the early phases of suckling in rat *(62)*. Furthermore, two novel peptides derived from preproTRH$_{178-199}$, preproTRH$_{178-185}$ and preproTRH$_{186-199}$ also increase during suckling in the PVN *(62)*. PreproTRH$_{178-185}$ was the most active prolactin secretagogue. In suckling experiments, where mothers were separated from their pups for 6 hours and then reunited for 45 minutes, suckling induced a fivefold increase in PVN preproTRH$_{178-199}$ and preproTRH$_{186-199}$, and a sixfold increase in serum prolactin compared to nonsuckling controls. While these data implicate these novel peptides in suckling-induced prolactin release, further experiments are required to rule out nonspecific effects of stress *(62)*.

The periaqueductal gray (PAG) expresses high levels of preproTRH mRNA *(63)*. Mature TRH *(64)* and TRH receptor binding *(65)* are present in moderate levels throughout the PAG. PreproTRH mRNA is strongly induced in the PAG during opiate withdrawal *(63)*. Fos-like immunoreactivity is greatly increased in the ventrolateral PAG during withdrawal *(66)*, and may mediate induction of preproTRH mRNA *(67)*. While TRH levels in the PAG remain unchanged during opiate withdrawal *(68,69)*, the levels of the N-terminal preproTRH$_{53-74}$ and preproTRH$_{83-106}$ peptides were elevated, and TRH was increased in the lateral hypothalamus, suggesting this region may play a physiological role in opiate withdrawal *(69)*. In addition, a reduction in the C-terminal peptide preproTRH$_{208-255}$, and no change in preproTRH$_{178-199}$, in opiate-withdrawal versus control animals was also observed *(69)*. Opiate withdrawal also increases preproTRH$_{83-106}$ in the lateral hypothalamus. We speculate that during opiate withdrawal, proTRH processing may be altered in several brain regions, resulting in increased levels N-terminally derived peptides (preproTRH$_{53-74}$ and preproTRH$_{83-106}$) and decreased levels of some C-terminally derived peptides (preproTRH$_{208-255}$). These results demonstrate that levels of various products derived from proTRH can be posttranslationally regulated in an independent fashion under altered physiological conditions. Thus, it is logical that neuroendocrine inputs into the PVN can affect proTRH processing as well.

TRH-Gly, is widespread and detectable throughout the CNS in a similar distribution to TRH, and is also present in the prostate, serum, spleen, adrenals, kidney, and gastrointestinal organs. Levels of TRH-Gly are upregulated by hypothyroidism and by thermal stress, following the pattern for TRH itself *(70)*. The pancreas contains TRH-Gly within β-cell secretory granules *(70)*. The ratio of TRH/TRH-Gly is highest in pituitary and hypothalamus, and much lower elsewhere in neural tissue. TRH-Gly is much better characterized than other proTRH-derived peptides discussed above. TRH-Gly is increased in several limbic regions following chronic elctroconvulsive shock treatment, including hippocampus, and pyriform cortex *(71)*.

Although many neurons in the PVN contain more than one peptide, TRH neurons are unique in being almost always unassociated with other known peptides *(72)*. This makes the regulation of proTRH-derived peptide biosynthesis very specific. TRH neurons in the PVN are located in a region where they can be regulated by a number of neuroendocrine inputs. TRH neurons are densely innervated by norepinephrine (NE)-containing axons that stimulate TRH secretion *(73)*. TRH neurons are also densely innervated by neuropeptide Y (NPY) neurons. In smaller numbers, SRIF and endogenous opioid peptides (EOP) terminals are also in contact with TRH neurons *(73)*. In vivo, these various neuroendocrine inputs may affect the levels of preproTRH mRNA and the posttranslational processing of proTRH by influencing the biosynthesis and maturation of the prohormone convertases (PCs) PC1 and PC2 *(74)*. Evidence for coordinated regulation of mRNAs for processing enzymes and their substrates has been documented in several cases *(75–77)*. By contrast, outside the hypothalamus TRH is colocalized with other substances. For example, in the descending bulbospinal pathway, TRH is colocalized with substance P (SP) and serotonin (5-HT) *(78)*. Regulation of these TRH neuronal systems is much less well characterized than the thyrotropic neurons of the hypohyseal-portal system.

TRH-synthesizing neurons in the rat PVN receive a large number of afferent neuroendocrine inputs. The majority of inputs to TRH neurons are derived from the diencephalon, telencephalon, and brainstem *(79)*. The paraventricular and medial parvocellular divisions of the PVN are densely innervated by NE-containing and epinephrine (E)-containing inputs from the medulla and pons *(80)*. Further, NE-containing neurons densely innervate the midregion of the external layer of the ME. These inputs activate tuberoinfundibular neurons. NE/E excitation of PVN TRH neurons mediates the rise in TSH in response to acute cold exposure or hypovolemia *(81–83)*. However, it has also been proposed that β1-adrenergic receptors mediate a phasic inhibitory regulation of TSH release. The data on NE/E modulation of TRH biosynthesis may be reconciled by an examination of how these inputs affect the posttranslational processing of proTRH, as well as examining their effects on PCs enzyme biosynthesis. Peripheral levels of T3, T4, or TSH may also influence NE/E effects on TRH biosynthesis and/or release.

The PVN also receives prominent dopamine (DA) inputs from the posterior and dorsal areas of the hypothalamus, the zona incerta of the subthalamic region, and the A14 region of the anterior hypothalamus *(73)*. In contrast to the NE/E system, DA inputs appear to inhibit TRH secretion, mainly at the level of the ME *(84)*. Augmentation of DA neurotransmission inhibits basal and/or cold-stimulated TSH release, while DA antagonism has the opposite effect, though some studies have failed to replicate these findings *(73)*. As well, TRH release may be indirectly inhibited by DA stimulated secretion of SRIF *(85)*. Conversely, DA stimulates TRH release from isolated hypothalamic fragments *(85,86)*, again reinforcing the need to examine TRH biosynthesis in both in vitro and in vivo systems. Within the HPT axis, thyroid hormones appears to modulate DA levels in the ME, and TSH increases DA ability to inhibit TRH *(87)*.

A wide array of neuropeptides including NPY, TRH itself, SRIF, EOPs, neurotensin (NT), and vasoactive intestinal polypeptide (VIP) have inputs to the PVN and/or external layer of the ME *(88–92)*. Other mediators including GABA and various cytokines also appear to regulate TRH or TSH secretion, but there is as yet no anatomical evidence to support a direct action on TRH neurons in the PVN and/or ME *(73)*. The arcuate nucleus is the major source for NPY fibers innervating the TRH neurons in the PVN *(93)*. Physiologically NPY is critical to integrating thyroid function, food intake, and thermoregulation *(94)*.

Previous work in starved rats has shown a decrease in hypothalamic, but not thalamic reticular, preproTRH mRNA, as well as decreased circulating TRH. This supports the concept that hypothyroidism produced after starvation is of hypothalamic origin *(95)*. Leptin, a recently discovered peptide hormone that is synthesized and released by adipose tissue, decreased in starvation. Absence of leptin is responsible for the obese phenotype of *ob/ob* mice, and administration of this hormone to these animals decreases plasma corticosterone, suggesting that leptin is capable of inhibiting the hypothalamic–pituitary–adrenal axis. In normal rats and mice leptin inhibits hypothalamic CRH release *(96)*.

Leptin may have an important role in the neuroendocrine regulation of the HPT axis *(97)*. During prolonged fasting in rats, low levels of T3 and T4 are observed, and TSH is in the low to normal range. This is due in part to suppression of preproTRH gene expression in PVN neurons. Since the decrease in thyroid hormone levels is blunted in mice and rats by systemic leptin, it has been proposed that the decrease in leptin detected during fasting alters the set point for feedback inhibition by thyroid hormones on the biosynthesis of preproTRH mRNA *(97)*. The mechanism of such leptin regulation of preproTRH biosynthesis is unknown. It is hypothesized that leptin has direct

actions on cell bodies in the arcuate nucleus, positively regulating POMC, and thus α-MSH, and negatively regulating NPY and the Agouti-related peptide *(94)*. NPY afferents on TRH neurons are inhibitory. In preliminary studies done in this laboratory, both leptin and α-MSH elevate preproTRH mRNA, proTRH, and TRH secretion, in primary hypothalamic cultures (unpublished results). Using the same primary cultures of hypothalamic neurons, leptin dose-dependently increases proTRH synthesis and TRH secretion. Immunocytochemical analysis reveals that approximately 40–50% of the hypothalamic cells are positive for the leptin receptor. Of these, approximately 10–15% colocalize with proTRH. These data suggest that the regulation of proTRH biosynthesis and TRH release in response to starvation includes direct regulatory actions of leptin and α-MSH on hypothalamic TRH neurons involved in HPT axis homeostasis *(98)*. In summary, leptin effects on proTRH biosynthesis include: a) an inhibitory action of leptin on NPY release from the arcuate nucleus, which in turn may reduce the inhibitory action of this peptide on TRH release from the PVN; b) a stimulatory action of leptin on α-MSH release from the arcuate nucleus, which may stimulate TRH release from the PVN; and c) a direct action of leptin on TRH neurons located in the PVN.

Biosynthesis and Processing of proTRH

After hormone precursors are synthesized on membrane-bound ribosomes, they are translocated into the lumen of the rough endoplasmic reticulum (RER) via a signal recognition peptide. These proteins generally have their hormone sequences flanked by a single, a pair, or four (tetra) basic amino acids *(99–101)*. During vectorial transport through the Golgi complex (GC) and beyond, the newly synthesized proteins are subjected to posttranslational modifications including glycosylation, phosphorylation, amidation, acetylation, and proteolytic conversion *(102,103)*. This endoproteolytic cleavage is produced at the C-terminal side of the single or paired basic amino acid residue(s) by subtilisinlike processing enzymes, the prohormone convertases *(101–107)*. Basic residue(s) are then removed by carboxypeptidase enzymes (CP) *(108,109)*. Further modifications can occur in the form of N-terminal acetylation, pyroglutamate formation or C-terminal amidation, which confers bioactivity to many peptides *(110)*. Finally, partially processed proteins reach the last compartment of the GC, the trans-Golgi Network (TGN). At the TGN, unprocessed or partially processed products are sorted to the regulated secretory pathway (RSP) *(111–116)* to be stored in immature secretory granules (ISG). Upon maturation, electron-dense SGs containing sorted products can fuse with the plasma membrane in response to an extracellular stimulation in a calcium-dependent manner, thereby releasing their contents into the external milieu *(117)*. Two pathways of unstimulated release are proposed for AtT_{20} cells, constitutive (nongranular) secretion and basal release from compartments that form after sorting into the RSP *(118)*. The mechanism whereby constitutive and regulated proteins are differentially sorted into separate vesicles after budding from the TGN is still under intensive investigation *(119,120)*.

Two hypotheses have been proposed to explain how proteins are selectively targeted from the TGN to the RSP. In the first hypothesis, proteins are sorted by passive aggregation, in which the proteins condense within forming ISG, thereby excluding other proteins from entering in the granule. This process occurs under acidic pH and high calcium concentrations *(121–126)*. However there are data suggesting that aggregation alone is not sufficient for sorting. Modifications of the chromogranin B sequence *(127)* can prevent the correct sorting of these peptides to the RSP, while their in vitro aggregation properties appeared unaltered. The insulin-like growth factor-1 *(128)* does not aggregate in the TGN, but is still sorted in the RSP. The second hypothesis, originally proposed in 1985 by Kelly *(129)*, involves *cis*-acting sorting-signals within a protein destined for the RSP that interact with membrane-bound sorting receptors. Sorting receptors, possibly located in the forming SG, direct segregation of the protein for further packaging into SGs. Protein aggregation within the ISG can occur in this model, but is more critical for product concentration than sorting *per se*. Evidence supporting this second hypothesis has come from experiments involving chimeric proteins *(130–135)*, where the fusion of constitutively secreted protein to a protein destined for RSP caused a rerouting of this protein to the RSP. Conversely, proteins that have their sorting signal domains modified may be misrouted from the RSP into the constitutive pathway, as demonstrated for POMC *(136)*, chromogranin A and B *(137)*, PC2 *(131)*, and glycine α-amidating monooxygenase (PAM) *(138)*. A related hypothesis is sorting by retention, in which all proteins are initially targeted to ISGs, after which proteins that do not belong in the RSP are removed to their final destination, *e.g.*, lysosomal enzymes *(119,139)*.

Recently it has been proposed that the membrane form of carboxypeptidase E (CPE), localized to the TGN, is a sorting signal receptor *(140)*. CPE is proposed to direct POMC, proinsulin, proenkephalin, but not chromogranin A, into the RSP *(141)*. Thus, CPE is a common sorting receptor for some, but not all, prohormones, and there must be other sorting receptors to direct trafficking of other proteins to the RSP *(141)*. However, Irminger et al. *(142)* have provided evidence refuting the claim that CPE is a sorting receptor for proinsulin. In those studies they used pancreatic islets isolated from CPE-deficient (*Cpefat/Cpefat*) and control (*Cpefat/+*) mice to examine whether the trafficking of proinsulin and insulin was affected. They found that CPE was not essential for the sorting of proinsulin to the RSP *(142)*. However, similar experiments with procholecystokinin in *Cpefat/Cpefat* mice indicate that CPE does

function as a sorting receptor *(143)*. Further experiments are needed to clarify this controversy.

Rat preproTRH is a 29 kDa polypeptide composed of 255 amino acids. This precursor contains an N-terminal 25 amino acid leader sequence, 5 copies of the TRH progenitor sequence Gln-His-Pro-Gly flanked by paired basic amino acids (Lys-Arg or Arg-Arg), four non-TRH peptides lying between the TRH progenitors, an N-terminal flanking peptide, and a C-terminal flanking peptide *(144,145)*. The N-terminal flanking peptide (preproTRH$_{25-50}$-R-R-preproTRH$_{53-74}$) is further cleaved at the C-terminal side of the arginine pair site to render preproTRH$_{25-50}$ and preproTRH$_{53-74}$, thus yielding a total of seven proTRH-derived peptides. Our current model of proTRH processing is depicted in Figure 1.

Rat, mouse, and human proTRH, similar to other peptide hormone precursors such as pro-enkephalin, contains multiple copies of one of its peptide products, in this case, the progenitor for TRH, Gln-His-Pro-Gly [Lee, 1988 #3458; Masanobu, 1990 #4198]. Like for many other secretory hormones, processing of proTRH then takes place within the RSP *(111,146,147)* to generate its secretion products. Two recently discovered serine proteases, which are members of the family of prohormone convertases, PC1 (SPC3) and PC2 (SPC2), related to subtilisin and the yeast processing enzyme Kex 2 (148–150) are the primary PCs involved in post-translational processing of proTRH *(2,74,147,151)* (Fig. 2). This processing is followed by the action of CPE to remove the basic residue(s) *(152)*. Gln-His-Pro-Gly is then amidated by the action of PAM, which uses the C-terminal Gly as the amide donor, and the Gln residue undergoes cyclization to a pGlu residue to yield TRH.

In this model of rat proTRH posttranslational processing *(74,146)* the 26 kDa precursor is cleaved at two mutually exclusive sites to generate the first intermediate forms (Fig. 1). One cleavage generates a 15 kDa N-terminal peptide (preproTRH25–151 or 157) and a 10 kDa C-terminal peptide (preproTRH154 or 160–255). An alternate cleavage generates a 9.5 kDa N-terminal peptide (preproTRH25–106 or 112) and a 16.5 kDa C-terminal peptide (preproTRH109 or 115–255). These cleavage steps occur in the TGN, prior to packaging into ISGs *(111,153)*, in agreement with similar studies of the cellular location of early processing for POMC and somatostatin (SRIF) *(112)*. However, in later stages of proTRH processing, while the 15 kDa N-intermediate peptide remains unprocessed in this compartment, the 16 kDa C-terminal intermediate was further processed at basic residues 206–207 to the 5.4 kDa C-terminal peptide. In contrast, the 15 kDa N-terminal intermediate appeared to undergo processing in a post-GC compartment, that is, the SGs. This observation strongly suggests that these two intermediates follow different paths of processing *(153)*.

Evidence supporting differential distribution for the N- and C-terminal peptides comes from recent immunocytochemical (ICC) and immunoelectron microscopy (IEM) studies using transfected AtT$_{20}$ cells encoding preproTRH cDNA and in primary cultures of hypothalamic neurons *(2,5)*. In transfected AtT$_{20}$ cells the data indicated that while the 15 and the 6kDa N-terminal intermediates, are located in the GC and TGN, end products including preproTRH$_{25-50}$, preproTRH$_{160-169}$, and TRH, are only present in SGs along the plasma membrane and in cell processes. On the other hand, the 16.5 and 5.4 kDa C-terminal peptides result in positive immunostaining in the GC, along the plasma membrane, and in cell processes. Thus, C-terminal intermediates appear to reach further along the RSP before processing than their N-terminal counterparts. This differential processing might serve as a mechanism to regulate the timing of production of peptides such as preproTRH$_{160-169}$, preproTRH$_{178-199}$ and preproTRH$_{53-75}$, and possibly TRH. For example, the 16.5 kDa intermediate, which is processed in the TGN, contains preproTRH$_{178-199}$, and preproTRH$_{160-169}$. A portion of such peptides, formed prior to their entry into SGs, might exit the cell via the constitutive pathway to maintain a basal level of release independent of TRH secretion. Similarly to transfected AtT$_{20}$ cells, the distribution pattern for N- and C-peptides derived from proTRH primary cultures of hypothalamic neurons were also different *(2)*. The contrasting staining patterns for the two antisera (N- and C-terminal) suggest the existence of a different peptide distribution for N-terminal versus C-terminal peptides, possibly, due to different intracellular routing of intermediates to SGs. However, no conclusive data are yet available to confirm this hypothesis.

Tissue-Specific Processing of proTRH Regulates the Output of proTRH-Derived Peptides

In recent years it became evident that tissue-specific processing of proTRH in the brain is a key mechanism to generate several different proTRH-derived peptides in addition to, or instead of, TRH. ProTRH processing products such as TRH involved in the regulation of the HPT axis are produced in the PVN. The largest concentration of hypothalamic TRH neurons outside of the PVN are found in the dorsomedial nucleus, lateral hypothalamus, and preoptic area, including medial, periventricular, suprachiasmatic, and the sexual dimorphic nucleus of the preoptic area *(10)*. In addition to the PVN, TRH neurons are present in many other regions of the CNS including regions in the diencephalon, telencephalon, mesencephalon, myelencephalon, and spinal cord. An extensive anatomic description of TRH neurons and TRH fibers in these tissues has been reported earlier *(10)*. In several areas of the brain where production of proTRH is found, TRH and proTRH-derived peptides are also detected, but the levels of TRH and other proTRH-derived peptides vary. For example, the reticular nucleus of the thalamus contains abundant preproTRH mRNA and several proTRH-derived peptides in their

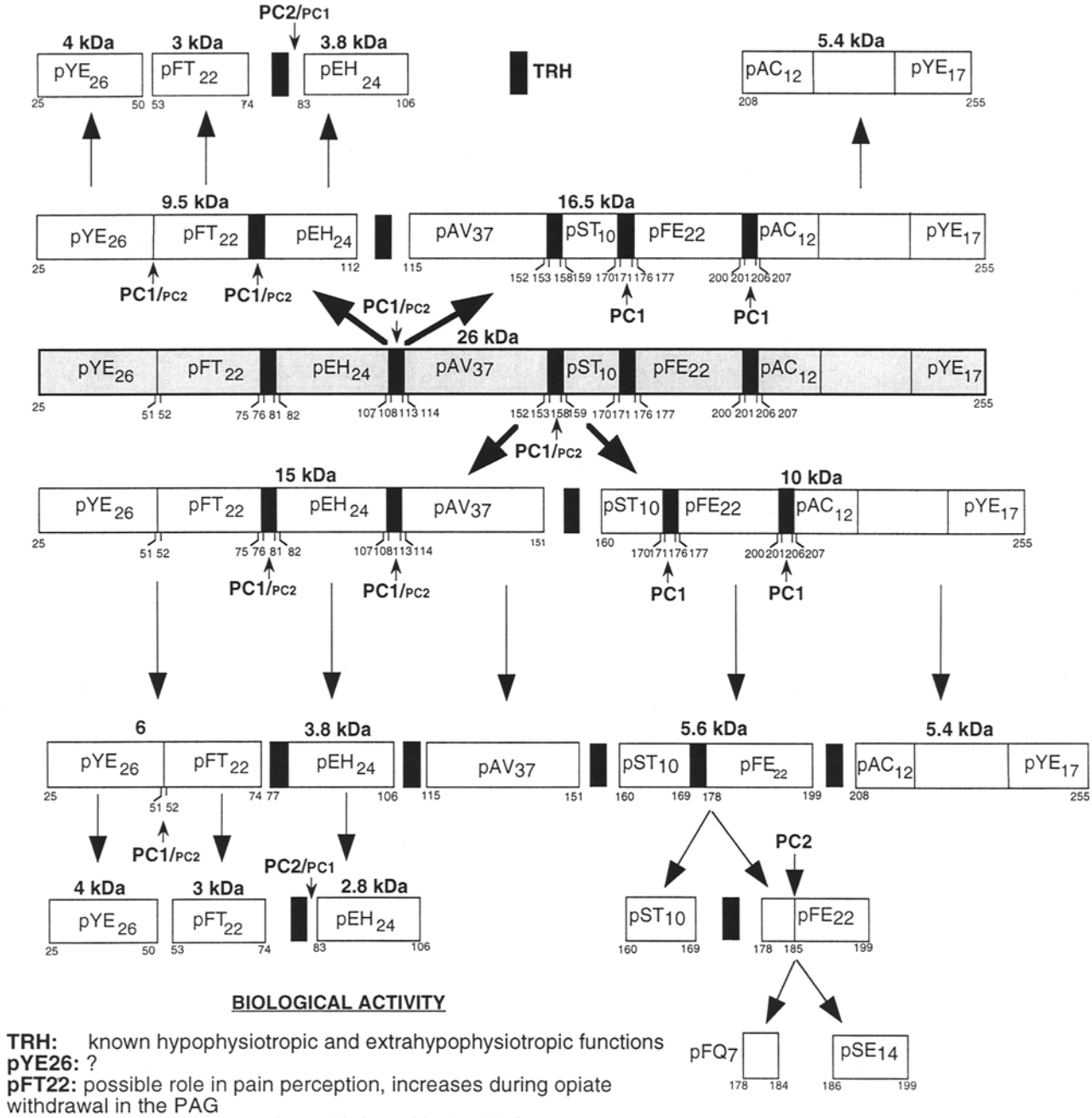

Fig. 1. Representation of the current model of rat proTRH processing, indicating the role of PC1 and PC2. The small arrows indicate PC1 and PC2 activity. Cleavages sites and direction of the processing cascade are indicated with longer arrows. Thicker arrows indicates that most of the initial cleavage of the intact precursor was produced at this site. The positions of paired basic residues are indicated by numbers. NonTRH peptides are indicated in the shaded proTRH molecule, and TRH is indicated by a black rectangle.

extended forms, but does not contain mature TRH *(154)*. Moreover, the N-terminal extended forms of TRH, TRH-preproTRH$_{160-169}$ and TRH-preproTRH$_{178-199}$ are major end products of proTRH processing in the olfactory lobe (OB) *(155,156)*, but not in the hypothalamus where proTRH is completely processed to its mature peptides and TRH *(155)*. In the ME, PVN, and preoptic area (POA), proTRH is fully processed to its mature forms, while in the OB less than

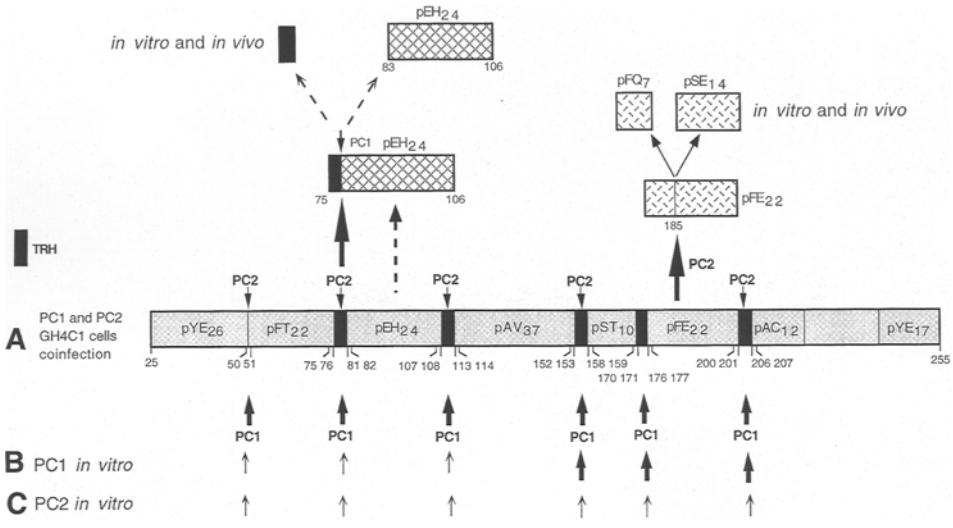

Fig. 2. Representation of rat proTRH and its cleavage by PC1 and PC2 as showed in our previous studies done *in vitro* and coexpression conditions. The arrows indicate the site of cleavages and whether they are major (thick arrow) or minor (thin arrow) sites for each enzyme.

60% of N-terminal preproTRH$_{25-50}$ is formed. Similarly, while in the OB the 10kDa C-terminal intermediate (see Fig. 1) is the main end product, in the ME and PVN this intermediate is fully processed to preproTRH$_{160-169}$ and preproTRH$_{178-199}$ (Fig. 1). Finally, in the POA partial processing of the 10 kDa peptide is observed, and the lateral hypothalamus contains lower levels of both N- and C-terminal proTRH-derived peptides as compared to the ME and POA (Nillni, unpublished data). These data show that even though the mRNA for preproTRH was present in all these tissues, the output of final products are probably dependent in great part of the presence of active PCs by regulating the prohormone processing *(5,157)*.

The PCs are a family of seven subtilisin/kexinlike endoproteases including furin, PC1 (also known as PC3), PC2, PC4, PACE4, PC5-A (also known as PC6-A), its isoform PC5-B (also known as PC6-B), PC7 (also known as LPC), PC8 (also known as SPC7) *(101,148–150,158–161)*. The structure of these serine proteinases resemble both the bacterial subtilisins and yeast kexin *(162–164)*. These enzymes cleave at the C-terminal side of single, paired or tetra basic amino acid residue motifs *(99)*, followed by removal of remaining basic residue(s) by carboxypeptidase enzymes (CPs) *(108,109)*. The selective expression of PC1 and PC2 in endocrine and neuroendocrine cells suggests they are significant in prohormone processing *(148,158,163,165)*. PC1 and PC2 have been shown to process proTRH *(74,147,151,157)*, proinsulin *(99,166,167)*, proenkephalin *(168)*, prosomatostatin *(169,170)* and POMC *(171,172)* to various intermediates and end products in coexpression experiments.

The distribution of neurons containing mRNAs encoding preproTRH, PC1, and PC2 in the PVN and other areas of the brain has been determined using *in situ* hybridization *(157)*. The glomerular layer of the OB and the lateral hypothalamus displays coexpression of preproTRH with PC2 mRNA, but not PC1, whereas in the tenia tecta coexpression of mRNA for preproTRH with PC1, but not PC2, is evident. On the other hand, the PVN displays preproTRH mRNA coexpression with both enzymes. Double *in situ* hybridization indicates that in the PVN, PC2 mRNA is present in 60–70% of TRH neurons, and PC1 in 37–46% of TRH neurons *(173)*. Even though these investigators found a trend for more coexpression of mRNA for preproTRH with PC2 than PC1, coexpression alone does not define which enzyme is more important in the processing of proTRH in vivo.

During the last few years, this laboratory has provided unequivocal evidence for the role of PC1 and PC2 in the processing of proTRH *(2,74,147,151)*. In coinfection experiments, where recombinant vaccinia viruses are used to coexpress PC1, PC2, PACE4, PC5-B, and furin together with rat preproTRH in constitutively secreting LoVo cells or in the regulated-secreting endocrine GH_4C1 cell line, RIA of LoVo-derived secreted products demonstrates that furin cleaves the precursor to generate both N- and C-terminal intermediates, while PC5-B does not produce any peptide. PC1, PC2, and PACE4 only produce N-terminal intermediates and less efficiently than furin. Interestingly, in LoVo cells furin cotransfection produces TRH-Gly at much greater levels than any of the other PC enzymes. Recent data indicate that furin, which is ubiquitously expressed in all tissues, may serve a role in processing of prosomatostatin within the constitutive pathway *(169,170)*. Since LoVo cells only contain the constitutive secretory pathway, these results suggest that proTRH can be processed to a certain extent without entry into the RSP. However, caution must be taken with this interpretation because under conditions of viral expression, the unusually high level of virus in coinfected cells can produce disruption of

cellular compartments. The products resulting from coexpression of preproTRH with either furin or PC1 are similar, in agreement with their similar specificity observed in a number of cell coexpression experiments *(168,170)* and in vitro data *(174)*.

In GH_4C1 cells, PC1, PC2, furin, PC5-B, and PACE4 produce both N-terminal and C-terminal peptides derived from proTRH. Significantly, TRH-Gly and TRH are produced in highest amounts by PC1, PC2, and furin. Further analysis of the cleavage specificity of PC1 and PC2 reveals that PC1 is primarily responsible for cleavage of the entire TRH precursor to mature TRH, as it can generate all products at significantly higher levels than PC2. While 7B2 is known to be involved in the maturation of PC2 *(163,175)* it does not augment PC2's ability to cleave proTRH to either N- or C- terminal forms. Subsequently, we have examined the role of PC1 and PC2 in the formation of prepro$TRH_{178-199}$ by coexpressing rat preproTRH cDNA with PC1, PC2 and 7B2 in GH_4C_1 cells *(62)*. PC1 effectively cleaved proTRH to immunoreactive forms recognized by anti-prepro$TRH_{178-199}$, while PC2 played a minor role, even in the presence of 7B2 (Fig. 1).

Even though PC1 displays a greater ability to process proTRH than PC2, PC2 can process certain regions of the proTRH sequence *(74)*. For example, in cells coexpressing proTRH and PC2, but not PC1, greater quantities of 2.8 kDa peptide (pEH_{24}, preproTRH_{83-106}) relative to 3.8 kDa peptide (TRH-pEH_{24}, preproTRH_{77-106}) are found, while the converse is true for PC1 coexpressing cells. These data suggest that PC2 may be important in generating TRH from this intermediate. In support of a physiological role for both PC1 and PC2, proTRH is coexpressed and colocalized with PC1 and PC2 in primary cultures of hypothalamic neurons *(74)*.

In addition, PC1 was able to generate two prominent moieties derived from proTRH C-terminal intermediate, a 5.6 kDa and a 2.6 kDa peptides which could be detected with an antibody against the prepro$TRH_{178-199}$ sequence. The latter is similar in size to prepro$TRH_{178-199}$. In cells co-infected with PC2 and proTRH, the 5.6 kDa peptide is not present, but the 2.6 kDa peptide and a smaller form of about 1.6 kDa are observed. Microsequencing analysis of prepro$TRH_{178-199}$ peptide incubated in vitro with purified PC2 demonstrates one specific cleavage at Arg_{185} (...Glu^{182}-Leu^{183}-Gln^{184}-Arg^{185}-↓-Ser^{186}-Trp^{187}-Glu^{188}-Glu^{188}-Lys^{189}...) generating two novel peptides, pFQ7 and pSE14 (Figs. 1 and 2).

These two novel peptides, pFQ7 and pSE14, are present in rat PVN, lateral hypothalamus and ME *(62)*. Thus, the antibody generated against the prepro$TRH_{178-199}$ sequence recognizes the 10 kDa peptide, a 5.6 kDa form that probably is prepro$TRH_{160-199}$ *(146)*, a 2.6 kDa peptide that is prepro$TRH_{178-199}$, and two smaller moieties of 1.6 and 0.84 kDa that are proposed to be pSE_{14} and pFQ_7, respectively (Fig. 1). Figure 2 shows a diagrammatic representation of rat proTRH and its cleavage by PC1 and PC2 as proposed from the most recent studies and compared with previous in vitro studies *(62,147,151)*.

Differential processing has been reported for other prohormones, and these differences relate to alterations in the expression of various PCs within different cell types. Proopiomelanocortin (POMC) is processed primarily to ACTH, β-endorphin and N-$POMC_{1-77}$ in the anterior pituitary (melanotrophs). In turn these products are further processed to α-MSH, β-$endorphin_{1-31}$, N-$POMC_{1-49}$ and α-MSH in the intermediate lobe and brain *(1)*. Differential processing of a common polypeptide precursor is dependent upon the processing enzymes expressed in each specific cell type. Proenkephalin, which contains seven identical copies of met-enkephalin, is processed to large intermediate forms in the adrenal medulla, whereas this precursor is cleaved primarily to the pentapeptide met-enkephalin in the brain *(3)*. The biological actions of SP depends on the enzymatic processing of its precursor by the processing enzymes prolyl-endopetidase to yield SP_{5-11}, and endopetidase 3.4.24.11, to yield SP_{1-7}. While SP_{1-7} acts as an analgesic, inhibits aggression and enhances learning and memory, SP_{5-11} enhances pain transmission, stimulates aggression, and blocks learning and memory *(4)*. In the brain, procholecystokinin (proCCK) is processed to produce only CCK8 amide, while in the gut the precursor is cleaved to larger molecules, such as CCK12, 22, 33, 38, 58, and 83 amide *(176)*. Transfection experiments have shown that proneuropeptide Y (proNPY) can be cleaved by cell lines expressing either PC1 or PC2, but proNPY is primarily processed by PC2 in superior cervical ganglia *(177)*. Thus, differential processing of neuropeptides including proTRH, proNPY, POMC, proCCK, SP, and proenkephalin provides a critical mechanism through which cells regulate the levels of specific peptides to fulfill different physiological requirements, a mechanism potentially more versatile than the alternative splicing of mRNA.

Carboxypeptidases remove remaining C-terminal basic residues from prohormone intermediates that are initially cleaved by PCs. Experiments with the *fat/fat* mouse model of CPE deficiency *(178)* support a role for CPE in the processing of proTRH *(152)*. Mice homozygous for the *fat/fat* mutation are obese, diabetic, and infertile. These mice have a missense (Ser to Pro) mutation at CPE residue 202 that abolishes enzymatic activity *(178)*. Hypothalamic TRH levels are depressed 65% in *fat/fat* mice relative to heterozygous controls. SDS-PAGE demonstrates hypothalami from both *wt/fat* and *fat/fat* mice contain moieties different from those of the *wt/wt* mice. Processing products also differ from the wild type mice. These data indicate that the *fat/fat* mutation produces qualitative changes in proTRH processing, and that CPE is involved in the later stages of proTRH processing. Further, since hypothalami from *fat/fat* mice contain immunoreactive TRH, additional CPs must also be able to process proTRH to TRH, assuming that the

TRH detected is not a cross-reactive non-TRH species. CPs such as carboxypeptidase D (CPD), with similar enzymatic properties to CPE, are also present in compartments of the secretory pathway, and are distributed in many tissues, including the brain *(108)*.

In *fat/fat* mice, levels of TSH, T3, and T4 were normal, suggesting that 34% of normal TRH levels is sufficient to maintain thyroid function. This last observation is important because it is hypothesized that the five identical progenitor sequences of TRH contained in the prohormone may not be processed to mature TRH at all times, and that only few of them may be needed to maintain thyroid function.

TRH and preproTRH160–160 Action on their Target Cells and Their Regulated Degradation

Full-length cDNA clones for the first TRH receptor (TRH-R1) have been identified in mouse *(179)*, rat *(180)*, and human *(181)*. Though there are splice variants for rat and mouse that differ at their C-terminus, functional differences between these isoforms, or characterization of their relative occurrence in tissues and under various physiological conditions, has not been determined *(182)*. A second TRH receptor, TRH-R2 has recently been cloned *(183,184)*. This G-protein coupled receptor is approximately 50% homologous to the TRH-R1 at the amino acid level, and modulates calcium influx upon binding TRH. The expression of TRH-R2 is unique from that of TRH-R1, being restricted to the CNS, and being enriched in the spinothalamic tract, dorsal horn of the spinal cord, pontine nuclei, and cerebellum. This distribution in the first two sites is suggestive of a role in sensory perception and antinoception, while the later two areas of expression suggest a role in motor control. The identification of TRH receptor species, be they subtypes or members of a new family, with distinct pharmacological and/or neuroanatomical profiles is critical if we are to utilize our growing knowledge of TRH functions in the development of clinically useful therapeutics.

Of particular interest to the potential clinical usefulness of TRH or TRH analogues is the phenomeneon of TRH receptor desensistization. Pituitary TRH receptors after several hours exposure to TRH display markedly reduced TSH, but not, prolactin release. The IP3 response to TRH displays homologous desenitization in as little as 10 seconds of TRH exposure in transfected HEK 293 cells *(185)*. This occurs by rapid uncoupling of the receptor, and a decrease in PPI-PLC activity. In the same model system, intracellular calcium mobilization displays heterologous acute desensitization, with effects on other receptors whose signal transduction also depends on calcium elevation *(186)*. While most G-protein coupled receptors undergo acute desensitization by phosphorylation *(187)*, this has not been demonstrated for TRH receptors. Neither a specific protein kinase, nor calcium concentration, have been clearly implicated in TRH receptor desnistization *(188)*.

A second principal mechanism for TRH receptor desensitization is agonist-induced internalization *(189)*. Up to 80% of TRH receptors are internalized by pituitary cells, with a half-time of 2–3 minutes *(189)*. Thus, this mechanism is utilized by TRH receptors to a greater extent than for many other G-protein coupled receptors, and may represent a significant mechanism for clearance of secreted TRH. However, recent studies indicate that desensitization does not depend upon internalization, that is, if internalization is blocked, receptor uncoupling can still mediate desensitization *(190)*. This is similar to the angiotensin II and muscarinic M3 receptors that are also coupled to G_q and G_{11}. While the TRH receptor undergoes internalization and recyclization without ligand binding, this "housekeeping" function is slow relative to TRH-induced endocytosis *(191)*. TRH receptor-ligand complexes are internalized in clathrin-coated vesicles *(191)*. A portion of the receptor is targeted to lysosomes, while the remainder is recycled to the cell surface. Similarly, the ligand may remain associated with the receptor to return to the cell surface, or if it dissociates intracellularly, will be degraded in lysosomes, or possibly, reach the cell surface as well *(192)*. Internalization of the TRH receptor is dependent upon sequence motifs within its C-terminus *(193)*, as well as sequences within the second transmebrane region or third intracellular loop that are necessary for G-protein coupling *(192)*. Further, optimal rates of internalization appear to require coupling to $G_{q/11}$ and PPI-PLC *(194)*.

Over longer periods of time, TRH receptor binding is also reduced by downregulation of receptor number. TRH receptor downregulation occurs in response to TRH, thyroid hormones, and agents that raises cAMP levels *(188,195)*.

Of the other proTRH-derived peptides, only prepro-$TRH_{160-169}$ has characterized receptor binding *(196)*. Receptors for prepro$TRH_{160-169}$ seem to be of a single class, with a higher affinity for [Tyr^0] pST_{10} (IC50 = 8.3 ± 1.2 n*M*) than the native pST_{10} (IC50 = 9.3 ± 1.2 μ*M*). Recent studies indicate that pST_{10} receptors co-segregate with S-100 protein positive cells in pituitary cultures, supporting their expression in the folliculostellate cells of the anterior pituitary. Binding sites for pST_{10} are developmentally regulated, with an increase from birth to weaning, then a gradual decline to adult levels at post-natal day 60 *(53)*. Signal transduction by these receptors is not yet characterized. Within the CNS, pST_{10} receptor binding is highest in pituitary, the hypothalamus, spinal cord, and OB, as well as the hippocampus *(197)*. Its receptor binding is also very high in peripheral tissues, being two-thirds of that in the pituitary, in urinary bladder and vas deferens, and in the heart and testis, at a level equivalent to the hypothalamus *(54)*.

Specific enzymes that degrade TRH are found in many tissues including the brain, spinal cord, pituitary, liver, kidney, pancreas, adrenal glands and blood. These enzymes

not only inactivate TRH, but act in concert with biosynthetic processes to determine the steady state levels of TRH, and its metabolites, CHP (also known as histidyl proline-diketopiperazine or His-Pro-DKP) and TRH-OH. An understanding of the biosynthesis and processing of proTRH is critical to appreciating how, when and where modulation of this central regulator of the HPT axis takes place during physiologically appropriate modulation of thyroid function. It is also critical to understanding the function of TRH and other proTRH-derived peptides in extrahypothalamic regions of the brain, or outside of the nervous system. Four key enzymes breakdown TRH: PAP 1, PAP II, and thyroliberinase give rise to the stable cyclized metabolite CHP, and prolyl endopeptidase gives rise to the deamidated free acid, TRH-OH *(198)*. In the CNS, the soluble PAP I and prolyl endopeptidase, and the membrane-bound PAP II, are the principal enzymes acting to metabolize TRH *(199)*. TRH degradation in serum and many peripheral tissues is through the serum enzyme thyroliberinase *(15)*. Pyroglutamyl aminopeptidase I (PAP 1) (EC 3.4.19.3) is a soluble cysteine protease that removes the N-terminal pyroGlu residue from TRH *(199)*. It also cleaves peptides such as LHRH, NT and bombesin. Prolyl endopeptidase (EC 3.4.21.26) is a soluble serine protease that cleaves on the carboxyl side of of the TRH proline residue to generate TRH-OH *(199)*. It also acts on other neuropeptides, including LHRH, NT and SP. These two enzymes are present in many of the same tissues, so that both CHP and TRH-OH can be generated from TRH.

Displaying greater substrate specificity is the ectoenzyme pyroglutamyl aminopeptidase II (PAP II) (EC 3.4.19.6) *(200)*. Like PAP I, this 260 kDa metalloenzyme removes pyroGlu from TRH. The distinguishing features of PAP II are its greater substrate specificity, and being membrane bound. PAP II is present in CNS synaptosomal fractions, in adenohypophyseal plasma membrane, and liver and serum particulate fractions. Highest activity is observed in the hippocampus and cerebral cortex. PAP II has been identified in many species, being highest in rabbit CNS, and in most cases retains similar features. However, its substrate specificity in bovine synaptosomes, where it has been extensively characterized, is not as narrow as in other species *(201)*. The localization of PAP II in the CNS is consistent with its proposed role in the degradation of synaptic TRH *(202)*.

Thyroid hormone regulates PAP II in the anterior pituitary but not in the brain *(203,204)*. In the PVN, TRH levels and PAP II activity do not correlate during pregnancy and lactation, indicating that PAP II is not the principal determinant of TRH levels *(205)*. PAP II activity does vary with the estrous cycle *(205)*. Further, in brain areas other than the hypothalamus PAP II activity decreases from days 9–20 postnatally coincident with increases in TRH and decreases in CHP *(206)*, indicating PAP II activity can be a critical determinant of TRH steady state levels in some tissues. In sum, it appears that PAP II is in areas under prominent endocrine control, such as the pituitary and PVN, subserves a different role from that in non-endocrine tissues. Thyroliberinase, a fourth TRH-degrading enzyme, present in serum, is similar to PAP II but does not have the transmembrane anchor of PAP II. Like PAP II, thyroliberinase displays greater substrate specificity that PAP I or prolyl endopeptidase *(207,208)*. Thyroliberinase may regulated by thyroid hormone; TRH half life ranges from about 2 minutes in the plasma of thyrotoxic animals to 6 minutes in hypothyroid animals. In the humans, the half life of TRH is similar *(15)*.

The physiological significance of the soluble enzymes PAP I and prolyl endopeptidase within the brain and spinal cord are unclear, since in the case of neurotransmitter inactivation, TRH is probably degraded outside the neuron by ectoenzymes located on the cell surface, or within lysosomes after endocytosis. Membrane-bound ectoenzymes that are specific for TRH, such as PAP II, are more logically located for hydrolysis of synaptically released peptides. Soluble enzymes are better situated to control degradation of TRH during its transport in the hypophyseal portal vessels, and systemic TRH. The exact mechanisms that control the amount of TRH that ultimately reaches the pituitary remain to be elucidated.

Conclusions

In this review an overview was presented of the current knowledge of proTRH biosynthesis, its processing, its tissue distribution, and the role of known processing enzymes in proTRH maturation. The neuroendocrine regulation of TRH biosynthesis, the biological actions of its products, and the signal transduction and catabolic pathways used by those products were also reviewed. ProTRH is widely distributed in the hypophysiotropic and extrahypophysiotropic areas of the brain. The widespread expression of proTRH, PC1, and PC2 mRNAs, with their overlapping distribution in many areas of the rat CNS, indicates the striking versatility provided by tissue-specific processing in generating quantitative and qualitative differences in nonTRH peptide products as well as TRH. A most striking example is the reticular nucleus of the thalamus, where PC1 and PC2 are not coexpressed with proTRH. TRH is not produced in this nucleus, indicating a central role for PC1 and PC2 in maturation to TRH. However, other proTRH intermediates are present in the reticular nucleus, suggesting PCs other than PC1 and PC2 might be involved in processing of proTRH for this particular region of the CNS *(157)*.

Evidence is presented suggesting that differential processing for proTRH at the intracellular level is physiologically relevant. The data indicate that PC1 is primarily responsible for most proTRH cleavage events. PC2 is involved in specific processing events that occur later in the secretory pathway, specifically in the formation of the sec-

ond TRH molecule from the N-terminal side of prepro-TRH_{83-106}, and the proteolytic cleavage of preproTRH$_{178-199}$ to generate the novel pFQ7 and pSE14 peptides. It is clear that control over the diverse range of proTRH-derived peptides within a specific cell is accomplished most from the regulation at the posttranslational level rather than the translational or transcriptional levels. Four examples supporting this hypothesis are presented in this review. A) proTRH processing in the PAG is regulated during the opiate withdrawal, so that levels of TRH remain unchanged, but other proTRH-derived peptides are induced, B) proTRH processing is regulated during suckling, where a selective yet dramatic increase in preproTRH$_{178-199}$ and prepro-TRH$_{186-199}$ peptides is observed, C) leptin selectively stimulates a subpopulation of hypothalamic neurons enhancing proTRH biosynthesis and TRH released, and D) in the absence of transcriptional effects, glucocorticoids induce differential processing of proTRH in both primary cultures of pituitary cells and transfected AtT_{20} cells encoding preproTRH cDNA.

A better understanding of proTRH-derived peptides role represents an exciting new frontier in proTRH research. These connecting sequences in between TRH molecules to form the precursor protein may function as structural or targeting elements that guide the folding and sorting of proTRH and its larger intermediates so that subsequent processing and secretion is properly regulated. The particular anatomical distribution of the proTRH end products, as well as regulation of their levels by neuroendocrine or pharmacological manipulations supports a unique biological role for these peptides. Some of these roles, such as for preproTRH$_{160-169}$, will be within the HPT axis, while many others will be unrelated to traditional thyroid function.

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