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The biosynthesis and processing of neuropeptides: lessons from prothyrotropin releasing hormone (proTRH)

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1. ABSTRACT

The biosynthesis of prohormone-derived peptides is a complex cellular process, which requires specific cleavage, sorting, and modifications of the peptides before the final generation of the bioactive products. In this review, we describe the current knowledge of the cell biology of a key prohormone: proThyrotropin Releasing Hormone (proTRH), which is the precursor of the TRH peptide. In particular, we focus on the biosynthesis of the hypophysiotropic TRH, which is produced in the hypothalamic paraventricular nucleus (PVN) and is the main activator of the hypothalamic-pituitary-thyroid (HPT) axis. Recently, we showed that the regulation of the biosynthesis of TRH in the PVN also occurs at post-translational level through coordinated changes in proTRH processing, by the action of the prohormone convertase (PC1/3 and PC2) processing enzymes. Such regulation, which represents a novel aspect in the regulation of the neuropeptide biosynthesis, ultimately would lead to a more effective processing of prohormones into mature peptides.

2. INTRODUCTION

The biosynthesis of prohormone-derived peptides is a complex cellular process, which requires specific cleavage, sorting, and modifications of the peptides within the Regulated Secretory Pathway (RSP) before the final generation of bioactive products. Interestingly, a large number of researches have focused on the expression of neuropeptide genes, as a first approximation of their regulation. However, in recent years it has become clear that, to fully understand the biology of neuropeptides, one must understand the regulation of processing, sorting and release of the prohormone gene products.

The prohormones are initially synthesized on membrane-bound ribosomes, by which they are translocated into the lumen of the rough endoplasmic reticulum (RER). The newly synthesized polypeptide is transported to the Golgi complex (GC), and then to the *trans*-Golgi network (TGN), which is recognized as the major branch point where trafficking pathways emanate (1, 2). During this

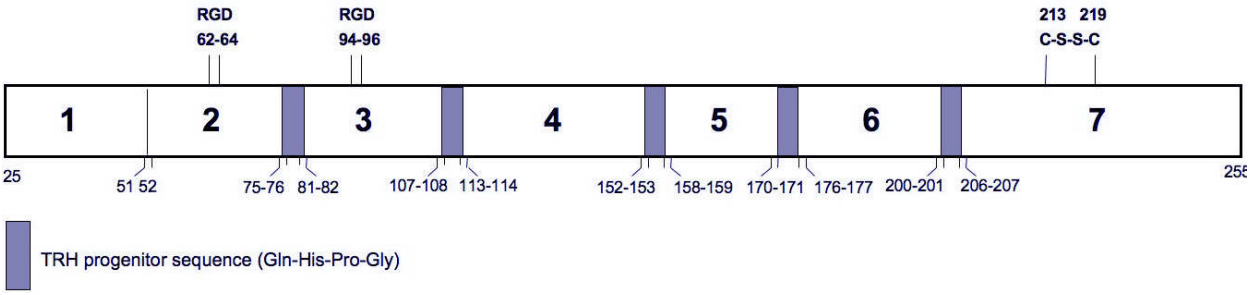

Figure 1. Schematic representation of the proTRH prohormone. TRH progenitor sequence (Gln-His-Pro-Gly) is indicated by a gray rectangle. Large numbers in the proTRH molecule indicate the different ProTRH-related peptides (1: preproTRH$_{25-51}$; 2: preproTRH$_{53-75}$; 3: preproTRH$_{83-106}$; 4: preproTRH$_{115-151}$; 5: preproTRH$_{160-169}$; 6: preproTRH$_{178-199}$; 7: preproTRH$_{205-255}$). Small numbers on the bottom of the proTRH molecule indicate the position of paired basic residues. Additionally, two potential sorting signals, the RGD (Arg-Gly-Asp) motif and a disulfide loop, are indicated on the top of the prohormone.

vectorial transport through the GC and beyond, the polypeptides are subjected to posttranslational modifications including glycosylation, phosphorylation, acylation and proteolysis conversion (3). Moreover, many events in the biosynthesis of peptides, such as cleavage of dibasic sequences, trimming of basic residues, and carboxy-terminal amidation, take place after the packing of the peptides in the secretory granules (SGs). Finally, mature SGs containing peptides are stored in the cell for release upon stimulation (3). All these processes are exquisitely synchronized in cells and may potentially be regulated according to physiological requirements. Recently, the research of these phenomena has given us some insight into which steps during the biosynthesis of neuropeptides can be regulated *in vivo*. In this review, we will describe the current knowledge of the cell biology of ProThyrotropin Releasing Hormone (proTRH), particularly it's processing and sorting. We will focus on the proTRH neurons of the hypothalamic paraventricular nucleus (PVN), since this is one of the most important places where its synthesis takes place. In particular, we will describe how the biosynthesis and processing of this prohormone can be regulated by physiological changes to more efficiently produce bioactive peptides.

3. PROTRH, A MULTIFUNCTIONAL PROTEIN

Rat preproTRH is a 29-kDa polypeptide composed of 255 amino acids widely distributed in many areas of the brain. This precursor contains an N-terminal leader sequence, five identical copies of the TRH progenitor sequence (Gln-His-Pro-Gly) flanked by paired basic aminoacid sequence, and seven proTRH-related peptides (Figure 1). TRH (pyro-Glu-His-ProNH_2) and other proTRH-derived peptides have demonstrated biological activities (4); however, only TRH itself has been extensively studied. The central function of the TRH produced in the PVN is to regulate the hypothalamic-pituitary-thyroid (HPT) axis. TRH is secreted to the portal vessel in the median eminence (ME) and stimulates the synthesis and secretion of thyrotropin (TSH) from the anterior pituitary. TSH, in turn, stimulates thyroid hormones (THs) release, which increase energy expenditure. TRH can also function as neurotransmitter acting on appetite control, thermoregulation, arousal, sleep, cognition, locomotion, antinociception, and psychological functions (4). In contrast to TRH, the biological activities of the other proTRH-derived peptides are poorly known. Evidence of specific actions has been suggested for preproTRH$_{160-169}$, which may enhance TSH release (5), and for preproTRH$_{53-74}$ and preproTRH$_{83-106}$ peptides, which are altered in various regions of the brain of rats experiencing morphine withdrawal (6). Also, rats undergoing suckling, which are hyperprolactinemic, have increased preproTRH$_{178-199}$ levels in the hypothalamic PVN (7). This peptide directly stimulates the release of prolactin by the pituitary, and also regulates a subset of dopaminergic neurons that are involved in the inhibition of pituitary prolactin release. Thus, it seems that preproTRH$_{178-199}$ involved in the secretion of prolactin by acting at two different levels. (Goldstein *et. al.*, in press). The existence of proTRH-derived peptides with both demonstrated and potential biological functions gives this molecule the characteristics of a multifunctional protein, similar to other prohormones, such as pro-opiomelanocortin (POMC).

4. BIOSYNTHESIS OF PROTRH

PreproTRH gene expression is continuously regulated in order to guarantee the production of prohormone levels according to the cellular requirements. The preproTRH promoter contains a TATA and GC box, required for the transcriptional initiation by RNA polymerase II, and several regulatory elements (8). THs act through thyroid hormone receptors (TRs) and mediate transcriptional regulation via interaction with DNA sequence in the preproTRH promoter, referred to as the thyroid hormone response element (TRE) (9). Proximal to the TATA box there is a specific TRE sequence called Site 4, which is critical for the binding of transcription factors, such as Phosphorylated cAMP Response Element Binding protein (P-CREB), and TRs (10). The signal protein Phosphorylated Signal Transducer and Activator of Transcription 3 (P-STAT3) also activates preproTRH gene expression by binding in the promoter to a site called SRE (STAT3-responsive DNA-elements) (11). Glucocorticoid receptor also regulates preproTRH gene expression through specific binding to a consensus sequence (8).

Following transcription, the biosynthesis of proTRH polypeptide follows the same prohormone

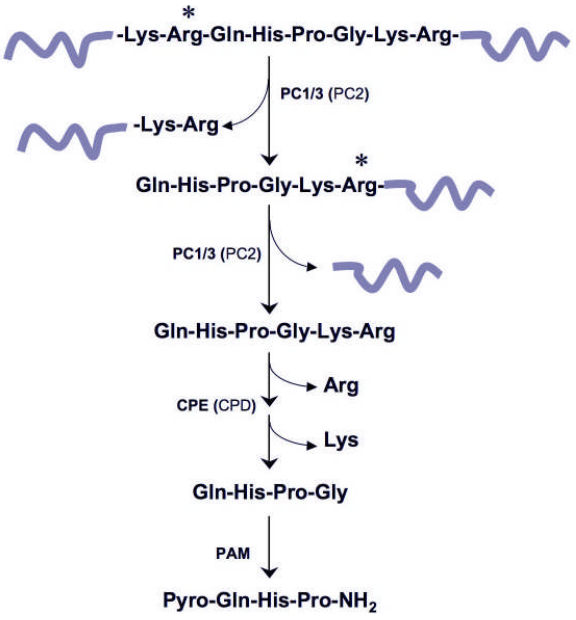

Figure 2. Schematic representation of the proposed post-translational processing of proTRH to generate the TRH peptide. Asterisks indicate the aminoacid where the PCs produce their enzymatic cleavages. Figure shows the enzymes primarily implicated in the processing of proTRH in bold, and the enzymes secondarily implicated in the processing in parentheses.

processing mechanisms described above, beginning with the mRNA-directed ribosomal translation that generates a large precursor called preproTRH. The signal sequence, or pre sequence, directs translocation of preproTRH into the RER after which the signal sequence is removed (4). The proTRH polypeptide precursor (26 kDa), which was identified for the first time in our laboratory, is found in small levels *in vivo* and *in vitro* (12). This suggests that proTRH is rapidly converted to smaller peptides by specific enzymes or, it is very unstable due to its intrinsic chemical characteristics (12). The proTRH is found in the RER, and in less quantity in the TGN, but is presumably not present in the immature SGs generated from the TGN (13). In fact, electron microscopy studies show that GC and immature SGs contain intact or partially processed proTRH, while mature SGs contain only final proTRH-related products (14, 15).

5. ENZYMES RESPONSIBLE FOR THE PROCESSING OF PROTRH

ProTRH is subjected to sequence-specific cleavages in a tissue and compartment-specific manner by processing enzymes (4). These include the prohormone convertases (PCs), which are responsible for the cleavage at the C-terminus of paired basic residues to generate shorter forms. These intermediate peptides are subsequently cleaved by exopeptidases, such as carboxipeptidase E or D (CPE or CPD, respectively), which remove the C-terminal basic amino acids (16). TRH progenitors are further amidated at their C-terminal side by the peptidylglycine α-amidating monooxygenase enzyme (PAM) and the Gln residue undergoes cyclization to a pGlu residue (4, 17). This modification is believed to be essential for peptide stability and to ensure specific recognition with their corresponding receptor (Figure 2).

5.1. Prohormone Convertases (PCs)

PCs are a family of seven subtilisin/Kexin-like endoproteases; the principal PCs acting in the RSP of neuroendocrine cells are PC1/3 and PC2 (18, 19, 20). PCs cleave at the C-terminal side of dibasic residues, leaving peptides with a pair of basic residues (Lys-Arg or Arg-Arg) extending from their C-terminus. All the PCs contain a proregion, a catalytic region, an adjacent domain termed P domain, and a C-terminal region. (19). Like their substrates, these enzymes undergo a maturation from larger precursor proteins (19). In the case of PC1/3, a rapid cleavage of the proregion occurs in the RER; the proregion occupies the active site of the zymogen preventing the premature cleavage of precursor prohormones (19). In the GC, where PC1/3 is highly concentrated, the high pH in this compartment favors the dissociation of the proregion and the 87 kDa-activated form of PC1/3 is able to initiate prohormone cleavages. In the mature SG, the enzyme is C-terminally cleaved into a 66-kDa form (likely intermolecularly), which is more active but has limited stability. It is believed that the acidic, calcium rich SG compartment irreversibly destroys the activity of this form of PC1/3 (19). Recently, an endogenous convertase binding protein, named proSAAS, which inhibits PC1/3 activity, was identified (21). PC2 activation is remarkably distinct from that of PC1/3, probably as a mechanism to control the different role of these enzymes, since PC2 cleaves precursors later than PC1/3 (20). PC2 exists in cells as a precursor for a long period of time; the proenzyme exits the RER as a complex with a neuropeptide essential for the maturation of PC2, named 7B2 (22). This neuropeptide is cleaved in the TGN and the autocatalytic, intramolecular processing of the PC2 propeptide is enabled. The removal of the propeptide from 75-kDa proPC2 results in the formation of active PC2 only if 7B2 has been associated with proPC2 (18, 19).

5.2. Carboxy Peptidases (CPs)

CPs remove remaining terminal basic residues from the prohormone intermediates that are initially cleaved by PCs. CPE has been proposed to be the carboxy peptidase associated with the biosynthesis of many peptides, including TRH (16, 23). CPE is also originally produced from a larger precursor, likely inactive. There are two forms of this enzyme, one membrane bound form of CPE (52-53 kDa) and one soluble form (50 kDa) (24). This enzyme is stored and released with the peptides contained in the SGs (24). CPD is another carboxy peptidase, which catalyzes the same reaction as CPE. This enzyme is localized primarily in the TGN, and in immature SGs (25), and seems to have a secondary role in the biosynthesis of some peptides (16).

5.3. Peptidyl alpha-amidating monooxygenase (PAM)

PAM is a copper-dependent bifunctional enzyme derived from a single gene that produces a variety of enzymatically active products through alternative splicing. Two independent PAM domains catalyze the two steps of

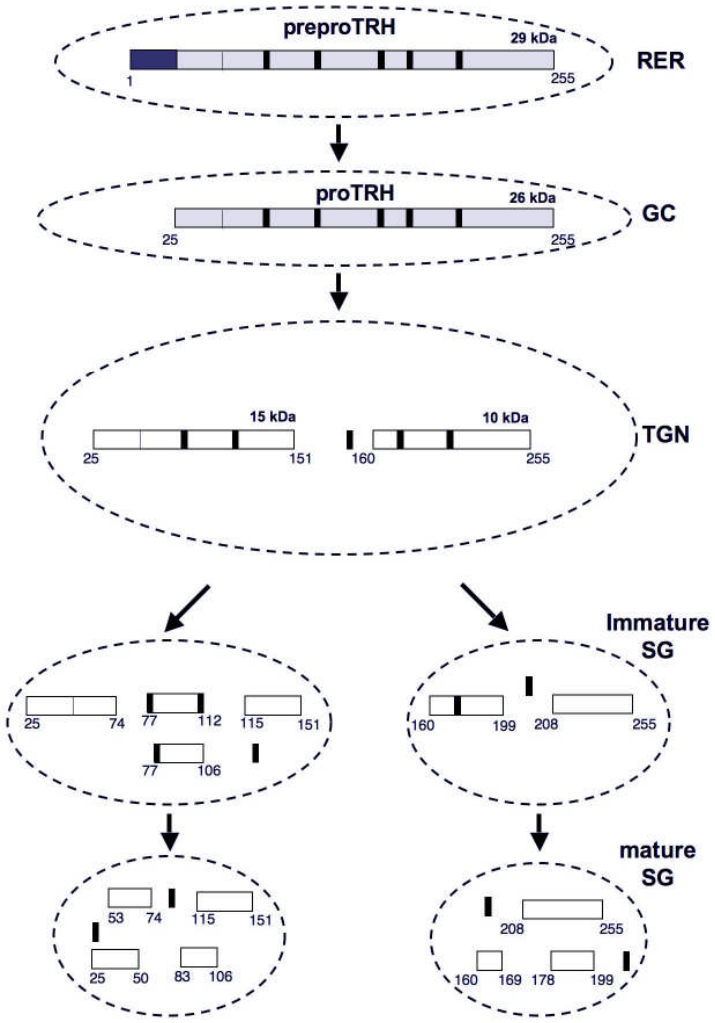

Figure 3. Representation of the proposed sorting of the proTRH-related peptides within the RSP. The figure shows a schematic representation of the major proTRH-related peptides contained in the different organelles of the RSP. For full explanation see the text.

the amidation reaction: a monooxigenase, which converts peptidyl glycine to peptidyl alpha-hydroxy glycine, and a lyase, which completes the conversion to amidated peptide and glyoxylate (17). PAM is subject to tissue specific endoproteolysis that generates independent soluble forms of each domain, as well as, a soluble bifunctional enzyme (17). This enzyme is also stored and released with the peptides from the SGs (26).

6. PROCESSING AND SORTING OF ProTRH

More than one decade ago, we developed the first model of post-translational processing of proTRH utilizing AtT20 cells expressing rat preproTRH (27). Using pulse-chase studies, we determined that the 26-kDa precursor is cleaved at two mutually exclusive sites. One cleavage generates a 15-kDa N-terminal peptide (preproTRH$_{25\text{-}151or157}$) and a 10-kDa C-terminal peptide (preproTRH$_{154or160\text{-}255}$); the alternative cleavage generates a 9.5-kDa N-terminal peptide (preproTRH$_{25\text{-}106or112}$) and a 16.5-kDa C-terminal peptide (preproTRH$_{109or115\text{-}255}$). This first step of processing is dependent of PC1/3 and begins in the TGN generating proTRH processing intermediates prior to packing into immature SGs (13, 14). In subsequent steps, the 15-kDa N-terminal intermediate of proTRH is processed to preproTRH$_{25\text{-}74}$, preproTRH$_{77\text{-}106}$ and preproTRH$_{115\text{-}151}$ while the 10-kDa C-terminal peptide produces preproTRH$_{160\text{-}199}$ and preproTRH$_{208\text{-}255}$. In the alternative cleavage of the 26-kDa precursor, the 9.5-kDa peptide generates preproTRH$_{25\text{-}50}$, preproTRH$_{53\text{-}75}$ and preproTRH$_{83\text{-}106}$ while the 16.5-kDa fragment produces preproTRH$_{160\text{-}199}$ and preproTRH$_{208\text{-}255}$ (4). All these cleavages are mainly produced by PC1/3; however, they can be produced by PC2 (28, 29). Some peptides are further processed as with preproTRH$_{160\text{-}199}$, which generates preproTRH$_{160\text{-}169}$ and preproTRH$_{178\text{-}199}$, or with preproTRH$_{77\text{-}106}$, which generates preproTRH$_{83\text{-}106}$ and a TRH progenitor. These final cleavages occur later in the vectorial transport within the RSP, and are mainly produced by PC2 (Figure 3) (28, 29). The important role of the PCs in

the biosynthesis of proTRH has been elucidated by the studies of PC1/3 and PC2 null mice. Analysis of ProTRH processing in PC1/3 null mice showed a substantial decrease in the biosynthesis of all proTRH-derived peptides including TRH and its proform, TRH-Gly, whereas PC2 null mice showed a minor defect in proTRH processing (Nillni *et al*, unpublished results). The finding that the knockout mice have processing profiles that are expected from the known biochemistry of PC1/3 and PC2 supports the notion that these two enzymes are necessary and sufficient for the biosynthesis of proTRH *in vivo*.

Our initial studies using a combination of brefeldin A (that blocks the RER to GC transport) and temperature blockade (that blocks formation of SGs) gave important information about the intracellular processing of proTRH (13). We demonstrated that the 26-kDa proTRH precursor is cleaved early in the RSP. The 16.5-kDa C-terminal intermediate is further cleaved in the TGN before packing into SGs; then, smaller peptides are produced in the SGs. Conversely, the 9.5-kDa N-terminal intermediate (or 15-kDa N-terminal intermediate in the case of the alternative cleavage) starts to be processed in the immature SGs. All the PC cleavages are followed by the removal of the paired basic residues by CPE, and CPD (16). Recently, we used the $Cpe^{fat/fat}$ mice, which have a mutation in CPE that makes this enzyme inactive and easily degradable (30), to demonstrate that CPE is primarily responsible for the generation of TRH (16). CPE is found in proTRH neurons, where it converts Gln-His-Pro-Gly-Lys-Arg to the TRH precursor, Gln-His-Pro-Gly (16). CPD mimics the actions of CPE, and is also present in proTRH neurons,; however, the role of CPD is secondary since the $Cpe^{fat/fat}$ mice contain in their hypothalamus 75% less mature TRH than control mice (16). For the final production of mature TRH, Gln-His-Pro-Gly is 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 (27).

The mechanism of sorting to the RSP and the nature of the protein motifs critical for this process are still a matter of debate (31). Several structural elements have been related to the sorting within the RSP. The N-terminal hydrophobic domain might represent a sorting signal for some proteins (32). Also, a disulfide loop in the N-terminal region was found to be essential for targeting some precursors to the RSP (33-35). In the case of POMC, the disulfide bond sequence would presumably bind to CPE (35), but whether this enzyme is a sorting receptor in the TGN is still disputed (36, 37). Dibasic sequences are numerous in prohormone precursors and could also be sorting signals (38, 39). Additionally, the RGD (Arg-Gly-Asp) motif has also been implicated in trafficking within the RSP (40). ProTRH contains 11 pairs of basic residues, one disulfide loop and two RGD motifs (Figure 1). We recently showed that the disulfide bond of proTRH is involved in the sorting of proTRH-derived peptides and in their retention in the SGs (41). We, also, showed that the initial processing of proTRH by PC1/3 in the TGN, and not a cargo-receptor relationship, is important for the downstream sorting events that result in the storage of proTRH-derived peptides in mature SGs (42). Interestingly, we found that the peptides derived from the C- and N-terminal sides of proTRH are located in different SGs *in vivo* and *in vitro*, indicating differential sorting of proTRH-derived peptides within the RSP (Perello *et. al.*, submitted manuscript). Therefore, we speculate that the first cleavage by PC1/3 triggers the exposure of sorting signals in the first proTRH intermediates, and initiates a differential sorting that determines the location of the final products in different compartments (Figure 3). Notably, specific protein motifs in the prohormone are sufficient to determine the sorting of proTRH-related peptides into the RSP, since they were also differentially located when proTRH was expressed in AtT20 cells. Finally, it important to point out that most of the work done on proTRH processing and sorting has been done in cultivated tumoral cells lines. However, this processing model appears to be very similar *in vivo*, as we have corroborated using hypothalamic micro dissection analysis (7, 43, 44).

7. PROTRH SYNTHESIZING NEURONS IN THE HYPOTHALAMIC PARAVENTRICULAR NUCLEUS (PVN)

ProTRH neurons are present in several hypothalamic nuclei including the preoptic area, anterior hypothalamus, dorsomedial nucleus, premammilary nucleus, and lateral hypothalamus (4). However, just the "hypophysiotropic" proTRH neurons, located in the hypothalamic PVN, regulate pituitary TSH secretion. The PVN is a symmetrical and triangular group of neurons located in the dorsal side of the third ventricle and composed of two parts: the magnocellular and the parvocellular parts (45). ProTRH neurons are located in the anterior, medial, and periventricular parvocellular subdivisions of the PVN, however, only the proTRH neurons located in the medial and periventricular region project to the ME (46, 47).

Three brain regions are known to innervate and regulate the hypophysiotropic proTRH neurons: the $A_{1,2}/C_{1-3}$ nuclear groups in the medulla, the hypothalamic arcuate nucleus (ARC), and the hypothalamic dorsomedial nucleus (DMN) (47). Axons coming from the medulla contain catecholamines, such as norepinephrine (NE), and cocaine and amphetamine regulated transcript (CART) (48). From the ARC, the POMC/CART neurons and the agouti-related protein (AgRP)/neuropeptide Y (NPY) neurons send fibers to the PVN (49, 50). The proTRH neurons in the parvocellular subdivision are heavily innervated by AgRP-containing fibers (51); in contrast, only one third of these proTRH neurons are innervated by POMC neurons (50). The hypothalamic DMN heavily innervates the proTRH neurons in the PVN; but, unfortunately, the neurotransmitters contained in these fibers are unknown (52).

8. REGULATION OF THE BIOSYNTHESIS OF PROTRH IN THE PVN

Several hormones, neuropeptides, and neurotransmitters regulate proTRH biosynthesis in the PVN. Clear effects have been shown for: alpha-melanocyte

stimulant hormone (α-MSH, which is a POMC-derived peptide), AgRP, NPY, CART, NE, THs, glucocorticoids, and leptin. Potent regulators of the biosynthesis of proTRH are the THs, which control the HPT axis activity at hypothalamic levels as part of the negative feedback action. Hypothyroidism increases preproTRH mRNA levels; conversely, TH administration inhibits the hypothyroidism-induced rise of preproTRH gene expression (53-55). TH/TRs complexes bind to the negative TRE in the preproTRH promoter regulating its transcription (56, 57). Recently, we showed that the plasma TH levels are inversely related to the biosynthesis of proTRH and TRH specifically in the PVN (44). Leptin is another potent regulator of the biosynthesis of proTRH. During fasting, when leptin levels are low, the biosynthesis of proTRH decreases in the PVN, resulting in low plasma levels of TSH and THs (58, 59). Leptin treatment reverses these changes, indicating that the activation of the HPT axis is a component of the leptin-induced increase of energy expenditure. Part of the regulation of proTRH neurons by leptin is mediated by a direct action of the hormone on the PVN (60). This is supported by the fact that ObRb is present in proTRH neurons in the PVN, and that leptin stimulates proTRH gene expression in these neurons via binding of STAT3 to SRE in the preproTRH promoter (11, 60, 61). Although it is accepted that glucocorticoids regulate proTRH biosynthesis, the experimental data is contradictory. Glucocorticoids increase proTRH at transcriptional and posttranslational levels in different *in vitro* systems; however, they suppress preproTRH mRNA in the PVN when used *in vivo* (62-65). It has been suggested that the direct positive regulation of glucocorticoid is overridden *in vivo* by an indirect negative regulation. Nevertheless, more studies are necessary to clarify these findings.

Neuronal inputs are also important regulators of the biosynthesis of proTRH in the PVN. Particularly, ARC-derived neuropeptides play a significant role in the regulation of proTRH neurons. The neuropeptides produced by POMC/CART neurons, α-MSH and CART, stimulate the proTRH neurons while NPY and AgRP neuropeptides, produced by the other population of ARC neurons, inhibit the biosynthesis of proTRH (49, 50, 66-68). Intracerebroventricular (icv) administration of α-MSH can fully restore the fasting-induced decrease of preproTRH mRNA in the PVN of starved rats, however, it only partially restores the fasting-induced decrease of THs (50). Similarly, CART icv administration completely restores the fasting-induced decrease of proTRH mRNA in the PVN in fasted rats; however, it does not restore the fasting-induced decrease of THs (49). In contrast, AgRP and NPY have a potent inhibitory effect on the biosynthesis of hypophysiotropic proTRH; when icv administered in fed rats, they induce a state of central hypothyroidism similar to the fasting-induced state (67, 68). It is believed that these ARC-derived peptides play a significant role in the indirect regulation of proTRH neurons by leptin. NPY, AgRP, CART, and POMC expression are all regulated by leptin *in vivo* (69-71). Leptin could then activate proTRH neurons through these inputs by two mechanisms: a) an inhibitory action of leptin on NPY and AgRP release, leading to a reduced inhibitory effect of these peptides on proTRH biosynthesis and/or b) a stimulatory action of leptin on α-MSH and CART, which then stimulate proTRH biosynthesis. P-CREB signaling is critical during these indirect leptin actions on proTRH neurons. α-MSH binds to the Gs-protein coupled melanocortin 4 receptor (MC4R), which signals by increasing P-CREB levels subsequently activating different genes, including preproTRH (11, 72). AgRP acts as a competitive antagonist or inverse agonist on the melanocortin receptors (73, 74), and blocks α-MSH-induced effects on TRH release (75). NPY action on proTRH neurons is mediated by Y1 and Y5 receptors (76), which couple to Gi-protein and, when activated, decrease P-CREB levels (77). Recently, using nuclear P-CREB staining and a pharmacological antagonist of MC4-R, we showed that the melanocortin system has a primary role in the leptin-mediated activation of hypophysiotropic proTRH neurons (43). The physiologic role of the directly leptin-activated proTRH neurons remains unclear, but interestingly they represent a different subset of neurons within the PVN (43).

Many studies implicate adrenergic input from the medulla as the main neurotransmitter mediator of the activation of the HPT axis in response to cold exposure (78). Early studies showed that the activation of α_1-adrenergic receptors in the ME level increase the release of TRH (79). Although, it has been shown that cold exposure increases preproTRH mRNA in the PVN (80); the specific role of NE on the biosynthesis of proTRH at this level has not been fully studied. In a recent study, we present evidence that NE stimulates preproTRH gene expression and proTRH biosynthesis in cultures of hypothalamic neurons (Perello *et. al.*, submitted manuscript). We also studied the role of NE *in vivo*, and found that β-adrenergic receptor mediates the cold-induced increase in the biosynthesis of proTRH in the PVN (Perello *et. al.*, submitted manuscript). P-CREB signaling could also mediate this response, since β-adrenergic receptors are well known activators of this signaling (81).

The DMN provides an important input to the hypophysiotropic proTRH neurons in the PVN; these innervations are the symmetric type suggesting an inhibitory role (82). Unfortunately, the transmitters through which the DMN control the proTRH neurons are unknown. However, it has been suggested that an important function of this nucleus is to mediate leptin action on the PVN. In support of this, a subset of leptin-responsive neurons in the DMN strongly innervates the PVN (83). Moreover, the DMN may mediate the regulation of proTRH neurons in the PVN by leptin via a multisynaptic ARC-DMN-PVN pathway. This idea is based on the fact that the DMN neurons, which innervate the PVN, are associated with terminal axons containing α-MSH likely from the ARC (84). Therefore, leptin regulation of proTRH neurons of the PVN is a complex system, which includes its direct action on these neurons, and several potential indirect pathways.

Finally, it is important to note that other inputs may also regulate the proTRH neurons in the PVN (15). Potential effects have been suggested for dopamine,

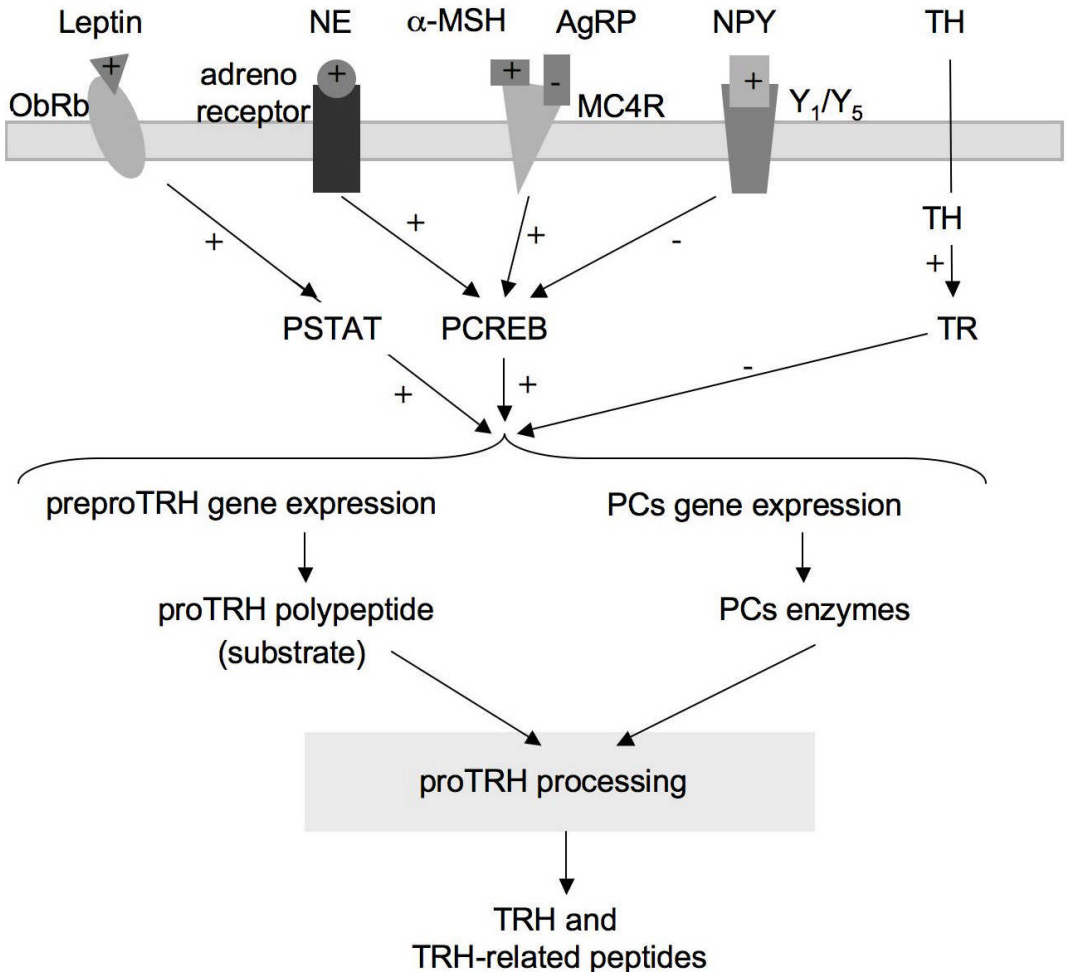

Figure 4. Current model of the regulation of proTRH processing by different factors. The figure shows a schematic representation depicting hormones, neuropeptides and neurotransmitters involved in the proTRH biosynthesis.

serotonin, TRH, histamine, somatostatin, endogenous opiod peptides (such as beta endorphin, enkephalin, and dynorphin), vasoactive intestinal polypeptide, gamma-aminobutyric acid, and cytoquines (such as interleukin 1 or interleukin 6).

9. REGULATION OF THE PROCESSING OF ProTRH IN THE PVN

Recently, we presented evidence supporting that regulation of hypophysiotropic TRH biosynthesis occurs at the post-translational level through coordinated changes in proTRH processing, particularly by the action of PC1/3 and PC2. Thus, we hypothesize that such regulation ultimately leads to more effective processing of pro-neuropeptides into mature peptides, such as TRH. Control of PC1/3 and PC2 function by hormones, neuropeptides, and neurotransmitters is another level at which the production of peptides can be properly regulated.

In a recent study, we demonstrated that thyroid status regulates proTRH processing, representing a novel aspect in the regulation of the HPT axis. Low iodine/PTU diet-induced hypothyroidism, in addition to increasing hypophysiotropic preproTRH mRNA and proTRH biosynthesis (53), increases the processing of proTRH prohormone. In the PVN of hypothyroid rats, we found an increase in the shorter forms of pro-TRH peptides, and an up-regulation in the levels of PCs (44). Conversely, hyperthyroidism induced by TH treatment decreases preproTRH mRNA and TRH levels in the PVN (55), as well as, decreases the post-translational processing of proTRH since proforms of proTRH-derived peptides accumulate, and the PCs are down regulated in this area (44). Likely, THs regulate proTRH processing by direct control of PC1/3 and PC2 expression in this specific hypothalamic nucleus. This is supported by recent findings that PCs promoters contain TREs, which can be negatively regulated by THs (85-87).

We also showed that leptin up-regulates PCs expression/protein biosynthesis in the PVN, and as a consequence the posttranslational processing of proTRH (88). We showed that leptin directly stimulates PC1/3 and

Table 1 Distribution of TRH and the immediate precursor TRH-Gly in the PVN of control, hypothyroid, and hyperthyroid rats

	TRH (fmol/μg)[1]	TRH-Gly (fmol/μg)[1]	Ratio TRH/TRH-Gly
Control	2.6±0.2	0.21±0.01	12.3±0.9
Hypothyroid	5.1±0.4 [2]	0.33±0.02 [2]	15.7±0.9 [2]
Hyperthyroid	1.2±0.2 [2]	0.16±0.01 [2]	7.2±0.8 [2]

TRH and TRH-Gly values were calculated using the specific TRH and TRH-Gly RIA assays, respectively. [1], represents the statistical values in fmol/μg of total protein for each peptide (mean±SEM). [2], represents statistical difference by a multiple comparison using a Newman-Keuls test ($P<0.05$ vs. control animals).

PC2 promoter-activities, likely via P-STAT3. In the PC1/3 promoter there is a known SRE sequence, while a putative SRE can be identified in the PC2 promoter using computer software (88). On the other hand, the leptin-induced increase in the PC levels *in vivo* is partially dependent on the activation of the melanocortin system, as we demonstrated recently (43). Interestingly, PC1/3 promoter contains two CREB response elements, which are transactivated by CREB-1 (89). Furthermore, P-CREB transcription factor could activate in a coordinated fashion the synthesis of preproTRH and PCs in some neurons of the parvocellular division of the PVN. Therefore, leptin can act on the proTRH neurons of the PVN directly (P-STAT) and indirectly via the melanocortin system (P-CREB) regulating in a synchronized manner the biosynthesis of proTRH and the PCs through both pathways.

The cold exposure-induced increase of proTRH levels in the PVN is also coupled to an up-regulation in the biosynthesis of PC1/3 and PC2. The up-regulation of these enzymes is mediated by the activation of β-adrenergic receptors (Perello *et. al.*, submitted manuscript). Similar to the melanocortin system, this activation may occur via the CREB response elements contained in the PC1/3 promoter (89). Changes in the processing of proTRH have been observed previously under other physiological conditions. The preproTRH$_{178-199}$ peptide and its processed forms increase in the PVN during suckling as a result of changes in proTRH processing (7). During opiate withdrawal in rats, the preproTRH mRNA, the preproTRH$_{53-74}$ and preproTRH$_{83-106}$ peptides, and the mature form of PC2 are increased in the periaqueductal grey, whereas the level of TRH remains unaltered (6, 90, 91). Thus, these data show other cases of a region specific regulation of proTRH processing in the brain. Figure 4 represents our current model of the regulation of proTRH processing by different factors.

In addition to the regulation of PCs, other steps in the biosynthesis of neuropeptides can be regulated. We found that proSAAS peptide increases in the ME of starved animals, indicating that low leptin signaling decreases PC1/3 levels and also decreases PC1 enzyme activity via proSAAS inhibition (88). In studies done with the Cpe$^{fat/fat}$ mice, we demonstrated that the deficiency of CPE affects TRH biosynthesis (16). However, CPE levels remain unaltered under different conditions, such as cold exposure, fasting, or changes in thyroid status (Perello *et al,* unpublished data), suggesting that this enzyme is not a checkpoint where the processing of proTRH is regulated. Conversely, PAM is believed to be a rate-limiting enzyme; thus, its levels could be subject to regulation under physiologic conditions (17). Interestingly, levels of PAM mRNA are increased in a coordinated fashion with the prohormone POMC mRNA in AtT20 cells (92). Accordingly, data from our laboratory indicate that PAM activity in proTRH neurons of the PVN is regulated by thyroid status. TRH and TRH-Gly levels increase under low iodine/PTU diet-induced hypothyroidism and decrease under TH-induced hyperthyroidism, as Table 1 shows. However, it is interesting that the ratio TRH/TRH-Gly, which could be a direct measure of PAM activity, changes differentially in these situations. In hypothyroidism, the ratio increases suggesting an increase in PAM activity, and conversely in hyperthyroidism the ratio decreases suggesting a decrease in PAM activity. Therefore, PAM activity may be responsive to THs levels indicating that this regulation may represent another level of control in the final production of mature TRH.

10. PERSPECTIVE

The specific regulation of the processing of prohormones is likely another key checkpoint where final amounts of bioactive peptides can be tightly regulated. We recently showed that regulation of the biosynthesis of POMC in the hypothalamus and in the medulla occurs at the post-translational level through coordinated changes in the PC enzymes (Perello et al, in press). This is consistent with other findings that indicate that similar regulation of the POMC/PCs occurs in seasonal adaptation in hamsters (93). Also, it has been postulated that leptin activates the N-acetylation of □MSH in POMC neurons, which could represent another posttranslational modification that carefully regulates the level of bioactive peptides (94); however, strong evidence supporting this possibility is lacking. Other hypothalamic prohormones, such as pro-corticotrophin-releasing hormone or pro-vasopressin have been shown to be co-regulated with the PCs (95). Therefore, we propose that this could be a common mechanism used by different types of cells to generate bioactive peptides in a more efficient way. Thus, the coordinated regulation of PCs may play an important role in neuroendocrine cells in maintaining a proper enzyme-substrate homeostasis and ensuring adequate processing of newly synthesized prohormones.

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