60 YEARS OF NEUROENDOCRINOLOGY

TRH, the first hypophysiotropic releasing hormone isolated: control of the pituitary–thyroid axis

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Abstract

This review presents the findings that led to the discovery of TRH and the understanding of the central mechanisms that control hypothalamus–pituitary–thyroid axis (HPT) activity. The earliest studies on thyroid physiology are now dated a century ago when basal metabolic rate was associated with thyroid status. It took over 50 years to identify the key elements involved in the HPT axis. Thyroid hormones (TH: T_4 and T_3) were characterized first, followed by the semi-purification of TSH whose later characterization paralleled that of TRH. Studies on the effects of TH became possible with the availability of synthetic hormones. DNA recombinant techniques permitted the identification of all the elements involved in the HPT axis, including their mode of regulation. Hypophysiotropic TRH neurons, which control the pituitary–thyroid axis, were identified among other hypothalamic neurons which express TRH. Three different deiodinases were recognized in various tissues, as well as their involvement in cell-specific modulation of T_3 concentration. The role of tanycytes in setting TRH levels due to the activity of deiodinase type 2 and the TRH-degrading ectoenzyme was unraveled. TH-feedback effects occur at different levels, including TRH and TSH synthesis and release, deiodinase activity, pituitary TRH-receptor and TRH degradation. The activity of TRH neurons is regulated by nutritional status through neurons of the arcuate nucleus, which sense metabolic signals such as circulating leptin levels. *Trh* expression and the HPT axis are activated by energy demanding situations, such as cold and exercise, whereas it is inhibited by negative energy balance situations such as fasting, inflammation or chronic stress. New approaches are being used to understand the activity of TRHergic neurons within metabolic circuits.

Key Words
- HPT axis
- TRH
- TRH receptor
- TSH
- PPII
- cold
- fasting
- stress
- metabolism
- prolactin
- energy balance

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A historical perspective on the hypothalamic control of the thyroid axis

The advancement of any scientific field requires the combination of creative new ideas with the development of technologies and knowledge in related areas; understanding the function of the hypothalamus–pituitary–thyroid axis (HPT) is no exception (Figs 1 and 2). Since the end of the 19th century, European physicians and surgeons associated neck swelling (thyroid enlargement, goiter), with iodine deficiency, cretinism, and myxoedema,

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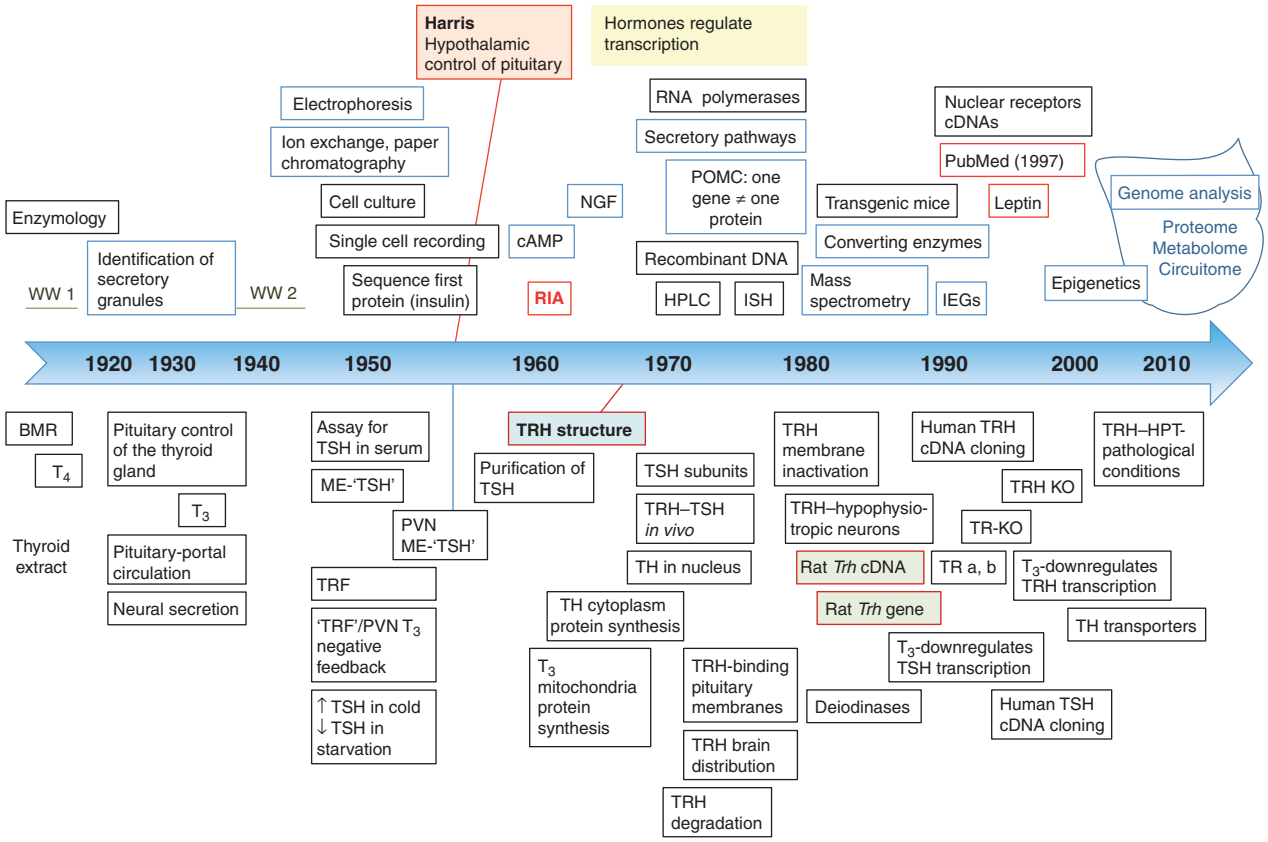

Figure 1

Time line. Figure depicts the principal discoveries that contributed to the actual understanding of TRH neurons and regulation of the hypothalamus–pituitary–thyroid axis (HPT). Above the blue line are marked some of the main findings in techniques or in cellular biology. Below are those related to the HPT axis. Space constraints makes it impossible to cite each piece of work, and some examples represent the ideas and paradigms of various authors. BMR, basal metabolic rate; IEGs, immediate early genes; ISH, *in situ* hybridization; KO, knock out; ME, median eminence; NGF, nerve growth factor; POMC, proopiomelanocortin; PVN, paraventricular nucleus; TH, thyroid hormones; TRF, thyrotropin-releasing factor.

defining hypothyroid conditions. Magnus-Levy (1895) was the first to demonstrate that respiratory metabolism was increased in hyperthyroidism and decreased in myxoedema. Indirect calorimetry allowed measurements of basal metabolic rate (BMR) and the evaluation of thyroid activity in clinical practice (Du Bois & Du Bois 1915, Harris & Benedict 1918). Soon it was recognized that stressful conditions such as fever, acidosis, or starvation modify BMR (Rowe 1920). In 1919, levothyroxine (3,3′,5,5′-tetraiodothyronine or T_4) was characterized, and then synthesized in 1926. Triiodothyronine (3,3′,5-triiodo-L-thyronine or T_3), which proved more active than T_4, was discovered 30 years later (reviewed in Tata 2013). Since RIAs were not available until the 1960s (Yalow & Berson 1959), thyroid function was initially assessed in animals and later in humans, by administering ^{131}I and measuring radioactivity in the neck at different times (Astwood & Stanley 1947), or by cytological methods (de Robertis 1948). The discovery of inhibitors of thyroid function, such as propylthiouracil (PTU), aided in the cure of hyperthyroidism (Astwood 1943). PTU became useful in researching thyroid hormone (TH) metabolism, and in the discovery of different deiodinases (Escobar del Rey *et al.* 1961, Visser *et al.* 1983). The inhibition of T_3-induced BMR activation in hypothyroid rats by cycloheximide helped to elucidate that the actions of T_3 require protein synthesis (Tata *et al.* 1962). The identification of TH receptors (THRs) followed (Tata 2013), unraveling the multiplicity of effects of TH on energy metabolism (Mullur *et al.* 2014). The pituitary control of thyroid activity had been recognized since the beginning of the 20th century, although the purification and identification of thyroid-stimulating hormone (TSH) spanned several decades (Magner 2014). Semi-purified TSH preparations from bovine pituitaries demonstrated a similar structure to other pituitary hormones. It is composed of two subunits (a and b) and contains complex carbohydrate moieties that are essential for bioactivity and clearance (Pierce *et al.* 1971, Weintraub

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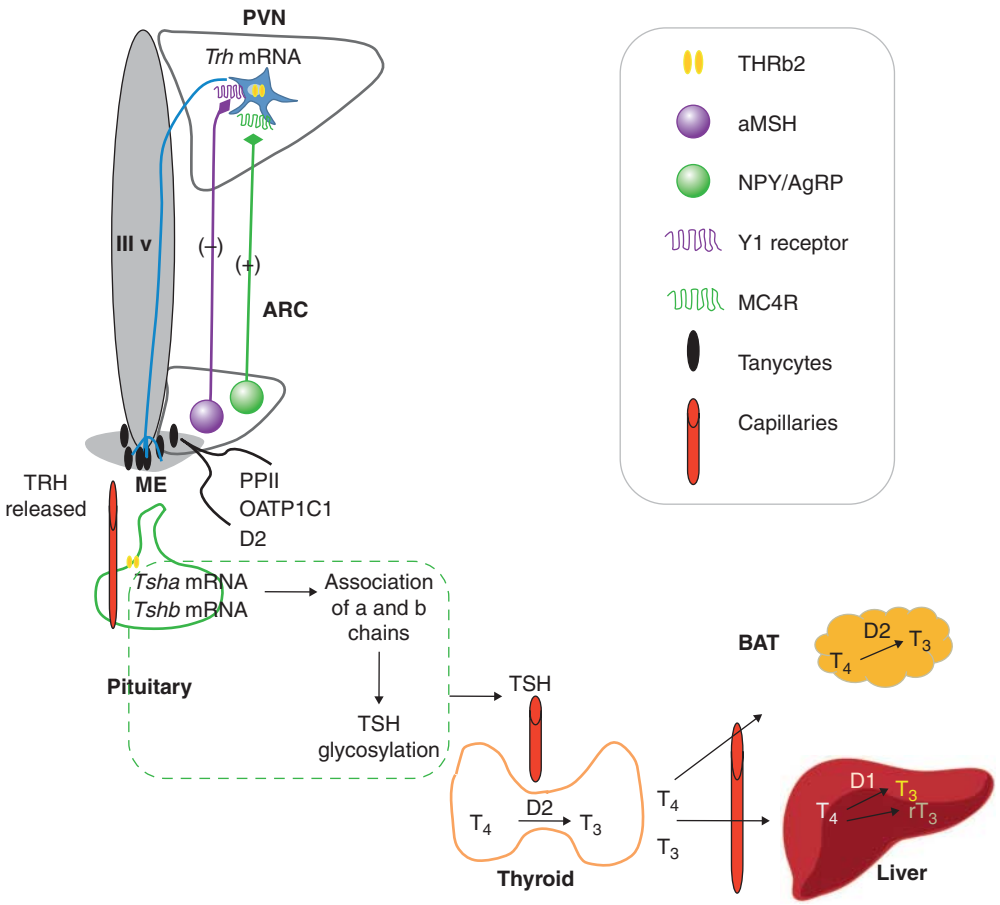

Figure 2
Elements involved in HPT regulation. At the level of the paraventricular hypothalamic nucleus (PVN), *Trh* mRNA is transcribed, its expression is regulated by multiple effectors, processed TRH is released from terminals localized at the median eminence (ME) in yuxtaposition with tanycytes that contain deiodinase 2 (D2) and pyroglutamyl peptidase II (PPII). In response to nutrient status, arcuate neurons synthesizing POMC/CART or NPY/AgRP project to the PVN and activate or inhibit (respectively) TRH neurons. Released TRH may be degraded by PPII before reaching portal vessels that transport it to the pituitary where it controls synthesis of TSHb and glycosylation of both TSH subunits (a and b) to form bioactive TSH. At the thyroid, TSH stimulates synthesis and release of T_4 that is modified at target tissues by deiodinases (e.g. D1 and D2).

et al. 1989). TSH extracted from bovine or human post-mortem pituitaries was used in research, RIA and clinic for almost three decades. RIA determinations of plasma TSH concentration facilitated the conclusive demonstration of the negative feedback effects of TH on TSH secretion from the pituitary (Reichlin & Utiger 1967) and, together with serum TH (total and free) quantification, the evaluation of thyroid status (Biondi & Wartofsky 2014). TSH stimulation tests made it possible to distinguish between primary and secondary hypothyroidism (Querido & Stanbury 1950). By the 1980s, the sequence of TSH subunits became available with the isolation of their cDNAs (Fiddes & Goodman 1981, Wondisford *et al*. 1988) and the clinical use of recombinant human TSH (hTSH), which eliminated the health risks associated with the use of contaminated hTSH isolated from post-mortem tissues (Weintraub & Szkudlinski 1999).

Physiological support for the existence of the hypothalamic control of pituitary–thyroid function started with the pioneering work of Uotila on pituitary-stalk sections (Uotila 1939) and was further substantiated by complementary approaches such as electrical stimulation, electrolytic lesions of median eminence (ME) or diverse hypothalamic nuclei, administration of hypothalamic extracts, and histological observations under different physiological conditions (Greer 1952, Brown-Grant *et al*. 1957). Diminished basal thyroid activity in rabbits was observed after pituitary-stalk transections had been made, and a piece of wax paper had been placed between sections to eliminate vascular regeneration (Brown-Grant *et al*. 1954).

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Discovery of TRH

From Harris' initial proposal that the master gland, the adenohypophysis (or anterior pituitary), was under the control of factors released from the hypothalamus to the portal circulation (Harris 1950), it took almost 20 years to identify the first hypophysiotropic molecule. Various groups attempted to characterize the thyrotropin-releasing factor (TRF), but failed to purify it to homogeneity. They made some valid conclusions such as its non-reactivity to ninhydrin which implied a blocked NH2 terminus (Schreiber *et al*. 1963), its localization to several brain areas, or variations in the TRF-bioactivity of tissue extracts from animals of different thyroid status (Shibusawa *et al*. 1956, Reichlin 1989). Hard and competitive work for over 10 years, around 1–5 million pig or ovine hypothalami, cumbersome chromatographic techniques, and some fortuitous findings by the groups of Schally and of Guillemin enabled the isolation of the tripeptide (pyro)Glu-His-Pro-NH_2, which was named thyrotropin-releasing hormone (TRH; Bøler *et al*. 1969, Burgus *et al*. 1969). The term 'factor' changed to 'hormone' when its structure was identified. An important breakthrough was the development of bio-assays to quantify pituitary hormones released *in vitro* (Guillemin & Rosenberg 1955). The peculiar N- (pyroGlu) and C-terminal (amide) residues, that delayed determination of TRH structure, proved essential for the biological activity of TRH, as chemical modifications were required to synthesize an active peptide based on the amino acid composition of the purified biologically active substance (Glu, His and Pro; Vale *et al*. 1973).

Once synthetic TRH became available, it was quantified by RIA in several tissue extracts, and detected not only in the hypothalamus but also in other brain areas, blood, and urine of several species (Jackson & Reichlin 1974, Winokur & Utiger 1974). Immunocytochemical techniques localized TRH in nerve terminals of the ME, in various hypothalamic nuclei as well as in various brain areas including the septum, nucleus accumbens or brain stem, where it plays a neuromodulatory role (Hökfelt *et al*. 1975, 1989, Lechan & Jackson 1982, Gary *et al*. 2003).

Metabolism of TRH

Biosynthesis

Soon after TRH chemical characterization, attempts began to elucidate its mode of synthesis. The initial work on the biosynthesis of neuropeptides, performed during the 1970s, was based on the incorporation of radioactive aminoacids, the availability of antibodies recognizing various forms, and sequential purification steps. Neurophysin and adrenocorticotropic hormone were found to be synthesized from precursor proteins (Mains & Eipper 1976, Gainer *et al*. 1977) in a similar manner to secretory proteins in other systems (Steiner *et al*. 1967, Kemper *et al*. 1972). These methods proved inadequate for TRH as incorporation of radioactive proline into the peptide was too low in the hypothalamic fragments used (McKelvy *et al*. 1975). The high concentrations of TRH in frog skin, and the knowledge that amidated peptides arose from glycine at their C-terminal end, allowed the isolation of a cDNA containing a partial sequence of the *Trh* precursor from a cDNA library screened using oligonucleotide mixtures containing the triplets that coded for Gln-His-Pro-Gly (Richter *et al*. 1984). This approach was unsuccessful in a hypothalamic rat cDNA library, probably because of the lower level of expression of *Trh* mRNA (Jackson 1989). The ingenious approach of synthesizing the peptide Cys-Lys-Arg-Gln-His-Pro-Gly-Lys-Arg-Cys, with an S—S bond linking the cysteines, left the middle portion of the molecule exposed to elicit an antibody able to detect this internal sequence. This antibody was used to identify the TRH precursor in an expression library of rat hypothalamic cDNAs, which isolated rat *Trh* cDNA (Lechan *et al*. 1986, Jackson 1989) and characterized the *Trh* gene (Lee *et al*. 1989).

The *Trh*-gene proximal promoter contains response elements (RE) to transcription factors whose binding was revealed by chromatin immunoprecipitation assays; for example, receptors for TH, or for glucocorticoid receptors (GR:GRE), CREB (CRE), cJun/cFos (TPA response element), STAT3, krueppel/Sp1, and GC-boxes for growth factor signaling (Joseph-Bravo *et al*. 2015). The protein codified by the rat (r) *Trh* gene is a precursor (pre-proTRH; 255 amino acids) containing five Gln-His-Pro-Gly sequences flanked by a pair of basic residues and cryptic peptides in between (Lechan *et al*. 1986). As for other neuropeptides (Loh *et al*. 2002), proTRH is processed in the secretory pathway through sequential enzyme activities: convertases, carboxypeptidase, pyroglutamyl cyclase, and peptidylglycine α-hydroxylating monooxygenase (Wu & Jackson 1988, Nillni 2010, Fekete & Lechan 2014).

Antibodies specific for proTRH, together with *Trh* cDNA, were used in immunocytochemical and *in situ* hybridization analyses that enabled the final identification of the paraventricular nucleus (PVN) as the hypothalamic nucleus with the highest expression of proTRH precursor (Lechan & Segerson 1989). Further

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studies demonstrated that TRH–hypophysiotropic cells are confined to the medial and caudal PVN of the rat (Fekete *et al*. 2000).

Inactivation

During the initial purification procedures it became evident that TRH was rapidly degraded in tissue homogenates (Redding & Schally 1969) and in plasma (Bassiri & Utiger 1972). Two soluble enzymes initiate hydrolysis of TRH and other peptides *in vitro*: proline endopeptidase (EC 3.4.21.26) cleaves the proline-amide bond; pyroglutamyl peptidase I (PPI; EC. 3.4.19.3), the pyroglutamyl-histidine bond. However, these soluble enzymes do not control intracellular TRH levels *in vivo* because TRH is stored inside secretory granules (O'Cuinn *et al*. 1990, Joseph-Bravo *et al*. 1998). A different pyroglutamyl peptidase was initially detected in serum and termed thyroliberinase because of its strict specificity for TRH (Bauer & Nowak 1979). Later, an enzyme with similar activity and biochemical characteristics was detected in the membranes of the anterior pituitary and in several brain regions, and named PPII (EC. 3.4.19.6) or TRH-degrading ectoenzyme (O'Connor & O'Cuinn 1984, Garat *et al*. 1985, Heuer *et al*. 1998). In the hypothalamus, PPII is expressed in neurons and in tanycytes whose cytoplasmic extensions reach the external layer of the ME, in proximity to TRH terminals (Joseph-Bravo *et al*. 1998, Sánchez *et al*. 2009). Cloning PPII (Schauder *et al*. 1994) led to its identification as a member of the M1 family of metalopeptidases and, by homology modeling and site-directed mutagenesis, interrogation of the structural determinants of its strict omega-peptidase specificity (Chávez-Gutiérrez *et al*. 2006). Because PPII is an integral membrane protein with the active site exposed on the cell surface (Charli *et al*. 1988), it is a prime candidate for TRH hydrolysis in the extracellular compartment, in particular before TRH reaches the ME–pituitary portal capillaries (Sánchez *et al*. 2009).

Release

TRH secreted from the ME enters the portal system to reach the pituitary. *In vivo* TRH release has been measured directly in portal blood of anesthetized animals, or by a push–pull cannula in the ME. However, these techniques are difficult to use in order to detect rapid changes in TRH secretion (Rondeel *et al*. 1992). *In vitro* systems were initially developed to study the mechanisms of neuropeptide secretion; incubates of the mediobasal hypothalamus, containing the ME, demonstrated TRH release by membrane depolarization through a Ca^{++} dependent mechanism consistent with exocytosis (Joseph-Bravo *et al*. 1979).

TRH at the anterior pituitary

TRH stimulates, *in vivo* and *in vitro*, not only the synthesis and release of TSH from thyrotrophs but also of prolactin (PRL) from lactotrophs, and in some species also of growth hormone (GH) from somatotrophs (Galas *et al*. 2009). The availability of radiolabeled TRH, and later of its more stable analog 3Me-His-TRH, facilitated the characterisation of the specific binding sites in the plasma membrane of the anterior pituitary (Labrie *et al*. 1972), in TSH secreting pituitary tumor cells (Grant *et al*. 1972), and in PRL secreting GH3 cells (Hinkle & Tashjian 1973). This receptor, TRHR1, has been characterized in various species. The sequence of mouse (m) TRHR1 corresponds to a seven transmembrane-spanning GTP-binding (G) protein-coupled receptor (GPCR; Straub *et al*. 1990); mTRHR1 cDNA has a high similarity in the protein-coding regions with orthologs in other mammals and its expression in anterior pituitary correlates with radioligand binding studies (Gershengorn & Osman 1996). A second TRHR (TRHR2) was later cloned and found to be expressed mainly in the brain (O'Dowd *et al*. 2000). $TRHR1^{-/-}$ mice pituitaries are devoid of any TRH-binding capacity, which suggested that TRHR1 is the only pituitary receptor (Rabeler *et al*. 2004).

TRH binds to TRHR1 at various residues of the extracellular (low binding affinity) and of the transmembrane (high binding affinity) domains. The extracellular site is proposed to be the initial place of interaction, which accounts for the low binding affinity and slow transformation to a tightly bound conformation with movement of TRH to the transmembrane site (Engel & Gershengorn 2007). In GH pituitary tumor cells, TRH signalling via TRHR1 is conducted through the activation of a Gq/11 protein and phospholipase C β1 mechanism: the production of inositol 3 phosphate and diacyl gycerol affects cellular calcium homeostasis (mobilizing intracellular pools) and activation of protein kinase C (PKC) (Drummond 1986). The interaction of TRH with TRHR1 induces rapid desensitization of the response due to multiple events (Hinkle *et al*. 2012). The ligand–receptor interaction induces receptor phosphorylation, within seconds, at multiple Ser/Thr sites in the cytoplasmic C-terminal tail by a GPCR kinase. TRH receptors bind to β-arrestin, internalize in clathrin-coated vesicles and accumulate in early sorting endosomes. They may

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transport to lysosomes or after TRH removal, dephosphorylate, and accumulate in recycling endosomes which reincorporate into the plasma membrane (resensitization). As b-arrestin is a scaffold for other signaling molecules, its interaction with the receptor permits cross talk with other pathways (Hinkle *et al*. 2012). At the turn of the 21st century the development of bioluminescence resonance energy transfer, and other techniques to detect intermolecular interactions, demonstrated the formation of the TRHR homodimers induced by TRH (Hinkle *et al*. 2012). TRH also provokes long-term transcriptional and posttranscriptional effects that diminish *Trhr1* mRNA levels in rat pituitary GH3 cells, at least in part, by stimulating *Trhr* mRNA degradation (Narayanan *et al*. 1992).

Regulation of HPT axis activity by TRH and negative feedback

The HPT axis is regulated by neuronal inputs that stimulate or inhibit PVN–TRH hypophysiotropic neurons. Of all TRH neurons expressed in the PVN, not all project to the ME. TRH–hypophysiotropic cells are enriched in the medial and caudal PVN of the rat but this differs in the PVN of the mouse and human (Guldenaar *et al*. 1996, Fekete *et al*. 2000, Fekete & Lechan 2014).

TRH–hypophysiotropic neurons receive afferents from multiple brain regions. Neurons from the arcuate nucleus transmit the nutritional status and the suprachiasmatic nucleus convey circadian cycle information, some neurons from the brain stem send information when external temperature drops (Fekete & Lechan 2014, Fliers *et al*. 2014, Joseph-Bravo *et al*. 2015). Stimuli that induce TRH–TSH release may coordinately increase *Trh* transcription.

A multifactorial control is exerted at various steps of the HPT axis. TRH stimulates TSH synthesis in pituitary cells by increasing mRNA levels of *Tshb* and *Tsha* (Shupnik *et al*. 1986). Transduction pathways involve Ca^{++}/calmodulin for TSHb activation or the PKC–MAPK pathway for TSHa (Hashimoto *et al*. 2000). TRH regulates the glycosylation pattern of TSH, which increases its biological activity and half-life (Weintraub *et al*. 1989, Szkudlinski *et al*. 2002).

TSH stimulates synthesis and release of TH which are transported in the blood by T_4-bound globulin, transthyretin or albumin, in different proportions depending on the species (Zoeller *et al*. 2007). More than 70% of TSH-stimulated TH release corresponds to T_4 (Maia *et al*. 2011). The peripheral conversion of injected T_4 has been recognized since the 1950s (Tata 1958), and was followed by characterization of the enzymes responsible (Silva & Larsen 1977, Visser *et al*. 1983, Gereben *et al*. 2008). The three identified deiodinases set the intracellular and peripheral levels of T_3: deiodinase type 1 (D1), the enzyme inhibited by PTU is mainly expressed in liver, kidney, pituitary, and thyroid, and converts T_4 to either T_3 or, reverse T_3. D2, expressed in brain, pituitary, thyroid, BAT, and heart has a higher affinity for T_4 than D1, and transforms T_4 to T_3; D2 is enriched in tanycytes and makes T_3 available to surrounding neurons in the hypothalamus. D3 is expressed in brain, placenta, and skin and inactivates T_4 and T_3. D1 and D3 are localized to the plasma membrane whereas D2 is localized to the membranes of the endoplasmic reticulum, which facilitates ready access to the nucleus for T_3 (Gereben *et al*. 2008). The activity and expression of these enzymes are modulated, in a cell-specific manner, by various effectors including TH; T_3 decreases *dio2* expression and increases that of *dio1* and *dio3*, whereas T_4 decreases the activity of D2 by increasing its ubiquitination and proteosomal degradation (Gereben *et al*. 2008, Abdalla & Bianco 2014). Peripheral conversion of T_4 to T_3 arises mainly from D2 in euthyroid, or via D1 in thyrotoxic animals (Maia *et al*. 2011).

TH feedback on HPT axis activity was conclusively demonstrated in the pituitary when TSH RIA became available (Reichlin & Utiger 1967, Reichlin *et al*. 1970). At the hypothalamus evidence was indirect, supported by the diminished goitrogenic effect of PTU in animals with lesions between the PVN and the ME, and the response to thyroidectomy in lesioned-PVN rats that presented with diminished TSH secretion (Greer 1952, Martin *et al*. 1970). It is now evident that the HPT axis is modified by the thyroid status in a concerted fashion at multiple levels. Hypothyroidism increases *Trh* mRNA levels in the PVN (Koller *et al*. 1987, Segerson *et al*. 1987), proTRH processing (Perello *et al*. 2006), TRH release from ME (Rondeel *et al*. 1992), TSH and TRHR1 synthesis in the pituitary (Shupnik *et al*. 1986, Schomburg & Bauer 1995), and TSH serum concentration (Biondi & Wartofsky 2014). In contrast, the expression of the TRH-degrading enzyme in tanycytes is decreased in hypothyroid animals (Lazcano *et al*. 2015). Opposite changes occur in hyperthyroidism (Supplementary Table 1, see section on supplementary data given at the end of this article; Chiamolera & Wondisford 2009, Costa-e-Sousa & Hollenberg 2012, Fekete & Lechan 2014, Fliers *et al*. 2014, Lazcano *et al*. 2015). The T_3-negative transcriptional regulation of *Trh* and *Tsh* occurs primarily through TRb2 (Abel *et al*. 2001, Chiamolera & Wondisford 2009, Sugrue *et al*. 2010), whose

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expression in the pituitary is down regulated by T_3, and modestly down-regulated by TRH (Lazar 1993). TH inhibit TSH secretion even faster than TRH or TSH transcription. This response may be related to the rapid up-regulation of expression and activity of the TRH-degrading enzyme by T_4 in tanycytes. Increased inactivation of TRH released from the ME could account for diminished TSH release, supporting the external layer of the ME as an ultimate critical control point in modulating TRH concentration on its passage to the portal system (Sánchez *et al*. 2009).

At a hypothalamic level, T_4 is taken up from the circulation by tanycytes that convert it to T_3 by D2; T_3 is then released in the surrounding neuropil and taken up by neurons (Tu *et al*. 1997). Several TH transporters have been identified recently. Among the best characterized in the brain is the monocarboxylate transporter 8 (MCT-8), which recognizes different TH, and is expressed in various tissues and cell types including neurons, endothelial cells, oligodendrocytes, astrocytes and tanycytes. Another is the organic anion-transporting polypeptide 1C1 (OATP1C1), which is found in tanycytes and endothelial cells. Its expression is modulated by TH and it has preferential substrate specificity for T_4 compared to other TH. Both participate in transporting TH across the blood brain barrier in mice but OATP1C1 does not in humans. Mutations in *SLC16A2*, the gene that encodes MCT-8, can produce severe neurological impairments in humans (Visser *et al*. 2011, Wirth *et al*. 2014).

The specificity of TH feedback effect on TRH expression for PVN–TRH hypophysiotropic neurons vs other hypothalamic neurons expressing TRH does not relate to an exclusive expression of THRs or TH transporters (Fekete & Lechan 2014, Joseph-Bravo *et al*. 2015). D3 is found in only 27% of TRH-immunoreactive varicosities present in the ME (Kalló *et al*. 2012). An hypothesis recently put forward is that T_3, transformed from T_4 by D2 in tanycytes, is taken up by TRH nerve terminals in the ME and transported in a retrograde fashion to the PVN, where it inhibits TRH transcription (Fekete & Lechan 2014).

Knock out (KO) animals for various elements involved in HPT axis regulation have revealed the critical steps in HPT axis function (Joseph-Bravo *et al*. 2015). The importance of the effects of TRH on TSH glycosylation and activity (Weintraub *et al*. 1989) has been demonstrated by comparing the phenotypes of TRH-KO, THRb-KO, and the double mutant. Increased TSH serum levels but reduced TSH bioactivity accounts for the low circulating T_4 concentration (Nikrodhanond *et al*. 2006), similar to that observed in humans with hypothalamic hypothyroidism (Beck-Peccoz *et al*. 1985), or in TRHR1$^{-/-}$ mice that have normal TSH levels but low circulating T_3 and T_4 concentrations (Rabeler *et al*. 2004). D1-, D2-, and D1D2-KO show compensatory mechanisms in the interplay between hypophysiotropic TRHergic neurons, pituitary TSH expression and release, which in combination maintain serum levels of T_3 stable despite altered serum concentrations of T_4 and TSH (Abdalla & Bianco 2014, Galton *et al*. 2014). D2-KO specifically in the pituitary produced contradictory results regarding TRH or TSH expression, albeit data coincide that these mice maintain constant T_3 serum levels (Fonseca *et al*. 2014, Luongo *et al*. 2015). MCT8-KO mice have increased *Trh* expression, which further confirms that TH uptake into tanycytes is required for negative feedback on *Trh* expression (Horn *et al*. 2013).

Energy homeostasis and the HPT axis

Negative energy balance

The effects of iodine deficiency or nutritional status on BMR and thyroid activity were observed a century ago (Hinz 1920) and later confirmed when it was found that TH serum concentrations were reduced during fasting or food restriction (Reichlin 1957, Palmblad *et al*. 1977, Harris *et al*. 1978). After fasting, TSH serum levels are low or normal, but ME–TRH release and *Trh* mRNA levels in the PVN decreased (Blake *et al*. 1991, Van Haasteren *et al*. 1995, Fekete & Lechan 2014). Pituitary *dio2*, *Thrb2*, and *Tshb* mRNA levels are diminished (Boelen *et al*. 2006), as well as hepatic D1 activity. In contrast, *dio2* hypothalamic expression and serum corticosterone are increased (Diano *et al*. 1998). Another element involved in the response to fasting is PPII activity in tanycytes which is up-regulated at a time (48–72 h) when the expression of *Trh* in the PVN tends to reinitiate (Lazcano *et al*. 2015). These changes differ from those observed in primary hypothyroidism. The discovery of the adipostatic hormone leptin (Zhang *et al*. 1994) helped unravel the mechanism of fasting-induced inhibition of the HPT axis. Leptin is released from adipose tissue proportionally to body fat and in response to caloric intake, while its serum levels decrease rapidly during fasting (Hardie *et al*. 1996). Leptin administration impedes fasting-induced inhibition of *Trh* mRNA levels in the PVN (Légrádi *et al*. 1997). In response to leptin, its receptor (LepRb) activates several transcription factors including the STAT3 which binds to the *Trh* promoter and increases *Trh* transcription (Guo *et al*. 2004). *Trh* mRNA

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levels in the PVN are increased by leptin, either directly through LepRb activation, or indirectly through afferents from the arcuate nucleus. In the arcuate nucleus, two neuronal groups synthesize orexigenic (neuropeptides Y (NPY)/Agouti-related peptide (AgRP)) or anorexigenic (pro-opiomelanocortin (POMC), precursor of alpha-melanocyte stimulating hormone (aMSH)/cocaine- and amphetamine-regulated transcript (CART)) neuropeptides. These NPY/AgRP and POMC/CART neurons are tightly regulated by metabolic signals such as leptin, insulin, or ghrelin. aMSH signals through the melanocortin receptor 4 (MC4R) that also recognizes AgRP but as an inverse agonist. TRH neurons receive afferents from aMSH, NPY, and AgRP neurons, the former stimulates and the latter two inhibit *Trh* mRNA levels (Fekete & Lechan 2014). aMSH induces CREB phosphorylation in TRH neurons *in vivo* and in hypothalamic neuronal culture where it increases *Trh* transcription (Harris *et al.* 2001, Sarkar *et al.* 2002). The analysis of mice lacking both MC4R and NPY demonstrates that fasting-induced suppression of the central arm of the HPT axis requires NPY, and that a second pathway based in the liver, that enhances the catabolism of TH during fasting, requires MC4R and NPY (Vella *et al.* 2011).

Non-thyroidal illness syndrome (NTIS) is a clinical condition that presents, as in fasting, with a low T_3 but normal or slightly decreased TSH serum levels, occuring during acute or chronic inflammation, and sepsis. The mechanisms involved differ to those produced by fasting. Despite low *Trh* mRNA levels, those of the arcuate nucleus POMC are not changed, and deiodinase activity is higher than that detected after fasting; in particular, for D2 in tanycytes and D1 and D3 activities in liver and muscle (Boelen *et al.* 2011, Fekete & Lechan 2014, Fliers *et al.* 2014). It has been proposed that while leptin is the main regulator of fasting induced changes in the HPT axis, deiodinase activity plays a major role during NTIS (Boelen *et al.* 2011).

Positive energy balance

In contrast to the relatively detailed knowledge about the central aspects of HPT axis regulation during energy deficit, less is known about regulation during energy excess. Although hypothyroid individuals tend to gain weight, obese individuals have normal or slightly enhanced total and free T_3 levels, which are postulated as an adaptation to the increased metabolic demands of increased body weight (Strata *et al.* 1978, Reinehr 2010). Diet-induced obesity (DIO) enhances HPT axis activity in male rats, as demonstrated by increased *Trh* mRNA levels in the hypothalamus/PVN and serum TSH concentration. This increase in HPT axis activity may be due to enhanced circulating leptin levels acting directly on PVN–TRH neurons, independently from POMC neurons, thus bypassing the drop of leptin sensitivity which occurs in the ARC during DIO, or through other circuits that maintain leptin sensitivity (Araujo *et al.* 2010, Perello *et al.* 2010). Likewise, mice fed a high fat diet for 7–20 weeks have an activated HPT axis, with higher hypothalamic *Trh* mRNA levels, and serum TSH concentration than mice on a control diet. This study also indicates that deiodinases activities adjust in tissue, time and obesity-tendency specific ways, contributing to metabolic responses to DIO (Xia *et al.* 2015).

Energy demands activate the HPT axis

Energy demanding situations such as hypothermia activate the thyroid (Dempsey & Astwood 1943, Brown-Grant *et al.* 1954). The cold response is blunted in pituitary-stalk operated rats (Uotila 1939) and after PVN-electrolytic lesions (Ishikawa *et al.* 1984). An acute cold exposure rapidly and transiently augments *Trh* mRNA levels in the PVN, followed by increased TSH in serum and T_4 at a later time (Zoeller *et al.* 1990, Uribe *et al.* 1993). Cold-induced TRH expression is independent of circulating TH concentration (Zoeller *et al.* 1990) or of nutritional status (Jaimes-Hoy *et al.* 2008), but is inhibited by a previous stress exposure (Uribe *et al.* 2011) or corticosterone injection (Sotelo-Rivera *et al.* 2014). Humans exposed to cold for over 60 h activate the HPT axis, which is not inhibited if food intake is reduced (Joseph-Bravo *et al.* 2015).

Other examples of HPT axis activation are observed in response to an acute increase in physical activity (Fortunato *et al.* 2008, Gutiérrez-Mariscal *et al.* 2012) or after 2 weeks of voluntary exercise in rats (Uribe *et al.* 2014). Wheel running diminishes food intake by 18% compared to sedentary animals. In the pair-fed group, body weight gain diminished to the same extent as the exercised. However, adipose tissue mass and leptin serum levels were reduced exclusively after exercise; *Trh* mRNA in the PVN and TSH serum levels diminished, compared to naïve rats, more in the pair-fed than in the exercised group; only pair-fed animals had low T_3 serum levels. The inhibition of the HPT axis caused by diminished food intake was thus partially compensated with exercise and the changes to all the parameters of the HPT axis correlated with distance run and loss of fat mass (Uribe *et al.* 2014). These results suggest that although TH and nutritional status modulate the basal

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state of the HPT axis, immediate energy demands may override leptin or TH signaling.

Stress interferes with HPT axis activity

Another important modulator of HPT activity long recognized is the inhibitory effect of stress. The differential effects of physical and emotional stress on HPT activity were elegantly shown by Harris's group, who compared thyroid activity after physical or emotional stress, in intact or adrenalectomized rabbits. A corticosterone injection, or stress, inhibits thyroid activity. However, only the effects of the emotional stressor (restraint) were avoided when pituitary-stalk sections were performed supporting an effect at hypothalamic level (Brown-Grant *et al*. 1957). Restraint indeed decreases rat *Trh* mRNA levels in the PVN and, as in other stressors, serum TSH (Du Ruisseau *et al*. 1978, Gutiérrez-Mariscal *et al*. 2012), but the effects of chronic stress depend on the type, intensity and duration (Armario *et al*. 1984). Because long-term stress affects many metabolic parameters that may regulate the HPT axis, direct cause-effects are difficult to discern (Joseph-Bravo *et al*. 2015).

Corticosterone affects the HPT axis. Injected into adrenalectomized rats for several days it inhibits PVN *Trh* expression (Kakucska *et al*. 1995) whereas, an acute injection is stimulatory. However, if injected 30 min prior to cold exposure, the cold-induced stimulation of PVN *Trh* expression or TSH serum levels is blunted (Ranta 1975, Sotelo-Rivera *et al*. 2014). Primary hypothalamic-cell cultures have provided information regarding potential regulators of the *Trh* promoter. TRH transcription is rapidly increased by agents that cause TRH release, such as noradrenaline or cAMP analogs that induce CREB phosphorylation and binding of pCREB to the *Trh* promoter. Corticosterone, which activates GR and its binding to GRE, also increases *Trh* expression, albeit less than cAMP analogs. However, if corticosterone and cAMP analogs treatments are combined, *Trh* transcription is no longer stimulated and pCREB or GR do not bind to their RE (Díaz-Gallardo *et al*. 2010), CREB phosphorylation is blunted and the catalytic subunit of phosphokinase (PKAc) interacts with GR in the cytosol, which explains the observed cAMP signaling interference induced by glucocorticoids (Sotelo-Rivera I, Cote-Vélez A, Díaz-Gallardo M, Charli JL, Joseph-Bravo P, unpublished observations). These *in vitro* results may explain why stress can alter PVN *Trh* mRNA response to an acute cold stimulus (Joseph-Bravo *et al*. 2015). Combining *in vitro* and *in vivo* paradigms will continue to provide important insights into the mechanisms involved in regulating the activity of the HPT axis.

Hypopysiotropic TRH neurons are involved in PRL release

Multiple effectors control PRL release. Soon after the discovery of TRH, evidence supported the hypothesis that TRH was one of the prolactin releasing factors. TRH stimulates PRL secretion either *in vivo* or *in vitro* (Jacobs *et al*. 1971, Tashjian *et al*. 1971). Suckling stimulates TRH biosynthesis in the PVN and release from the ME (Fink *et al*. 1982, Uribe *et al*. 1993, Van Haasteren *et al*. 1996, Sánchez *et al*. 2001). TRH antisera inhibit suckling-induced PRL release (de Greef *et al*. 1987). While dopamine exerts a tonic inhibition on PRL release *in vivo*, its release into the portal blood is inhibited by suckling, an event which potentiates TRH-induced PRL secretion (Martinez de la Escalera & Weiner 1992). However, TSH is neither released by suckling, nor PRL by cold exposure (Uribe *et al*. 1993, Van Haasteren *et al*. 1996, Sánchez *et al*. 2001). This discrepancy may be explained by CART which inhibits PRL release and its expression is upregulated in hypophysiotropic TRH neurons by cold but not by suckling (Sánchez *et al*. 2007). TRH and TRHR1 KO mice have shown that while TRH is necessary to sustain PRL secretion during lactation, pups from KO dams grow normally, suggesting that TRH is not essential for suckling-induced PRL release (Rabeler *et al*. 2004, Yamada *et al*. 2006).

Contrary to data showing that anterior pituitary PPII does not regulate the response of thyrotroph response to TRH, there is evidence that in lactotrophs the intensity of TRH action is under PPII control. PPII is expressed in lactotrophs and its knockdown or inhibition enhances TRH-induced PRL release (Cruz *et al*. 2008). PPII expression and activity are enhanced *in vivo* by TH and down-regulated by estrogens (Schomburg & Bauer 1995, 1997). In anterior-pituitary cultured-cells, PPII activity is rapidly enhanced by the removal of dopamine and addition of TRH (Bourdais *et al*. 2000). These results suggest that PPII is controlled by signals that shape PRL secretion in response to TRH; regulation of PPII may in turn alter PRL release.

Although many studies show that TRH acts directly on lactotrophs, evidence that in hypothalamic slices TRH provokes a transition from phasic to tonic firing of the tuberoinfudibular dopaminergic neurons that control PRL secretion (Lyons *et al*. 2010) indicates additional mechanisms that link TRH and PRL secretion.

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Perspectives for the 21st century

DNA recombinant techniques permitted the development of strategies that helped to characterize the various regulatory steps of the HPT axis. Transfected cells, KO and transgenic animals have provided important information, although data do not always correspond to what would be expected from the known physiology of the HPT axis (Joseph-Bravo *et al*. 2015). These discrepancies may be due to the redundancy of effector molecules or their receptors, and compensatory effects during development. TRH neurons are considered an important participant in energy homeostasis (Lechan & Fekete 2006, Levin 2007, Hollenberg 2008). This is further substantiated by recent findings on increased *Trh* and brain derived neurotropic factor (*Bdnf*) expression in lean animals compared to their fat counterparts; BDNF is an important participant in brain plasticity and metabolism (Byerly *et al*. 2009, Cao *et al*. 2011). Defining the circuits in which TRH neurons are involved under different circumstances now seems to be feasible with the combined techniques of Cre-recombinase, opto-genetics, pharmaco-genetics, proteomic, and genomic analysis. A recent example is the demonstration with opto- and chemo-genetic tools that a TRH projection from the PVN onto AgRP-ARC neurons drives hunger in mice (Krashes *et al*. 2014).

New evidence supports the hypothesis that environmental threats (nutrition, toxins) or stressful situations alter the programming of adult HPT axis activity (Joseph-Bravo *et al*. 2015). Considering compelling new data on epigenetic changes due to stress or other factors, and the effects of endocrine disrupting chemicals, important considerations are required in the maintenance of experimental animals. Epigenetic modifications alter the gene expression of various elements that may modify HPT axis activity. For example, early life stress increases the methylation of hippocampal GR and hence its expression, diminishing the inhibitory feedback that glucocorticoids exert during a response to stress (Turecki & Meaney 2014, Joseph-Bravo *et al*. 2015). The opposite is observed when raising animals in enriched environments (Cao *et al*. 2011). Development may also be affected by endocrine disruptors which contaminate water and food: experimental animals and cell cultures are kept in plastic bottles, cages and plates that leach endocrine disruptors (Préau *et al*. 2015). To obtain more reproducible data and help us understand neuroendocrine physiology, some standards, additional to those recently established (Bianco *et al*. 2014), are urgently needed. Neuroendocrine research should also include gender and age differences as, to date, most research has been performed in young adult male rodents.

Supplementary data
This is linked to the online version of the paper at http://dx.doi.org/10.1530/JOE-15-0124.

Declaration of interest
Authors are academic staff at UNAM with nothing to declare.

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Prominent Reviews
We apologize to those authors whose excellent work is not cited here due to space constraints. However, this list of reviews have been selected as the most prominent reviews on the history of TRH and the HPT:

Abdalla & Bianco 2014
Biondi & Wartofsky 2014
Boelen *et al*. 2011
Chiamolera & Wondisford 2009
Costa-e-Sousa & Hollenberg 2012
Fekete & Lechan 2014
Fliers *et al*. 2014
Gary *et al*. 2003
Gereben *et al*. 2008
Greer 1952
Hinkle *et al*. 2012
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Lazar 1993
Lechan & Fekete 2006
Magner 2014
Maia *et al*. 2011
Martinez de la Escalera & Weiner 1992
Mullur *et al*. 2014
Nillni 2010
O'Cuinn *et al*. 1990
Reichlin 1989

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Reinehr 2010
Szkudlinski *et al*. 2002
Tata 2013
Turecki & Meaney 2014
Visser *et al*. 2011
Weintraub *et al*. 1989
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