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Advances in TRH signaling

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Abstract The activity of the hypothalamus-pituitary-thyroid axis (HPT) is coordinated by hypophysiotropic thyrotropin releasing hormone (TRH) neurons present in the paraventricular nucleus of the hypothalamus. Hypophysiotropic TRH neurons act as energy sensors. TRH controls the synthesis and release of thyrotropin, which activates the synthesis and secretion of thyroid hormones; in target tissues, transporters and deiodinases control their local availability. Thyroid hormones regulate many functions, including energy homeostasis. This review discusses recent evidence that covers several aspects of TRH role in HPT axis regulation. Knowledge about the mechanisms of TRH signaling has steadily increased. New transcription factors engaged in TRH gene expression have been identified, and advances made on how they interact with signaling pathways and define the dynamics of TRH neurons response to acute and/or long-term influences. Albeit yet incomplete, the relationship of TRH neurons activity with positive energy balance has emerged. The importance of tanycytes as a central relay for the feedback control of the axis, as well as for HPT responses to alterations in energy balance, and other stimuli has been reinforced. Finally, some studies have started to shed light on the interference of prenatal and postnatal stress and nutrition on HPT axis programing, which have confirmed the axis susceptibility to early insults.

Keywords TRH · TRH degrading ectoenzyme · Thyroid · Early life stress · Energy homeostasis · Median eminence · Tanycyte · Hypothalamus · TRH-receptor · Thyrotropin

1 Introduction

Thyrotropin releasing hormone (TRH) is synthesized in various brain areas and in several hypothalamic nuclei [1]. The hypophysiotropic TRHergic neurons are those localized in the paraventricular nucleus (PVN) of the hypothalamus that send projections into the external layer of the median eminence, from where they release TRH into the portal vessels [2]. In rat, TRH hypophysiotropic neurons are localized in the medial and caudal zones of the PVN. TRH neurons of the anterior part of the PVN send projections to other brain areas [3]; in mouse PVN hypophysiotropic TRH neurons are located in the anterior-medial zones [2]. The PVN TRH neurons receive afferents from various brain areas that transmit signals from the internal milieu or from the environment, regulating not only TRH release from the nerve terminals located in the median eminence but also, TRH synthesis. Neurons in the arcuate nucleus sense a variety of hormones related to nutritional status due in part to a relatively permeable blood-brain barrier (BBB), and receive nerve inputs from hypothalamic and brain-stem nuclei engaged in energy balance. Afferents from the arcuate contact TRHergic neurons of the PVN where they release either neuropeptide Y (NPY) and agouti related protein (AgRP), or the pro-opiomelanocortin (POMC)-derived peptide, α-melanocyte stimulating hormone (αMSH), and cocaine-amphetamine related transcript (CART) [2, 4]. Environmental signals such as temperature or light are transmitted to the PVN TRH neurons through afferents arising from the brain stem or from the suprachiasmatic nucleus, respectively [1, 5]. Albeit less characterized, the limbic system activated in response to

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stressful-emotional stimuli conveys information onto PVN TRH neurons [6]. In addition, the hypophysiotropic TRH neurons are one of the targets of the feedback regulation that thyroid hormones apply on the central arm of the HPT axis [2], and respond to changes in other hormonal inputs such as circulating glucocorticoids [1]. The hypophysiotropic TRHergic neurons decode thus multiple neuronal, immune and hormonal signals that regulate the synthesis and /or release of TRH [1–8].

The TRH gene is transcribed and translated in the neuronal cell body into a protein precursor that contains repeated gln-his-pro-gly sequences. The precursor is compartmentalized with convertases, carboxypeptidase E, glutaminyl cyclase and peptidyl-glycine α-amidating monooxygenase in secretory granules that are transported to nerve terminals; the time taken for protein synthesis, and granule transport (0.5–1 um/s) to nerve terminal takes at least 2 h [1, 2, 9, 10] (Fig. 1a). Nerve terminals containing TRH are concentrated at the external layer of the median eminence, in close proximity with the cytoplasmic extensions of β-2 tanycytes, and with the portal hypothalamus-pituitary capillaries [11] (Fig. 1b, c). Both α and β tanycytes are the locus of enzymes involved in HPT regulation. One of these enzymes is deiodinase 2 (D2), which converts T_4 to T_3 (Fig. 1c, d) [1, 2, 12]. Another is TRH degrading ectoenzyme (*Trhde*), also named pyroglutamyl peptidase II (PPII), according to its catalytic activity [13, 14]. The expression of PPII in β2 tanycytes, including its cytoplasmic extensions into the median eminence, suggests that the protein is localized at the cell surface, near TRH terminals and able to degrade TRH released into the intercellular space of the median eminence. Consistent with this hypothesis, the specific activity of PPII is relatively high in the median eminence, and PPII inhibition increases TRH recovery from the extracellular medium of median eminence incubated *in vitro*. The peripheral injection of a specific inhibitor of PPII enhances TSH levels in cold stressed rats, which supports that PPII controls the amount of TRH that reaches the anterior pituitary [13]. The narrow specificity of PPII suggests that TRH is its only substrate *in vivo*, and that PPII may be critical for the control of the amplitude/duration of TRH effects. PPII is also expressed in the pituitary (in lactotrophs), and the liver expresses a soluble isoform released into the circulation (termed thyroliberinase) [15, 16].

When TRH reaches the pars distalis of the pituitary, it regulates cells that express TRH receptor-1 (TRH-R1), such as the thyrotrophs (Fig. 1c), lactotrophs and a fraction of somatotrophs. These cell types are subject to a multifactorial regulation that determines the extent and specificity of their response to TRH [17–19]. In thyrotrophs, TRH causes the release of TSH and simultaneously, the synthesis of *Tshb,* and of TSHα, the glycoprotein subunit α (CGA) common to other hypophysiary hormones [20]. TRH also regulates the pattern of glycosylation of TSH that confers full biological activity at the TSH receptor in the thyroid gland [21]. Binding of TRH to TRH-R1 receptor, which is coupled to Gq/11 protein, activates phospholipase C, mobilizes calcium and activates protein kinase C [19]. Several cell lines or primary cultures have aided to identify the transduction pathways involved in TRH signaling: IP3 mobilizes intracellular calcium causing TSH release; TRH-stimulated *Tshb* synthesis involves the Ca-calmodulin pathway; for *Tsha* the PKC-MAPK pathway [17, 19]. Studies with cell lines, such as GH3, have also shown the relevance of intermittent occupancy of TRH receptor, which is consistent with ligand-induced down-regulation of TRH receptor [19, 22]. TRH binding to TRH-R1 promotes receptor phosphorylation, an event that favors its endocytosis; additionally, its mRNA is down regulated by TRH and TH, and upregulated by estrogens [19]. The ability of TRH to regulate the expression of *Tshb* may depend upon either the pituitary architecture, and/or paracrine factors, since it can be lost in cell culture [23].

TSH is also synthesized in the pars tuberalis of the pituitary (Fig. 1c, e), where it is not regulated by TRH [24]; instead, the photoperiod controls, through melatonin, the synthesis of TSH, not only in birds but also in several mammals including some mice strains [25, 26]. Tanycytes express the TSH-receptor (TSH-R1); primary ependymal cell cultures respond to TSH stimulation, via cAMP or phosphorylated ERK pathways, stimulating *Dio2* expression [27], thus increasing the amount of T_3 available to hypothalamic neurons. TSH released in a constitutive manner from the pars tuberalis also reaches the circulation; it differs in its N-glycosylation pattern from that derived from the pars distalis, and forms protein-complexes with immunoglobulin or albumin that impede its bioactivity in the thyroid gland [28].

2 Feedback control of TRH output from the hypophysiotropic neurons

The activity of hypophysiotropic TRHergic neurons is set by the negative feedback control exerted by thyroid hormones (TH), by neuronal and hormonal influences responding to the nutritional status, and by inputs activated upon acute immediate demands. Drastic situations as fasting or food restriction, inflammation, hypo- or hyper-thyroidism, change the activity of the TRH neurons and the expression of TRH either directly or indirectly throughout the combined action of distinct effectors. In the case of TH feedback, several factors and cellular components are involved.

Negative feedback by thyroid hormones is exerted at multiple levels [1, 7, 17, 29, 30]. Site-4 of *Trh*-gene promoter binds (Fig. 1a) thyroid hormone receptors (TRs) as monomer, homodimer, or heterodimer with retinoid acid receptor X (RXR) [30]. T_3 inhibits *Trh* transcription through either TRα or TRβ in transfected cells but *in vivo*, TRβ2 is the principal receptor involved in T_3 feedback effects at the PVN [31]. T_3

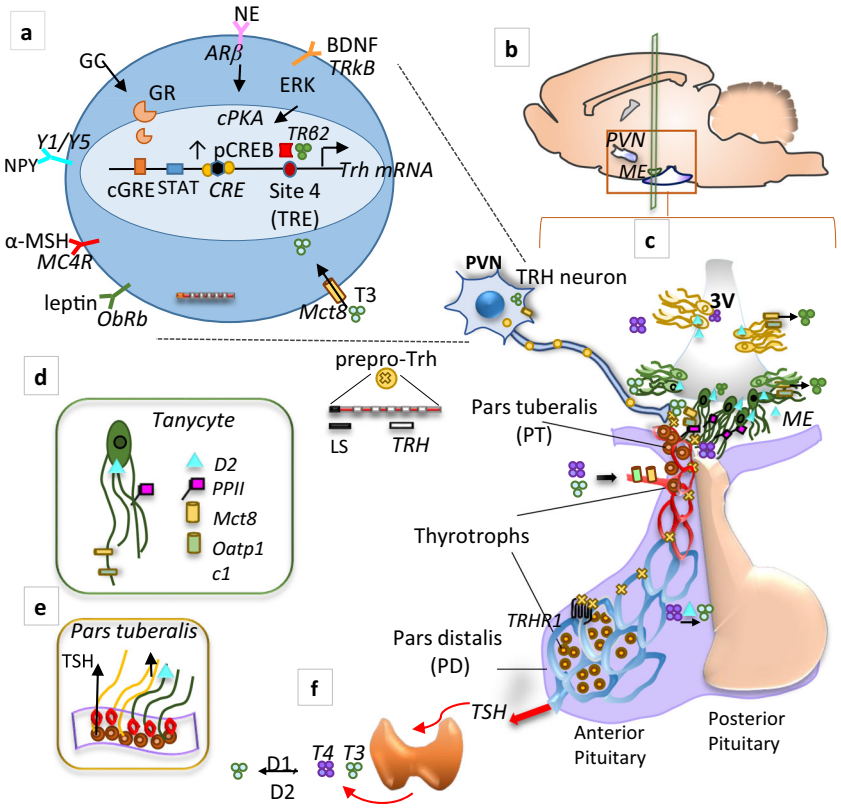

Fig. 1 Central regulation of the hypothalamic-pituitary-thyroid axis in the rat. Panel **a** depicts the multifactorial regulation of TRH gene transcription in the parvocellular neurons of the paraventricular nucleus (PVN) of the hypothalamus. Leptin, through the Ob-Rb receptor and the phosphorylation of Stat3, can directly activate the TRH promoter. α-melanocyte-stimulating hormone (α-MSH), through MC4R, noradrenaline (NA) via the beta adrenergic receptor (ARβ) and through protein kinase A (PKA), and brain-derived neurotrophic factor (BDNF) via the tyrosine kinase receptor B (TrkB) and extracellular regulated kinase (ERK) phosphorylate CREB (cAMP response element binding protein), which interacts with the CREB response element (CRE). Neuropeptide Y (NPY), acting via Y1/Y5 receptors, inhibits TRH synthesis. Additionally, when the glucocorticoid receptor (GR) is activated by glucocorticoids (GC), it binds as heterodimer with c-Jun to a composite glucocorticoid response element (cGRE). GC signaling may interfere with PKA signaling blocking CREB phosphorylation, pCREB and GR binding to *Trh* promoter hence inhibiting TRH's transcription. T3 inhibits TRH gene expression through the nuclear receptor TRβ2 that binds to site 4, a functional thyroid hormone response element (TRE). **b**: Hypothalamic-pituitary-thyroid axis activity is regulated by hypophysiotropic thyrotrophin-releasing hormone (TRH) neurons located in the medial and caudal zone of the paraventricular nucleus (PVN) of the hypothalamus.**c:** TRH is synthetized from the precursor prepro-TRH; it contains an N-terminal amino acid leader sequence (LS, represented in black) followed by an N-terminal flanking peptide, five copies of the TRH progenitor sequence (represented in white) flanked by paired basic amino acids (Lys-Arg or Arg-Arg), and criptic peptides (orange). Pro-TRH is compartmentalized into secretory granules, and processed through various enzymatic steps to biologically active TRH while the secretory granules are transported to the nerve terminal. The third ventricle is lined with ependymal cells and tanycytes; some α tanycytes (yellow) and β tanycytes (green) are depicted. TRH-nerve terminals make contact with the β2 tanycyte processes in the median eminence. β2 tanycytes express pyroglutamyl peptidase II (PPII), that specifically degrades TRH regulating the amount of TRH transported, through the portal vessels, to the pars distalis (PD) of the anterior pituitary gland. TRH binds to TRH receptor type 1 (TRHR1) in thyrotrophs and stimulates the synthesis and secretion of TSH. PD-TSH acts on the thyroid gland stimulating the synthesis and secretion of thyroid hormones, T_4 and T_3; they enter the cells through membrane transporters such as MCT8 or OATP1C1, and are then modified in a cell-specific manner by deiodinases type 1, 2 or 3 (D1, D2, D3). Tanycytes processes transport T_4 from portal capillaries and blood vessels in the arcuate nucleus or from the cerebrospinal fluid (CSF). Hypothalamic T_3 is produced locally from T_4 by action of D2 expressed in tanycytes; TRH nerve terminals at the median eminence take up T_3 and transport it to the cell body and inhibit *Trh* transcription. The expression of PD-*Tsh* is also controlled by negative feedback of thyroid hormones. **d**: some of the molecules expressed in tanycytes, such as D2, PPII, and the thyroid hormone transporters (Mct8 and Oatp1c1), contribute to regulate the HPT axis activity. **e**: schematic representation of thyrotrophs cells in the pars tuberalis (PT) that synthesize TSH in response to variations in the photoperiod and not to TRH; this TSH differs from PD-TSH in their type of glycosylation, PT-TSH does not interact with the thyroid TSH receptor, instead it stimulates D2 in tanycytes. β1 and β2 tanycytes establish contact with portal capillaries and secretory cells of the pars tuberalis. **f:** illustrates TSH action at the thyroid gland promoting the secretion of T_4 and T_3, T_4 being further transformed into T_3 in target tissues by D1 and D2

promotes the binding of TR, histone deacetylases and corepressor NCoR to the *Trh* promoter; however, the mechanism of negative regulation by T_3 remains elusive [30–34].

TR may interact with various other transcription factors extending, by this cross-talk, the action of other regulators [35–37]. Effects of the heterodimer TR:RXR depend on the proportion

and subtype of RXR (a or b); *in vivo*, RXRb enhances T3-repressive action [38]. Retinoids cause central hypothyroidism in humans and rat, and inhibit *in vitro* TRH transcription [39]. As TH induce the synthesis of retinaldehyde dehydrogenase in tanycytes, retinoic acid availability to the nearby neurons may modify the effects of TH-TR in TRH neurons [40]. Besides RXRb, TR also heterodimerizes with PPAR and the endogenous concentration of each transcription factor directs the resultant inhibition [41]. Another family of transcription factors, the LXRα or β which are activated by oxysterols, bile acids and fatty acids [42], heterodimerize with RXR or with TR, and chromatin immunoprecipitation analyses detect LXR:TR binding to the *Trh* promoter; TR binds to either RXR or LXR but not to both simultaneously [43]. Support of LXR involvement at PVN level is provided by experiments with LXRαβ knock down with shRNA in newborn pups, or with LXR-KO mice that show activated HPT axis in spite of high TH circulating levels; *Trh* expression is increased in the PVN, as well as that of many TH responsive genes in target tissues [43, 44]. LXR has been proposed as an important transcription factor involved in multiple metabolic pathways, whose dysregulation might play an important role in the metabolic syndrome [36, 42, 45]. Cholesterol metabolites and fatty acids could, in principle, activate LXR and contribute to repress *Trh* and *Tsh* transcription, providing possible mechanistic answers to the link between obesity and HPT dysfunction [46, 47].

Entry of thyroid hormones into the brain is facilitated by transporters present at the blood-brain barrier such as the monocarboxylate transporter 8 (MCT8, exclusive for TH, and other transporters as the organic anion-transporting polypeptide 1c1 (OATP1c1); both are expressed in blood vessels and tanycytes, while neurons contain mainly MCT8 [48, 49]. Transport of T_4 from brain blood vessels is facilitated by OATP1c1 in rodents [50]; T_4 is taken up by tanycytes and transformed to T_3 by deiodinase type 2 [48]. Although D2 activity is rapidly inhibited by T_4 in several tissues, by increased degradation through the ubiquitin-proteasome pathway, this does not occur in tanycytes [51]. Increased D2 expression and activity in tanycytes leads to increased T_3 available to hypophysiotropic TRH neurons and TRH synthesis down-regulation. Since D2 is not detected around the third ventricle at the level of the PVN [52], it has been hypothesized that T_3 enters TRHergic neurons at the median eminence level, and is transported by retrograde flux to the soma [2]. D3, the enzyme that degrades T_3, is detected in axons of neurons in the median eminence but very few of these are TRHergic [53]. TRH neurons thus sense T_3 directly [2]. *Dio3* mRNA has not been observed in rat or mice tanycytes in basal conditions [54], but only after T_3 treatment [55]. In fact, in several organs D3 is hardly detected but induced under various conditions [56]. In organotypic cultures of medial-basal hypothalamic slices, rich in tanycytes, the stimulatory effect of T_3 on *Dio3* expression is inhibited by TSH; as mentioned above, TSH stimulates synthesis and activity of D2 in tanycytes [40]. Thus, the balance between D2 and D3 sets up T_3 levels, depending on the particular loci where T_3 acts [57].

Hypothyroidism increases the expression of *Trh* in the PVN, and of *Dio2* in tanycytes, TSH synthesis and release from the pituitary, and decreases the expression of *Mct8* in the median eminence [2, 29, 48, 55], whereas changes in the expression of arcuate peptides are controversial [55, 58]. PVN *Trh* mRNA values increased by hypothyroidism are normalized by peripheral administration of T_4, also of T_3 since when T_4 levels are low T_3 may enter into the brain from the circulation [55, 59–61]; a microarray analysis revealed 110 genes up or down regulated by T_3 treatment in the hypothalamus [55].

T_4 administration inhibits not only *Trh* transcription but also the expression of convertases involved in processing TRH-precursor to TRH [30, 62]. In addition, the thyroid status modulates tanycyte *Trhde* expression, and PPII activity in the median eminence, which are rapidly increased in response to thyroid hormone administration [13, 63]. In mice, the enhancement of PPII expression in tanycytes by T_4 injection precedes the reduction of *Trh* mRNA levels in the PVN and is dependent on D2 [64]. Thyroliberinase, the soluble form of PPII synthesized in the liver and released to the circulation from where it likely reaches the portal vessels, is also upregulated by thyroid hormones [65, 66]. These results indicate that β2-tanycyte PPII and thyroliberinase are regulated accordingly for feedback, and may contribute to adjust the HPT axis, although a formal demonstration is missing.

At the anterior pituitary level, T_3 inhibits the expression of *Tshb*, *Tsha* and *Trh-r1*; in contrast, it upregulates PPII activity [67, 68]. The feedback effects of thyroid hormones at the pituitary level are further complicated due to the differential effects of other players that modulate TSH synthesis and are themselves targets of TH, as TRH or TRH-R1 [7, 69, 70]. Although it was originally thought that pituitary PPII contributes to the negative feedback that TH exert on the thyroid axis, failure to detect PPII expression or activity in thyrotrophs, or to modify the effect of TRH on TSH secretion with either a PPII antisense oligonucleotide or a PPII inhibitor in cell culture, casts doubt on its role in feedback control at pituitary level [15, 71].

3 Negative energy balance and the central regulation of the HPT axis

Two situations that inhibit the thyroid axis have deserved considerable attention, fasting and the non-thyroidal illness syndrome (NTIS); these are characterized by low *Trh* expression in the PVN, and low TSH and T_3 serum concentrations [2, 8, 72]. Injections of lipopolysacharide (LPS) produce an acute inflammation and, as NTIS, induce cytokines and NFkB, which upregulate D2 in α and β tanycytes [73–75]. The effects of inflammation extend to the TH transporters; an LPS

injection decreases significantly the mRNA levels of *Mct8* and *Oatp1c1* in brain blood vessels but not in astrocytes or neurons, which may lead to a reduction of TH passage through the blood-brain barrier [76]. Quantification of TH levels at the cellular level is not yet possible and therefore, many of these assumptions remain untested.

In conditions of negative energy balance, the activity of the HPT axis is inhibited. Fasting or food restriction leads to profound decreases of the serum concentration of leptin, which upregulates the activity of NPY/AgRP neurons in the arcuate nucleus, peptides that inhibit the expression of *Trh* in the PVN [2, 77]. In addition, negative energy balance inhibits POMC neurons and the release of αMSH in the PVN, a peptide that stimulates the synthesis and release of TRH from hypophysiotropic neurons [2, 78]. A leptin injection, or the icv injection of αMSH, counteracts the decrease in PVN *Trh* mRNA levels induced by fasting [2, 79]. Thus, the leptin drop, relayed through these arcuate nucleus neurons, contributes to a reduction of PVN TRH neurons activity, and of *Trh* mRNA expression in the PVN. Furthermore, in addition to this indirect pathway, leptin can activate directly *Trh* expression [77]. Fasting also targets tanycytes, where it increases *Dio2* expression and activity, presumably through the combined effect of increased corticosterone and decreased leptin serum concentrations [2, 80]. Parallel to fasting-induced increase in *Dio2* expression, in male rats PPII expression in tanycytes and activity in the median eminence, and thyroliberinase activity are also increased, whereas that of anterior pituitary PPII is not [63]. Thus, tanycyte PPII and/or thyroliberinase may engage in the adjustment of the thyroid axis in response to fasting, and/or maintain its long-term inhibition.

4 Positive energy balance and the central regulation of the HPT axis

Many circumstances, albeit not all, that affect the activity of POMC or NPY neurons coincide with altered *Trh* expression; conditions that stimulate energy expenditure increase αMSH and TRH content in the PVN [1, 17] (Supplementary Table 1). An interesting dichotomy stems from studies related to obesity, since obesity is correlated with reduced expression of *Pomc* and with increased expression of *Npy* although in diet induced obesity, resistance to gain weight in different mice strains correlates with POMC [81]. Feeding rats with a high fat diet (HFD) increases body weight and the activity of the HPT axis: *Trh* mRNA levels, processed peptide content in the PVN as well as concentrations of circulating TH are increased in response to the HFD; these changes have been attributed to a direct effect of increased leptin on TRHergic neurons [82]. However, the same group has recently reported that inhibition of the activity of the nutrient-sensing enzyme Sirtuin 1 (silent mating type information regulation 2 homolog 1 [Sirt1]), which is increased in the hypothalamus of obese subjects and correlates with inhibited energy expenditure, causes in HFD-fed rats increases in mRNA levels of *Pomc* and of carboxypeptidase E in the arcuate nucleus, and peptide levels of αMSH and TRH in the PVN, parallel to reduced body weight gain and increased oxygen consumption (in high-fat fed but not in lean rats) [83].

Differences in the propensity to gain weight under a HFD led to classify rats as obesity resistant (OR) or obesity prone (OP); OR rats express higher levels of *Pomc*, and lower of *Npy*, than OP rats [84]. A similar study performed in mice shows that obesity resistance to HFD correlates with a normal activity of the HPT axis during 7 weeks. In contrast, OP mice have increased *Trh* and *Tsh* expression, normal hypothalamic *Dio2* expression and activity or TH circulating levels, but altered expression of several T_3-target genes in liver; both OR and OP mice have similar alterations by 27 weeks [85]. The increase in *Trh* and *Tsh* expression in the obesity prone mice seems to contradict their involvement in energy expenditure, a conclusion supported by several reports indicating that hypothalamic expression of *Trh* and *Bdnf* is higher in lean than in obese animals [86, 87]. Furthermore, a recent search of the genetic background of young adults with extreme obesity found, in the exome data, a set of genes with novel mutations (indels, nonsense and missense) which are predicted as deleterious for protein function. The TRH gene was among the genes identified by this criterion [88]. These results exemplify that basal levels of TRH or TSH measured at a given time point might not correlate with either leptin, hypothalamic D2, or arcuate peptides, nor with metabolism in TH target tissues.

The information presented so far partially supports the conclusion that hypophysiotropic TRHergic neurons are energy sensors that are inhibited in conditions of energy deficit as starvation or food restriction. In conditions of positive energy balance as in diet induced obesity, variations in experimental protocols, and the complex etiology of the disease in humans, complicate the possibility to reach a definitive conclusion. It is now evident that plastic changes are induced by metabolic cues causing synaptic rearrangements in the circuits that sense and control energy balance; for example, the melanocortin system is highly susceptible of adaptation to extreme metabolic conditions [89]. Either fasting or obesity are dynamic physiological states that alter the function of various organs, they promote the release of corticosterone, cause inflammation, revealing many more players yet to be identified. In summary, these numerous actors may affect the activity of TRHergic neurons in a multifactorial fashion [90] representing a challenge for future investigations.

5 Responses of hypophysiotropic TRH neurons to acute stimuli

Energy demanding situations as cold exposure increase very rapidly (15–60 min) PVN *Trh*-mRNA levels [1, 2, 91–94].

They also enhance convertase expression in the PVN [95]. Simultaneously, within minutes, the pre-existing TRH stored in median eminence terminals is released. An increase of TSH concentration in serum is detected at 30–60 min, followed by an enhancement of serum T_4 concentration. T_4 is converted to T_3 by D2 at target tissues: in brown adipose tissue (BAT) for example, D2 activity is stimulated by sympathetic afferents; in turn, T_3 increases uncoupling protein 1 (UCP-1) transcription and maintains thermogenesis [1, 96]. Increased TH levels exert feedback inhibition to normalize the activity of the axis and achieve homeostatic equilibrium. Regulation of deiodinase activities and TH effects are target specific [97]. Even if cold-exposure is maintained, PVN *Trh* mRNA levels normalize by the second hour [91, 98]. The rapid transcriptional response of TRHergic neurons to neuronal stimuli is elicited by the almost immediate increase in CREB phosphorylation. Stimuli that enhance CREB phosphorylation, for example by PKA or ERK activators in hypothalamic or neuroblastoma cells, increase pCREB binding to CRE-2 and stimulate TRH synthesis (Fig. 1b); [99–101]. The relevance of pCREB for *Trh* expression has been demonstrated *in vitro* [102], or *in vivo* in response to αMSH and other arcuate-derived peptides [103]. The transient cold-induced increased *Trh* expression may relate to the transiency of the kinetics of CREB activation, and to non-cell autonomous effects such as the counter-regulatory effects of corticosterone.

As reported for chronic stress, acute stress inhibits *Trh* synthesis and the activity of the HPT axis [1]. An acute stress previous to a cold exposure blunts the cold-induced stimulation of the HPT axis, effect reproduced by a corticosterone injection 1 h before cold stimulation which interferes with the increase in *Trh* mRNA levels; likewise, corticosterone blunts the enhancement of *Trh* mRNA levels by cAMP analogs in hypothalamic cells [96]. These results underscore the importance of timing in intracellular activation, transport and inactivation of transcription factors for the transcriptional control of *Trh* expression, as previously shown for CRH neurons [104]. The interfering effects of glucocorticoids on PKA signaling occur before transcription; neither pCREB nor GR bind to their response elements in the TRH gene proximal promoter when hypothalamic cells are co-incubated with dexamethasone (or corticosterone) and forskolin (or cAMP analogs) [96, 101]. Recent experiments have revealed that this interference is not due to the recruitment of deacetylases to the TRH promoter, but occurs by a protein:protein interaction between activated GR and catalytic PKA that diminishes their translocation into the nucleus in cultured cells, and decreases the phosphorylation of CREB. *In vivo*, the number of PVN TRHergic cells expressing pCREB increases after 1 h of cold exposure, but not if animals were previously injected with corticosterone (Sotelo et al., 2016 unpublished). These recent findings contribute to untangle the early interactions between transcription factors that regulate *Trh* gene expression. However, many more remain to be elucidated since for example, the CRE site is flanked by GC sites and SP1/Krüppel response elements [101] which may be activated by insulin and, depending on the type of Krüppel factor activated, it could have stimulatory or inhibitory actions on CREB binding or on *Trh* transcription [105].

6 Integrated responses of the HPT axis

As consequence of a meal or temporal food deprivation, leptin, insulin, ghrelin and other hormones activate or inhibit the POMC or AgRP/NPY neurons that control the HPT axis activity. These stimuli are likely transient, but necessary for maintaining homeostatic metabolic environment. Given the nocturnal nature of laboratory rodents, and that most experiments are performed during light hours, results may vary depending on the time frame [92]. Furthermore, not all effectors have been characterized and therefore, consideration of the different variables involved provides a framework to analyze and interpret the functioning of the HPT axis. How an acute response is modified by the situation of the animal at a particular time deserves attention. For example, several reports suggest that exercise inhibits the HPT axis activity; conditions of forced or exhaustive exercise decrease the levels of circulating TH levels, and it has been proposed that it is due to the increase in circulating glucocorticoids or interleukin 6 [106]. The effect of increased physical activity on the thyroid axis probably depends on the extent of the energy demand and individual reserves; 20–30 min of intense treadmill exercise causes a rapid increase in serum T_4 concentration with later decreases in T_3 serum concentration [107], while growth hormone (GH) is gradually increased, dependent on the increases in TH as a treatment with propyl-thiouracil avoids GH increase together with its lipolytic effects [108]. We have shown that intermittent exercise provided by voluntary wheel running increases *Trh* expression and activates the HPT axis proportionally to the amount of exercise performed during the last period or the 2 weeks the experiment lasted, and to the loss of white adipose tissue; lipolysis provides the required fuel that balances the diminished food intake [109]. The HPT axis appears stimulated in spite of lower serum leptin concentrations than those of the pair-fed group [109]. These assumptions are valid when data are compared to a pair fed group that was given the amount of food consumed by the exercised group and not, to naive rats fed ad libitum [109]. Since exercise is intermittent its impact on central pathways is probably so, which differs from longer-term forced situations like training exercise. Voluntary wheel running might resemble an active-mode of life, whose effects are in contrast to the sedentary style that animals encounter in an un-enriched cage [110].

In conclusion, we have provided examples of steady state conditions in hypophysiotropic PVN TRH neurons activity that reflect a balance between thyroid hormone feedback, nutrition status, and the stimulatory or inhibitory influences of

the circadian cycle. These studies indicate that at a given time TRHergic neurons respond to an incoming stimulus depending on the state of the circuit engaged, which is modulated by previous influences.

7 Genetic alterations in TRH signaling

Central hypothyroidism arises from dysfunction at hypothalamic or pituitary levels. Congenital causes are being traced to mutations in the genes coding for elements involved in TRH signaling. Although adult TRH knockout mice have central hypothyroidism and a decrease in the bioactivity of TSH [111], in humans congenital causes have not been attributed to hypothalamic gene mutations [112, 113]. Only one *Trh* mutation has been characterized in obese young adults [88]. However, several mutations related to central hypothyroidism correspond to TRH-receptor or to other elements down the chain of participants in the activity of the HPT axis [113–115], aside from those that involve combined pituitary pathologies. Thus, rare biallelic TRH-R mutations, including a nonsense mutation and an in-frame deletion, have been associated to isolated congenital hypothyroidism with reduced circulating T_4 and absent TSH response to exogenous TRH [116, 117]. Recently, a deleterious missense TRH-R mutation associated with congenital hypothyroidism mapped to the second transmembrane helix drastically reduces the ability of the receptor to signal through the Gq/11dependent pathway [115]. These results are consistent with data showing that the ablation of TRH-R1 expression in mice produces a profound central hypothyroidism [118].

A genome wide association study indicates a significant association between polymorphisms (rs16892496 and rs7832552) of the TRH-R1 gene and lean body mass in humans. Subjects carrying the theoretically highest expressing (TT) genotype in the rs7832552 single nucleotide polymorphism [according to [119]; see below] had higher lean body mass compared to the other subjects [120]. An additional study in older women is consistent with an association of the rs16892496 polymorphism with fat-free mass, and suggests additionally an association with muscle strength [121]. Recently, the functionality of some of these mutations was assessed with luciferase gene reporter assays in cell culture; these mutations show diminished activity compared to the other alleles [115, 119]. Thus, a functional link may exist between TRH-R1 gene polymorphism and lean body mass.

8 Altering the programming of the HPT axis

Many more problems than those found by genetic alterations are being detected, which are due to interference on developmental programming of the HPT axis, which set its function at adulthood. The transcriptional regulatory networks that at different stages of development define cellular and circuit phenotypes are being identified [122–124]. Some are relevant for hypothalamic TRHergic neurons [123], but whether they relate to hypophysiotropic PVN neurons is not always known [125, 126]. The development of the components of the HPT axis follows different patterns for which their susceptibility for perturbations extends to various periods. The thyroid gland matures until the second half of pregnancy in humans, and by embryonic (E) day 17.5 in rodents. The fetus depends on mother's TH delivered through the placenta and the function of the offspring thyroid at birth correlates with that of their mother [127, 128]. Small variations may affect the HPT axis set point [128]. The maturation of neuroendocrine hypothalamus differs between altricial and precocial animals; in rodents it occurs mainly during the postnatal period, whereas in sheep or primates it essentially takes place before birth [129]. However, both pre- and postnatal periods share a sensibility to metabolic and nutritional alterations [129, 130]. Direct effects on the programming of the HPT axis likely depend on time of insult, given the existence of critical periods in the development of the various elements involved.

In rodents, hypothalamic neurogenesis peaks at E12 [PVN: 12–14; ARC: 12–16]; in the rat PVN, TRHergic neuroendocrine neurons are born during the embryonic day 12 [131] and E12.5 in mice [123]. Expression of PVN-*Trh* increases during lactation as do that of other elements of the HPT axis, such as hypothalamic PPII activity, pituitary TRH receptors, serum concentrations of TSH, TH, and thyroid hormone receptor (Trb1) in the PVN [132–134]. TRH-induced TSH release appears at postnatal day (PND) 5–7 [135, 136]. At birth, D1 is present in liver and pituitary, but T_4 to T_3 conversion is low in hypothalamus and increases during lactation [137]. In the rat, *Dio2* expression is intense at PND 15 in the lateral parts of the median eminence and the lining of the lower portion of the third ventricle [138]. TH feedback effects on *Trh* mRNA levels in the PVN, TRH-induced TSH release, and pituitary-TSH biosynthesis, are not detected until the 2nd postnatal week, in parallel to increased serum T_4 concentrations [139–141]. The mechanisms of the delayed appearance of the feedback control of the HPT axis have been clarified in the chicken, that mature near birth (hatching) as humans do. In this species, the limiting step that explains the time gap between the onset of HPT function and TH-mediated negative feedback is the increase of D2 activity in the hypothalamus (including in the MBH tanycytes). This increase allows a T_3 gradient sufficient to establish the set point of the HPT axis, T_3 acting on *Trb2* whose expression is instituted earlier [142]. Rodent tanycytes appear to differentiate from a subpopulation of radial glia [143, 144]. In the rat, tanycytes are first detected at E18 [145], but most are generated during the first postnatal week. This contrasts with the pattern observed in human hypothalami that express *Dio2* since mid-term with almost disappearance around birth and raising again by day 2 [146]. The

description of the developmental pattern of D2 activity in rodent tanycytes is lacking but it could permit to test the hypothesis put forward in the chicken.

Another important aspect of the physiology of the hypophysiotropic TRH neurons is the maturation of the connections between ARC and PVN that are formed at PND8–10 [147]. The fetus and newborn have a positive energy balance and leptin is a strong participant in the adequate axonal growth and wiring of these nuclei [129, 147]. Besides leptin, glucocorticoids, insulin and ghrelin control the activity of POMC and NPY/AgRP neurons [129]. Perturbations in the perinatal environment modify the programming of these arcuate neurons, with long-term effects that potentially will affect the activity of PVN-TRH neurons. Some mechanisms involved in the long-lasting effects of early metabolic dysregulations are being elucidated; for example, the reduced adult *Pomc* gene expression in prenatally over-nourished rats is due to the epigenetic changes (hypermethylation) in the promoter; other epigenetic changes have been identified in the leptin or glucocorticoid receptor genes [130, 147, 148].

The mother's nutritional status can alter fetal programming and lead to metabolic disturbances in the adult. The hypothalamic cyto-architecture is affected in offspring from malnourished mothers, their offspring showing propensity for obesity and leptin resistance [129]. Small babies at birth, or offspring from malnourished mothers have dysfunctional HPT axis. Protein malnutrition during pregnancy diminishes T_3 serum levels and TSH response to cold exposure, possibly because TRH release is inhibited [149, 150]. The opposite situation also has strong deleterious effects on the development of the offspring, as maternal obesity programs it for obesity. In humans, maternal obesity alters the serum concentrations of thyroid hormones and her fT_3 serum levels associate with those in the fetus and with the degree of obesity [151]. Multiple models have been studied in an attempt to understand the mechanisms that lead to metabolic programming of obesity [129, 130]. In rodents, offspring from obese mothers have increased number of neurons expressing orexigenic peptides (NPY/AgRP), as well as leptin resistance and altered intra-hypothalamic connectivity [147], changes that may influence development of the HPT axis or its postnatal or adult responses. In post-weaning offspring of HFD-fed rats, the amount of TRH precursor protein is increased in the PVN, together with serum TT_3 and fT_4 concentrations; white adipose tissue mass and leptin serum concentration are increased together with decreased expression of nuclear factors responsible for transducing leptin signaling in the arcuate (pSTAT3 and SOCS3), suggesting leptin resistance [152]. Overfeeding pups during lactation, from obese mothers, amplifies the hypothalamic changes observed in offspring from obese mothers [153]. In non-human primates under HFD during pregnancy fT_4 levels are normal, but their 130 day old fetus do show differences; they have low expression of hypothalamic TRH as well as of genes responsible of thyroid hormone production [154]. In contrast, obese women and their neonate child have altered TH levels [151, 155].

Stress is another very important threat against optimum development. Endogenous glucocorticoids fluctuate during pregnancy; well-timed surges promote the development and maturation of multiple organs including brain, pituitary and thyroid; administration of high concentrations at wrong stages of development may have life-long physiological consequences, such as altered HPA functioning [156]. Stress can also induce hypothyroidism in the mother and in consequence the deficient hormone availability affects brain development of the offspring [127]. Injection of corticosterone to a pregnant rat from E18-E21 decreases core body temperature and *Trh* mRNA levels in the PVN of female offspring but not males. Although this suggests a gender specific effect, decreased TRH precursor protein amount is detected in PVN fibers of both genders; furthermore, serum T_3 concentration diminishes only in males but that of TT_4 in both sexes at PND14 while levels are normal by PND60 [157]. The apparent difference in gender response remains to be analyzed under a situation of energy demand, since estrogens have multiple effects on the HPT axis including its response to cold exposure [158], and gender differences in stress response are observed early in life [159].

Since other components of the HPT axis present postnatal maturation, long-lasting alterations may also be provoked by postnatal factors. Postnatal effects are produced by changing the nutritional status. Overfeeding only during lactation by limiting the size of litter, or feeding the mother with HFD, induces obesity in the offspring at adulthood that present several symptoms of hypothyroidism and leptin resistance including low *Trh* expression in the PVN, and of TSH in the pituitary [153, 160]. Early life stress produces long life changes in metabolism and the stress response of the offspring [161–163]. Based on the initial observations that maternal behaviour predisposes the offspring to these alterations, a model of maternal separation (MS) has been implemented leading to the characterization of the degree of methylation of several stress-responsive genes [162]. Varying degrees of grooming and leaking lead to early changes in circulating T_3 and altered hippocampal GR expression which plays a central role in the negative feedback control of the HPA axis [164, 165]. Since the activity of the HPT axis is modulated by acute or chronic stress [1], and because we have shown that stress or corticosterone injections blunt the response of the axis to cold exposure [96], we recently studied the effects of MS on basal and fasting-induced expression of HPT axis activity markers. mRNA levels of PVN-*Trh* and of PPII in tanycytes were modified by MS in a sex-specific manner. Furthermore, the response to 48 h fasting was partially blunted in MS-males, which could alter their adaptability to energy demanding situations [166].

9 Environmental insults and the HPT axis

It has been recognized for long time that cigarette smoke alters the activity of the HPT axis of the offspring, whether exposed in uterus or afterwards [167]. Recent work contributes to the understanding of the mechanisms involved; nicotine administered to lactating mothers since the 2nd postpartum day causes in the adult offspring increased fat mass and circulating leptin concentration, with symptoms of leptin resistance. In addition, it provokes tertiary hypothyroidism evidenced by low *Trh* expression in PVN, TSH concentration in pituitary, and altered deiodinase activity in liver and BAT [168].

The multiple dangers imposed by chemicals in the environment have become now evident. Many of them cause endocrine alterations for which they are now recognized as endocrine disruptors. Their degree of toxicity varies depending on whether they coincide with a critical period of development, as they can bind to hormone receptors and thus affect hormone signaling [169, 170]. Recent reviews cover the variety of compounds ranging from cosmetics, insecticides, plastic derivatives, and flame retardants that affect the thyroid axis at different levels including the expression of *Trh* receptor [171–175], although to our knowledge, data on *Trh* expression are still lacking.

10 Conclusions

In the last years, there has been a steady increase in the knowledge of the mechanisms involved in TRH signaling and its central role in modulating the HPT axis. Among the new data, we can pin-point the mechanisms that control the transcription of the *Trh* gene, with the identification of new transcription factors engaged in *Trh* gene expression, as well as how signaling pathways define the dynamics of TRH-neurons response to acute and/or long-term influences. A better, albeit yet incomplete, description of the response and relationship of TRH neurons activity with positive energy balance has also emerged, as well as putative intermediary mechanisms. The importance of tanycytes as a central relay for the feedback control of the axis, as well as for HPT responses to alterations in energy balance, and other stimuli has been reinforced. Tanycytes seem critical for hypophysiotropic TRH neurons activity, but also to control the extracellular fate of released TRH through the activity of the TRH degrading enzyme. A relationship between polymorphism in the TRH-R gene and lean body mass underlines the critical role of TRH signaling in health. In addition, some studies have started to shed light on the interference of maternal and postnatal stress and nutrition on HPT axis programming, and confirmed the axis susceptibility to early insults, including the devastating effects of endocrine disruptors.

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Compliance with ethical standards

Conflict of interest statement The authors declare no conflicts of interest.

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