Regulation of TRH neurons and energy homeostasis-related signals under stress

Patricia Joseph-Bravo, Lorraine Jaimes-Hoy and Jean-Louis Charli

Departamento de Genética del Desarrollo y Fisiología Molecular, Instituto de Biotecnología, Universidad Nacional Autónoma de México (UNAM), A.P. 510-3, Cuernavaca, Morelos 62250, Mexico

Correspondence should be addressed to P Joseph-Bravo
Email
joseph@ibt.unam.mx

Abstract

Energy homeostasis relies on a concerted response of the nervous and endocrine systems to signals evoked by intake, storage, and expenditure of fuels. Glucocorticoids (GCs) and thyroid hormones are involved in meeting immediate energy demands, thus placing the hypothalamo–pituitary–thyroid (HPT) and hypothalamo–pituitary–adrenal axes at a central interface. This review describes the mode of regulation of hypophysiotropic TRHergic neurons and the evidence supporting the concept that they act as metabolic integrators. Emphasis has been be placed on i) the effects of GCs on the modulation of transcription of *Trh in vivo* and *in vitro*, ii) the physiological and molecular mechanisms by which acute or chronic situations of stress and energy demands affect the activity of TRHergic neurons and the HPT axis, and iii) the less explored role of non-hypophysiotropic hypothalamic TRH neurons. The partial evidence gathered so far is indicative of a contrasting involvement of distinct TRH cell types, manifested through variability in cellular phenotype and physiology, including rapid responses to energy demands for thermogenesis or physical activity and nutritional status that may be modified according to stress history.

Key Words
- HPA
- HPT
- stress
- metabolism

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Introduction

The tripeptide pglu-his-proNH2 was isolated from hypothalami and named according to its endocrine function: 'thyrotrophin-releasing hormone' (TRH; Boler *et al.* 1969, Burgus *et al.* 1969). TRH is synthesized in the neuronal cell bodies of many brain regions from a precursor protein, prepro-TRH (ppTRH), and processed to yield the biological active peptide (Lechan *et al.* 1986, Lechan & Segerson 1989, Nillni 2010). Several hypothalamic nuclei synthesize TRH (Fig. 1), but the TRH neurons that control thyrotrophin (TSH) release are the hypophysiotropic neurons present in the mid–caudal paraventricular nucleus (PVN) (anterior–medial PVN in mouse), whose axons project to the median eminence from where TRH is released into the portal vessels of the hypothalamo–pituitary system (Fekete & Lechan 2014; Fig. 1). In humans, hypophysiotropic TRH neurons are present in the medial region of the dorsocaudal portion of the PVN (Fliers *et al.* 2014).

The amount of released TRH that reaches the pituitary is modulated in the median-eminence extracellular space by the activity of the TRH-degrading ectoenzyme (pyroglutamyl peptidase II (PPII)) present on tanycytes, a group of ependymal cells lining the third ventricle whose end-feet are in proximity with TRH terminals and portal vessels (Sánchez *et al.* 2009). In the pituitary and upon binding to its receptor (TRH-R1), TRH controls the release

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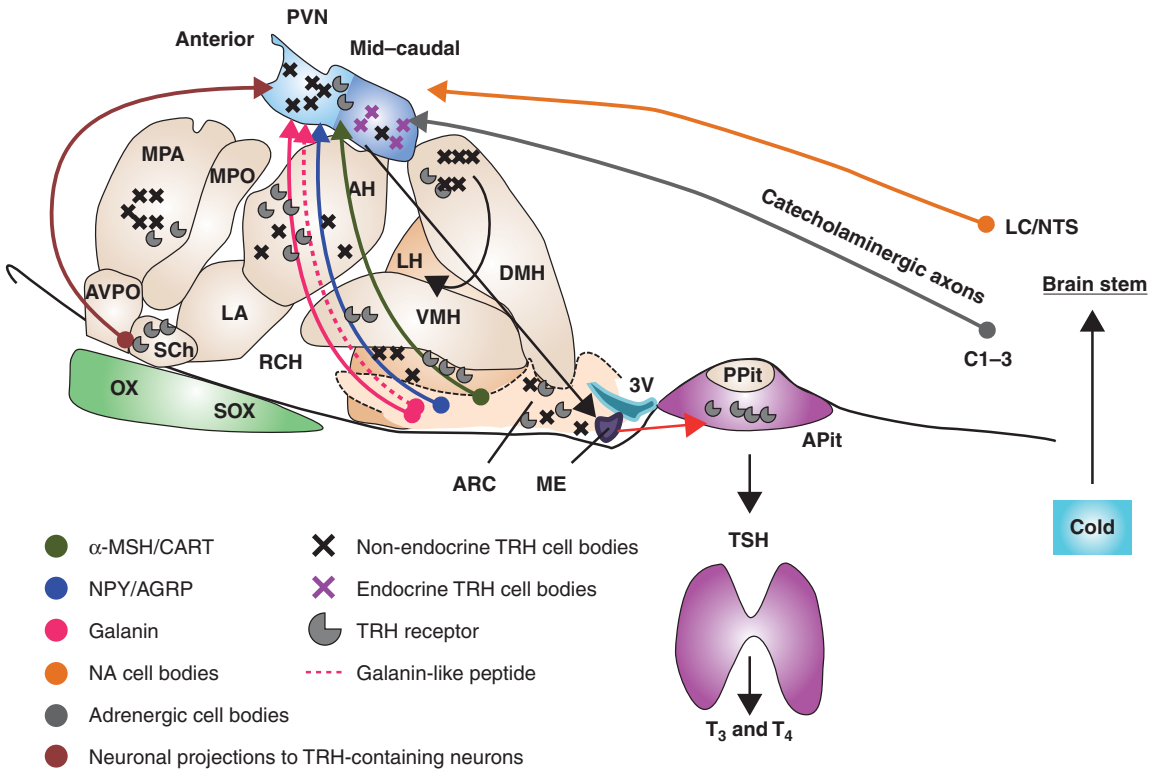

Figure 1
Schematic localization of TRH endocrine and nonendocrine cell bodies and TRH receptor fields in the hypothalamus and pituitary and of afferents to TRH neurons. TRH-synthesizing neurons in the rat PVN are represented in light purple, the anterior PVN and the mid–caudal PVN in dark purple. Arrows indicate afferents originating from the ARC and the brainstem that regulate the hypothalamic–pituitary–thyroid axis. AGRP co-localizes with NPY in the ARC (orexigenic peptides, blue arrow); NPY innervation to hypophysiotropic TRH neurons originates mainly from the ARC, and some (25%) from the brainstem. Hypophysiotropic TRH neurons receive afferents from ARC neurons synthesizing anorexigenic peptides cocaine–amphetamine regulated transcript (CART)/α-MSH and CART expressed in adrenergic C1–3 regions in the medulla. ARC neurons expressing the orexigenic peptide galanin innervate the anterior PVN parvocellular subdivision (aPVN), mid and caudal PVN, and GALP only aPVN (pink arrows). Catecholaminergic inputs arising from the brainstem play a key role in regulation of the HPT axis in response to cold. Both noradrenergic (LC and NTS, orange arrows) and adrenergic (C1–3 regions, brown arrow) axons establish contacts with TRH neurons in the PVN (Fekete & Lechan 2014). AH, anterior hypothalamic area; ARC, arcuate hypothalamic nucleus; AVPO, anteroventral preoptic nucleus; DMH, dorsomedial hypothalamic nucleus; GALP, galanin-like peptide; LA, lateroanterior hypothalamic nucleus; LC, locus coeruleus; LH, lateral hypothalamic area; ME, median eminence; MPA, medial preoptic area; MPO, medial preoptic nucleus; NTS, nucleus solitary tract; OX, optic chiasm; Ppit, posterior lobe of pituitary; PVN, paraventricular hypothalamic nucleus; RCH, retrochiasmatic area; SCh, suprachiasmatic nucleus; SOX, supraoptic decussation; VMH, ventromedial hypothalamic nucleus; 3V, third ventricule. The figure is based on Paxinos & Watson (2005) (The Rat Brain, 5th Edn): Fig. 78 (lateral 0.4 mm) and coronal sections (Figs 24–33; Bregma −1.4/−3.8) to delineate approximate location of hypothalamic nuclei; PVN division in Simmons & Swanson (2009)).

of TSH and its transcription and posttranslational modifications, such as glycosylation, that define the bioactivity of TSH (Chiamolera & Wondisford 2009). Under certain physiological conditions, TRH modulates prolactin or growth hormone synthesis and release (Galas *et al.* 2009). The concentration of pituitary TRH-R1 is regulated by TRH and several other hormones (Chiamolera *et al.* 2012, Hinkle *et al.* 2012). Released TSH controls several steps of the synthesis of thyroid hormones (TH) at the thyroid gland, increasing serum levels of thyroxine (T_4) and to a minor degree, 3,5,3′-triiodothyronine (T_3). Deiodinases 1 or 2 (D1, D2) convert T_4 to T_3 in various tissues and their activity is differentially regulated in a condition- and tissue-specific manner (McAninch & Bianco 2014). TH have crucial roles in basal metabolic rate, thermogenesis, lipid and carbohydrate metabolism, indicating that the hypothalamus–pituitary–thyroid (HPT) axis is an important player in energy homeostasis (Hollenberg 2008, Fliers *et al.* 2014, McAninch & Bianco 2014, Mullur *et al.* 2014).

The activity of the HPT axis is stringently regulated by neuronal stimuli impinging on TRH neurons and by the negative-feedback effects of TH on TRH and TSH synthesis and release (Hollenberg 2008, Fekete & Lechan 2014,

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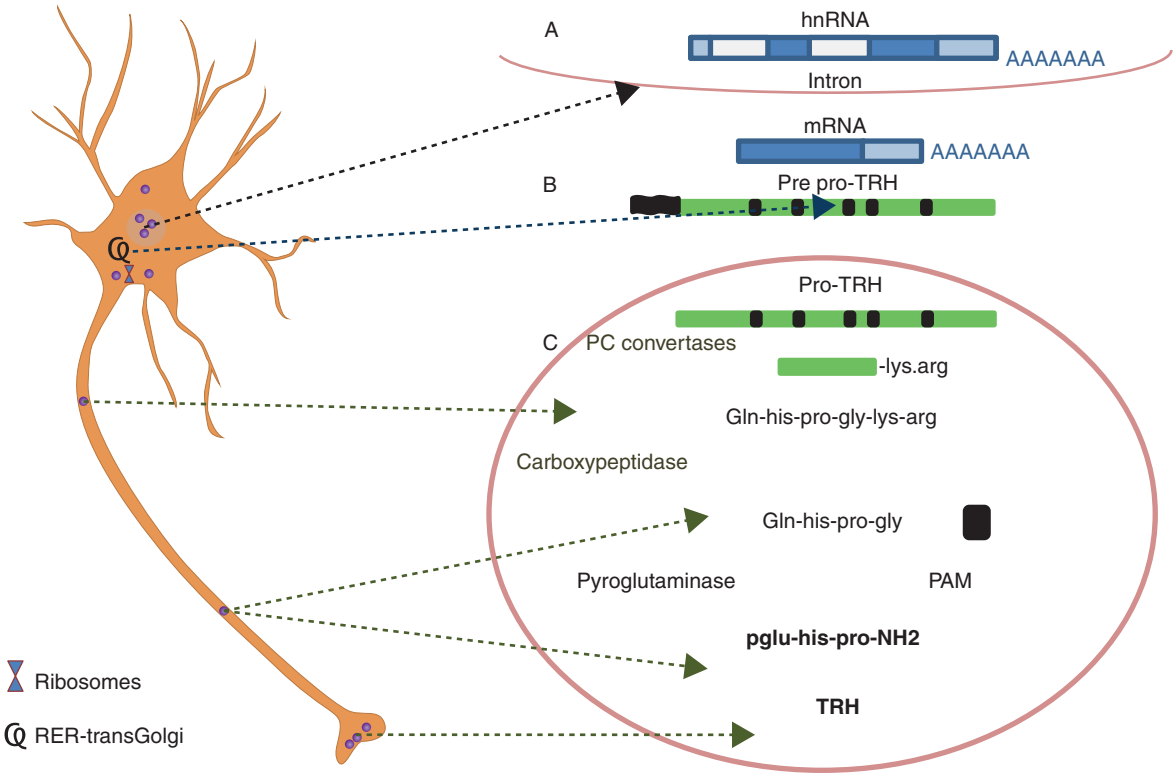

Figure 2
Schematic representation of TRH synthesis. (A) The primary transcript is synthesized in the nucleus as prepro-TRH heterogeneous nuclear RNA (hnRNA) which contains two introns and five exons. (B) After splicing, mature RNA is transported to the cytosol, binds to ribosomes, begins transcription of prepro-TRH mRNA, the leader sequence is cleaved, synthesis of pro-TRH continues with ribosomes linked to rough endoplasmic reticulum (RER) and precursor is transported inside the ER. (C) At the transGolgi pro-TRH may suffer a first cleavage by protein convertase 1 (PC1), proTRH is compartamentalized in secretory granules with the rest of processing enzymes: PCs cleave at the carboxy end of a pair of basic residues, a carboxypeptidase cleaves the basic residues leaving the immediate precursor of TRH: gln-his-progly (black squares) and cryptic peptides (green); pyroglutaminase converts gln to pyroglu and peptidyl-glycine-α-amidase (PAM) leaves the amino group of the glycine bound to the carboxyl end (forming the amide group) and cleaves the rest of the pro-carbon moieties. Processing occurs as the secretory granule is transported to the nerve terminal (Nillni 2010).

Fliers *et al.* 2014). T_3 downregulation of *Trh* mRNA levels occurs specifically in the hypophysiotropic neurons of the PVN and not in other hypothalamic nuclei expressing *Trh* in rat or mouse (Segerson *et al.* 1987, Sugrue *et al.* 2010). The neuronal concentration of T_3 in the PVN is controlled by D2-conversion of T_4 in tanycytes, the presence of T_3 transporters, and the activity of neuronal D3 that inactivates T_3, converting it to rT_3 (Fliers *et al.* 2006, Fekete & Lechan 2014). The most commonly used sensor of activation of TRH neurons has been the measurement of *Trh* mRNA levels. Rapid (<2 h) changes in TRH tissue concentration, mostly concentrated at nerve endings have been used as an indirect marker of TRH release because at later times TRH content represents the resultant of synthesis and release but not of degradation because the intracellular peptidases do not modulate TRH levels within secretory granules (Rondeel *et al.* 1991, van Haasteren *et al.* 1995, Charli *et al.* 1998, Aguilar-Valles *et al.* 2007).

TRH biosynthesis

The *Trh* gene encodes a protein that contains 5–8 (depending on the species, Wallis 2010) repetitions of the gln-his-pro-gly sequence (Lechan *et al.* 1986). Like other neuropeptides, the precursor ppTRH is synthesized in the neuronal soma at the endoplasmic reticulum (Fig. 2B) where it is cleaved to proTRH, compartmentalized at the transGolgi into secretory granules that travel together with processing enzymes to the nerve endings where most of TRH is found (Nillni 2010) (Fig. 2C).

Trh mRNA is transcribed from a unique gene (Fig. 2A); the proximal promoter equivalent to 250 bp upstream of the TATA box contains several response elements (REs) that

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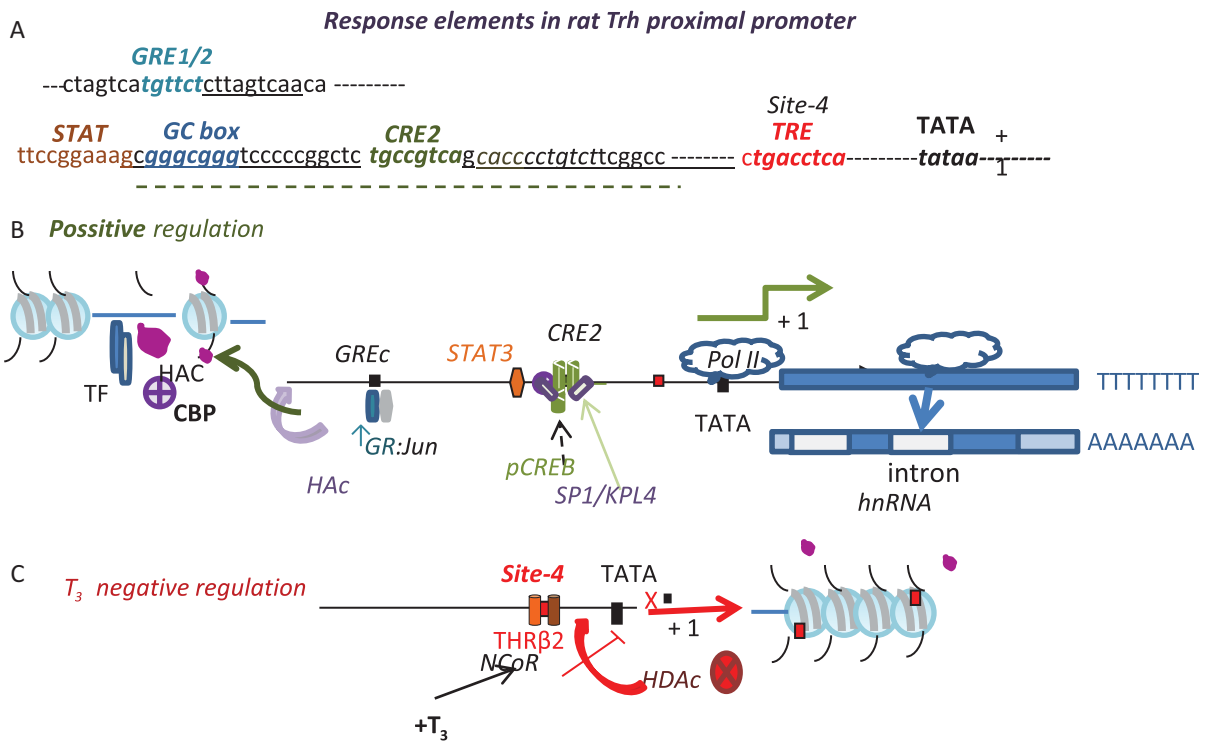

Figure 3
Sequences of response elements for transcription factors proven to be involved in regulating *Trh* transcription. (A) T_3 binds to thyroid hormone receptor THRβ on site 4 or THRE; corticosterone to GR-cJun on composite GRE (GRE1/2–AP1); leptin to STAT3, and pCREB on CRE; SP1 or KPL4 bind to extended CRE (green dashed line) (Lee *et al*. 1988, Díaz-Gallardo *et al*.2010*a*,*b*, Cote-Vélez *et al*. 2011). (B) Stimulation of transcription has been observed after treatment with noradrenaline, BDNF, and ERK, PKA activators that stimulate phosphorylation of CREB which binds to CRE; PKA activators also induce binding of SP1; pCREB recruits CBP with acetylase activity that releases the complex of histones and chromatin allowing binding of polymerase II (Pol II) and transcription machinery to proceed with transcription. This mechanism may occur with other transcription factors that present stimulatory activity such as STAT3 or GR-cJun. (Díaz-Gallardo *et al*. 2010*b*, Cote-Vélez *et al*. 2011). (C) Transcriptional repression of *Trh* transcription may occur by T_3 binding to THR on site 4, recruiting corepressors and deacetylases that compact chromatin avoiding binding of polymerase and basal transcriptional machinery (Hollenberg 2008, Díaz-Gallardo *et al*.2010*a*).

provide binding sites for specific transcription factors (TFs) whose activity is modulated by various signals (Lee *et al*. 1988; Fig. 3A). Chromatin remodeling constitutes the first step in transcriptional regulation; recruitment of specific TF, coregulators, and histone acetylases loosens the DNA from histones allowing initiation of transcription by RNA polymerase II (Pol II; Fig. 3B); certain TFs recruit histone deacetylases that compact chromatin repressing transcription (Gadaleta & Magnani 2014; Fig. 3C).

The negative regulation of TRH and TSH synthesis has long been recognized, but the mechanism is still under study (Segerson *et al*. 1987, Guissouma *et al*. 2000, Hollenberg 2008, Chiamolera *et al*. 2012, Vella *et al*. 2014). T_3 binds to TH receptors (THRs) THRα1, or THRβ1 and THRβ2, that are expressed and regulated in a tissue-specific manner; the same receptor may stimulate or inhibit transcription of multiple proteins in the same tissue (Ramadoss *et al*. 2014). The THR RE in the *Trh* promoter (site 4, Fig. 3A) binds THRs as homo- or hetero-dimers with retinoid X receptors (RXRs) *in vitro*; in transiently transfected cells, unliganded receptors activate *Trh* transcription whereas repression requires T_3 to be bound to the receptor (Guissouma *et al*. 2000, Hollenberg 2008, Chiamolera & Wondisford 2009; Fig. 4). Mice with knock-outs of the different THRs were used to demonstrate THRβ2 to be the most important receptor involved in downregulation of *Trh* transcription by T_3 (Abel *et al*. 2001, Chiamolera & Wondisford 2009); however, transfection of *Thrβ*-KO mice with either THRβ1 or THRβ2 rescues T_3-induced TRH expression, but only THRβ1 is able to activate in a ligand-independent manner (Dupré *et al*. 2004). Chromatin immunoprecipitation (ChIP) from stably transfected GH4C1 cells with *Trh* promoter detects acetylated histones 3 and 4, THRβ, and bound Pol II; T_3 treatment transiently recruits deacetylases (HDAC2, 3), diminishes Pol II binding and, after 24 h, also THRβ

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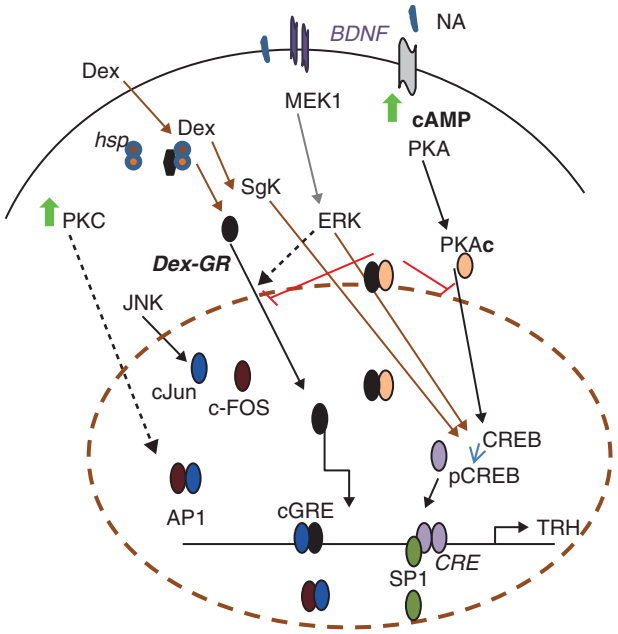

Figure 4
Model of rapid regulation of *Trh* transcription by extracellular signals. The transcription of the pro-*TRH* gene is rapidly increased by activators of CREB phosphorylation and by glucocorticoids. Noradrenaline (NA), BDNF, and dexamethasone or corticosterone, through protein kinase A (PKA), the ERKs MEK/ERK, and serum-glucocorticoid kinase (SgK), induce the phosphorylation of CREB; pCREB enhances transcription through an interaction with the CRE2 site. Activation of the glucocorticoid receptor (GR) by ligand binding releases it from a multiprotein complex; it is phosphorylated and transported to the nucleus where the GR:Jun heterodimer binds to a composite GRE (cGRE). A cross talk between the two pathways alters the individual transcriptional responses; glucocorticoids interfere with PKA stimulation of *Trh* transcription by a protein–protein interaction that impedes binding of pCREB or GR to their REs (Cote-Vélez *et al.* 2008, 2011, Díaz-Gallardo *et al.* 2010*b* and references therein).

(Umezawa *et al.* 2009). THRβ2 binds to the *Trh* proximal promoter region of chromatin from hypothalamic cells incubated 1 h with T_3 (Díaz-Gallardo *et al.* 2010*a*), as well as deacetylase (HDAC3), whereas Pol II and histone acetylases diminish compared with controls (Sotelo-Rivera *et al.* 2012). Chromatin from hypothalami of newborns has THRβ or RXR bound to TRH promoter which decreases after 20 h of T_3 treatment (Decherf *et al.* 2013). These results support the hypothesis that T_3–THRβ recruitment of deacetylases that may compact chromatin and inhibit Pol II binding and *Trh* transcription (Fig. 3C). Whether THRβ remains bound to DNA *in vivo* after longer T_3-treatments awaits resolution (Ramadoss *et al.* 2014).

Neuronal stimulation activates kinases such as PKA, PKC, and MAPK/ERK that rapidly enhance *Trh* transcription or mRNA levels in several cell systems (Uribe *et al.* 1995*a*, Pérez-Martínez *et al.* 1998, Harris *et al.* 2001, Cote-Vélez *et al.* 2005, 2008). The *Trh* proximal promoter contains a phosphorylated-CREB (pCREB) binding site (CRE2: positions −101/−92 in rat) that is protected, along with adjacent 5′ GC box and 3′ CACC sequences, by nuclear extracts of cAMP-stimulated hypothalamic cells (Fig. 3A; Díaz-Gallardo *et al.* 2010*a*,*b*); these adjacent sites are recognized by SP1/Krüppel-like factors (Kpl; Ren *et al.* 1998, Díaz-Gallardo *et al.* 2010*b*, Pérez-Monter *et al.* 2011). Deleted or mutated CRE2 or CACC sites resulted in a decrease of 50–80% in basal and 50% in forskolin-induced transcription (the latter also with mutated GC box; Cote-Vélez *et al.* 2011). Multiple-intracellular signals phosphorylate CREB in the nucleus, pCREB binds to its REs and interacts with coactivators (some with histone–acetylase activity) increasing transcription (Fig. 3B; Altarejos & Montminy 2011). SP1 binding may increase the stability of pCREB on CRE2, as has been proposed for a nonconical sequence (Lundblad *et al.* 1998) or, depending on the TF of the SP/KPl family, cause interference. ChIP assays revealed other REs, such as the STAT-binding site, responsive to leptin stimulation (Guo *et al.* 2004). A composite glucocorticoid RE (cGRE), formed from a half-site GRE next to an AP1 RE, binds the glucocorticoid receptor (GR) as a heterodimer with cJun after dexamethasone stimulation (Cote-Vélez *et al.* 2005, Díaz-Gallardo *et al.* 2010*b*; Fig. 3A).

Caveats regarding biosynthesis studies

Before continuing with this review we would like to stress some problems worthy of consideration when studying the regulation of TRH biosynthesis, given the diversity of experimental paradigms used. Cell lines are homogenous but with a particular set up that differs from the physiological situation, they are usually incubated with drugs for long periods and under steady conditions in contrast to *in vivo* situations where events such as clearance and diffusion play important roles. Transient transfections omit the regulatory steps related to chromatin remodeling (Gadaleta & Magnani 2014). Primary culture of embryonic hypothalamic cells (Uribe *et al.* 1995*a*, Harris *et al.* 2001), or transfected hypothalami of newborn pups (Guissouma *et al.* 2000), involve a mixture of multiple hypothalamic nuclei whose developmental windows, afferents and receptors, might differ to those exclusive to the PVN; furthermore, they miss the developmental effects of gonadal hormones (McCutcheon & Marinelli 2009). Knockout animals provide important information (Supplementary Table 1, see section on supplementary data given at the end of this article), but early-compensatory responses may lead to altered homeostatic states and/or the animals may have phenotypes that result from the simultaneous

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manipulation of many organs that contribute, for example, to altered TH levels (Astapova & Hollenberg 2013); however, they revealed the redundancy of important regulatory molecules (Chiappini *et al.* 2013). Notwithstanding the problems mentioned, these distinct approaches have provided important information for identifying potential modes of *Trh* transcriptional regulation.

The HPT axis in energy balance

Circuits involved in energy balance overlap with those involved in food intake. Afferents from neurons of the brain stem or of the arcuate nucleus (ARC) convey nutritional information to the PVN and other hypothalamic nuclei. ARC neurons detect circulating hormones levels modulated by the nutritional and energy status (ghrelin, leptin, insulin, glucose, and fatty acids) and activate or inhibit neurons that express orexigenic (neuropeptide Y (NPY) and agouti-related peptide (AgRP)) or anorexigenic (pro-opiomelanocortin (POMC) and cocaine amphetamine-regulated transcript (CART) peptides) (Schneeberger *et al.* 2014); *Trh* expression is inhibited by orexigenic and stimulated by anorexigenic peptides (Fekete & Lechan 2014). Table 1 summarizes data on mRNA levels of *Trh* and several ARC peptides, as well as on serum hormones in rodents studied under different paradigms. Caution is required to distinguish measurements in the hypophysiotropic area (approximately 50% of parvicellular PVN neurons (Simmons & Swanson 2009)) normally determined by *in situ* hybridization (ISH) of *Trh* mRNA in mid–caudal PVN, against total PVN by analysis in dissected tissue (Fig. 1; Fekete & Lechan 2014).

TRH–hypophysiotropic neurons are the central integrator of the HPT axis, considered to be metabolic sensors as they decode neuronal and hormonal signals related to the energy status (Lechan & Fekete 2006, Hollenberg 2008). THs have multiple targets responsible for energy distribution and expenditure (Klieverik *et al.* 2009, Mullur *et al.* 2014). Negative energy balance such as fasting, food restriction, or pathological situations, including infection or critical illness, decreases serum levels of TH, and TSH to some extent, as well as of *Trh* expression in hypophysiotropic TRH neurons. In humans, these conditions are recognized as nonthyroidal illness syndrome (NTIS). The principal mechanism of fasting-induced decreased *Trh* expression involves a drop in leptin serum concentrations that lowers α-MSH and CART stimulatory tone, and enhances the inhibitory effect of NPY/AgRP on TRHergic neurons (Fekete & Lechan 2014). Results from animal models of infection or inflammation support the hypothesis that the mechanism causing NTIS primarily entails increased D2 activity in tanycytes and reduced D1 and D3 in liver and muscle, changes probably produced by cytokines and not by corticosterone (Fekete & Lechan 2014, Fliers *et al.* 2014). The effects are more pronounced in chronic situations where multiple factors intervene, including feeding status. In humans, postmortem analyses of critically ill patients showed low T_4 and T_3 liver concentrations and reduced *TRH* mRNA expression, supporting inhibition of the HPT axis at central level (Fliers *et al.* 2014).

The effects of energy excess on TRHergic neurons are less clear. Body-weight gain (approximately 20%) induced by high-fat diet (HFD), or ovariectomy, is associated with increases in *Trh* expression in the PVN; both conditions augment circulating leptin levels, although only HFD increases TH serum concentrations in rat (Perello *et al.* 2010). Leptin rapidly activates TRHergic neurons, indirectly in mid-PVN through its effects on ARC neurons and directly in caudal-PVN with high concentration of leptin receptors (Huo *et al.* 2004, Perello *et al.* 2006). Leptin's direct effect is further supported by the exclusive increase in *Trh* mRNA in caudal PVN of ovariectomized rats (Uribe *et al.* 2009). The response of TRHergic neurons to feeding conditions differs in obese and lean rodents (Perello *et al.* 2010); under basal conditions *Trh* mRNA levels in the PVN are similar in obese and in lean Zucker rats; however, fasting decreases *Trh* expression more drastically in obese rats than in lean rats, a difference overridden by adrenalectomy (Duclos *et al.* 2005). A different allostatic state in obesity, related to a particular state of receptors causing leptin or insulin resistance, could be the basis of these differences.

Events involved in the regulation of energy balance such as fat metabolism, thermogenesis, body weight, food intake, and fat distribution is sex-dependent. Estrogen modulates the expression of several orexigenic and anorexigenic peptides and hormones (Brown & Clegg 2010). Male rats are more sensitive to insulin and females to leptin; compared with males, females adapt more efficiently to energy deficits, are more susceptible to HPT inhibition by food restriction, have lower pituitary expression of TSHβ and serum TSH associated with higher levels of T_3, and reduced hepatic activity of D1 under basal conditions (Cizza *et al.* 1996, van Haasteren *et al.* 1996, Valle *et al.* 2005, Marassi *et al.* 2007). Estrogen stimulates thyroid function, increasing iodide uptake and thyroid peroxidase activity which would increase TH biosynthesis (Lima *et al.* 2006). The expression of *Trh* in the PVN varies during the estrous cycle with highest levels at diestrous 2

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Table 1 Regulation of the HPT axis under chronic or acute stress. The HPT axis is differentially regulated depending on the origin of stress (physical or psychogenic), the duration (acute or chronic), and the animal's energetic and hormonal status

Rodent strain	Acute treatment	Chronic treatment	Food ltk	BW or body fat	Hypothalamic TRH peptide	PVN (mRNA)		Arc (mRNA)					TSH	TT_4	TT_3	Cort	References
						Trh	*Crh*	*Agrp*	*Npy*	*Cart*	*Pomc*	*Bdnf*					
Lou/C rat		vs Wistar		↓		+ ↑		* ↑	* ↑	* –	+ ↓	+ ↑	↑	↓	–	↓	Mitchell *et al.* (2006) and Veyrat-Durebex *et al.* (2013)
S–D		64 h fast				+ * ↓		* ↑	* ↑				↓	↓	↓	↑	Rondeel *et al.* (1992) and Sánchez *et al.* (2008)
		Refeeding				* 24 h ↑		* 4 h ↓	* ↓				24 h ↑	↑	↑		
S–D		HFD	↑	↑	↑	+ ↑							↑ 3 h	↑ & FT_4	↑ & FT_3		Perello *et al.* (2006, 2010)
		Leptin i.c.v.			↑ 3–6 h												
Wistar		FR 7 days (80%)		↓	↑	+ ↓	+ ↓		+ ↓ Y1R				–	↓	↓	↑	Jaimes-Hoy *et al.* (2008) and de Gortari *et al.* (2009)
		DIA 7 days	↓	↓	–	+ ↑	+ ↓ and		+ – Y1R				↑	–	↓	↑	
		DIA/CRH-R2 antagonist				+ ↓	CRHr2						↓	↓	–		
C57BL/6J		(−) Leu (7 days)		↓		↑ Regulated by CREB							↑		↑		Xia *et al.* (2015)
		(−) Leu + anti-TRH		–											↓		
CFY rat		Protein free diet (12 days)		↓		* ↓							↓		↓		Shi *et al.* (1993)
Wistar		MWM trained 5 days				+ ↑	+ ↑						–			↑	Aguilar-Valles *et al.* (2005, 2007)
Wistar		14 days: exercise vs pair-fed	–	Fat: ↓	–	* + ↑							↑	–	↑	↓	Uribe *et al.* (2014)
Wistar		OVX		↑	–	+ ↑							–		–	↑	Uribe *et al.* (2009)
		OVX + E_2		↓	–	↓							↑		–	↑	
Wistar		Rimonabant															
	1 dose		↓	↓		+ ↑	+ ↑		↓	–	↓						Rorato *et al.* (2013)
		Daily for 7 days	–	–		–	–		–	–	–						
C57BL/6J		AMIO i.p.:															
		WT	↓			* ↓							↑	–	–		Rosene *et al.* (2010)
		D2KO				–							–	–	–		
Wistar	L-NAME s.c.				↑ 1 h	# ↑ 2 h							↓ 1 h			–	Uribe *et al.* (2011)
S–D	Ethanol i.p.				↓ 6 h	* ↑ 6 h PVN							↓ 1 h	↓ & FT_4	↓ & FT_3		Zoeller *et al.* (1995)

Table 1 *Continued*

Rodent strain	Acute treatment	Chronic treatment	Food Itk	BW or body fat	Hypothalamic TRH peptide	PVN (mRNA)		Arc (mRNA)						TSH	TT_4	TT_3	Cort	References
						Trh	*Crh*	*Agrp*	*Npy*	*Cart*	*Pomc*	*Bdnf*						
S–D, Wistar	Cold 4 °C 1 h					+ * ↑								↑		↑ 3 h	↑	Uribe *et al.* (1993), Zoeller *et al.* (1995), Sánchez *et al.* (2001) and Gutiérrez-Mariscal *et al.* (2012)
	Suckling + 30 min cold					* ↑								↑			–	
	CORT i.p. + 1 h cold vs veh. cold exposed					–								↓	↓	–	↓	
Wistar	Open field test				–	+ ↑ 15 min								↑ 30 min	↑ 1 h		↑	Gutiérrez-Mariscal *et al.* (2012)

Symbols: (*) quantified by ISH (medial PVN); (+) semi-quantified by RT-PCR (total PVN); (#) quantified by northern blot (total PVN). α-MSH, melanocyte-stimulating hormone alpha; AMIO, amiodarone; Arc, arcuate nucleus; Cort, corticosterona; CREB, cAMP response element binding; CRHr2, corticotrophin releasing hormone type 2 receptor; D2KO, deiodinase 2 knockout; DIA, dehydration-induced anorexia; E_2, 17β-estradiol; Food Itk, food intake; FR, food restriction; HFD, high-fat diet; ISH, *in situ* hybridization; L-NAME; N^{ω}-nitro-L-arginine methyl ester; Leu, leucine; MWM, morris water maze; OVX, ovariectomized; POMC, proopiomelanocortin; PVN, paraventricular nuecelus; Rimonabant, type 1 endocannabinoid receptor blocker; S–D, Sprague–Dawley; Y1r, neuropeptide Y type 1 receptor.

(Uribe *et al.* 1991). However, the effects of estrogen and the consequences of ovariectomy on serum TH and TSH concentrations are controversial and depend on the paradigm studied and age and previous gonadal status (Lima *et al.* 2006, Marassi *et al.* 2007, Uribe *et al.* 2009). Consistent with role of TRH in prolactin secretion, *Trh* mRNA levels increase, parallel to prolactin serum concentration, during the lactation period and in response to suckling (Uribe *et al.* 1993). As lactation progresses, levels of *Trh* mRNA decrease whereas those of prolactin rise; removal of pups normalizes *Trh* mRNA expression proportional to weaning time and inversely proportional to serum corticosterone levels (Uribe *et al.* 1991, 1995*b*).

Stress and the HPT axis

Stress was defined by Selye as the nonspecific response of the body to any demand (Selye 1976). This definition has been revised (Koolhaas *et al.* 2011). The type of stress defines the nature of the response; physiological (systemic) stressors such as cold, pain, and disease involve neuronal circuits that usually decode stimuli at the level of the brain stem, whereas psychogenic stressors are motivated by previous experiences and involve limbic areas such as the amygdala, hippocampus, and frontal cortex; all directly or indirectly converge at the PVN and activate the sympathetic nervous system and the hypothalamus–pituitary–adrenal (HPA) axis (de Kloet 2014, Myers *et al.* 2014). The response of the HPA axis is characterized by increased synthesis and release of corticotrophin-releasing hormone (CRH) from the PVN, adrenocorticotrophin hormone from the pituitary, and glucocorticoids (GCs) from the adrenal cortex. In rodents, paradigms of psychogenic stressors include restraint, chronic defeat stress, or isolation; their responses differ according to the controllability of the stressor, whether the animal senses a previously encountered stressor (homotypic) that causes habituation or a new one (heterotypic) to which a hyperreaction may occur (Koolhaas *et al.* 2011, Myers *et al.* 2014).

Stress, whether chronic or acute, modifies eating patterns and metabolism, and is considered to be at the base of the metabolic syndrome (Charmandari *et al.* 2005, Maniam & Morris 2012). GC excess or deficiency leads to several metabolic problems (Rose & Herzig 2013). As these hormones act in multiple organs, it is difficult to distinguish direct effects from indirect effects in paradigms such as adrenalectomy or thyroidectomy and hormone replacement. Chronic stress may increase or decrease food intake in humans whereas in animals this usually diminishes unless they are offered a

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palatable food, although abdominal adiposity increases (Dallman *et al*. 2007). Chronic GC administration increases *Npy* and *Agrp* expression, the activity of their synthesizing neurons, alters the melanocortin system; and increases food intake; it causes multiple metabolic changes such as insulin resistance, visceral fat accumulation, high serum leptin levels (Dallman *et al*. 2007, Maniam & Morris 2012) and decreased *Trh* expression in the PVN and TSH serum concentrations in rat and in human (Kakucska *et al*. 1995, Alkemade *et al*. 2005). The GC-induced increase in NPY expression could contribute to GC-inhibitory effect on *Trh* mRNA levels in the PVN. Patients with Cushing's disease present hypothyroidism and *Trh* expression in the PVN is reduced in postmortem brains of patients that received GC treatment or suffered major depression (Alkemade *et al*. 2005).

Various forms of long-term psychogenic stress decrease TSH and TH serum levels in rats (Armario & Castellanos 1984, Servatius *et al*. 2000), but reports on *Trh* expression are scarce. Constriction injury of sciatic nerve decreases *Trh* mRNA levels in the whole hypothalamus as well as serum TH levels (Kilburn-Watt *et al*. 2010). Foot shock (14 sessions/day) decreases total and free T_4 and T_3 serum concentrations without affecting *Trh* in the PVN, whereas *Agrp* mRNA levels in the ARC increase (Helmreich *et al*. 2005); a milder form of stress, such as 60 min daily restraint for 2 weeks, does not affect *Trh* mRNA expression in the PVN nor TSH or TH serum levels (Uribe *et al*. 2014). The intensity of the stressors thus seems to determine HPT activity and *Trh* expression; however, despite significant changes not being observed in the latter two paradigms, levels of *Trh* mRNA correlate negatively with those of corticosterone and positively, with body-weight changes.

Situations of increased energy expenditure such as exercise and cold are also considered stressful. Cold exposure rapidly activates the autonomic nervous system and the HPT and the HPA axes. Thermogenesis is achieved primarily by the activity of brown adipose tissue (BAT) and the uncoupling proteins through adrenergic stimulation in concert with TH and lipolysis (Mullur *et al*. 2014). Three-day cold exposure of rats increases BAT D2 and its thermogenic activity, D2 increases in skeletal muscle after 10 days and, total and free T_3 increase whereas total T_4 decreases at both times, indicating increased peripheral TH conversion (Louzada *et al*. 2014). Whether long-term cold exposure is constant or intermittent, the metabolic and behavioral effects (increased food intake or physical activity) vary because adaptive changes occur, although data on the effects of chronic cold exposure on TRH expression are lacking and changes in TSH or TH serum levels depend on the condition studied (Silva 2011, Yoo *et al*. 2014). Human beings adapt to prolonged exposure to a cold environment by increasing metabolic and physiologic responses; after moving to the Antartic, energy requirements increase, as well as T_3 production, clearance and distribution, TRH-stimulated TSH release, TSH and thyroglobulin serum levels, while altered total and free TH levels may depend on altitude differences (Palinkas *et al*. 2007). When exposed for 60 h to 50% energy restriction, humans loose considerable body weight but do not decrease TH or TSH serum levels as in temperate conditions, which indicates that cold stimulation overcomes the inhibition produced by food restriction (Case *et al*. 2006).

Exercise is proposed to be a chronic stressor that inhibits HPT and activates HPA axes (Mastorakos & Pavlatou 2005). This depends on the type of exercise, i.e. extenuating or forced versus voluntary (as wheel running; Steinacker *et al*. 2005, Stranahan *et al*. 2008). Compared with naïve sedentary controls, 2-week voluntary running reduces food ingestion (18%); the decreased energy intake in the sedentary pair-fed group causes similar changes to those caused by 40% food restriction for 25 days in rats, decreased levels of serum leptin, TSH and T_3, BAT-D2, and liver-D1 activities, and increased corticosterone (Araujo *et al*. 2009). Exercise blunted several of the changes produced by the 18% reduced food intake, i.e. *Trh* diminished 30% instead of the 50% seen in the pair-fed rats, and neither T_3 or deiodinases activities were altered; however, white adipose tissue (WAT) mass diminished in exercised rats whose serum leptin concentration decreased much more than that of the pair-fed rats; furthermore, the amount of exercise performed correlated positively with T_3 and *Trh* mRNA levels and negatively with WAT mass (Uribe *et al*. 2014). Exercise thus overrides the signals of energy deficiency, such as low serum leptin concentration; the intermittent activation of PVN–TRH neurons and of TH release (seen after an acute increase in physical activity, see below) may guarantee maintenance of TH levels for adequate fuel supply to oxidizing tissues, such as released fatty acids from WAT (Klieverik *et al*. 2009, Weber 2011). Hypophysiotropic TRHergic neurons may therefore be included in a central homeostatic circuit modulated by exercise, which by maintaining adequate TH-induced lipolysis or serum glucose levels through PVN-sympathetic and parasympathetic hepatic activity guarantee energy supply to metabolic tissues (Fliers *et al*. 2014).

TRH expression in the hypophysiotropic neuron at a given time depends thus on the concentration of T_3, THR and co-regulators, nutritional status, stress history, and the extent of neuronal and hormonal influences that sense the

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'basal' state of the organism and regulate TRH synthesis and release – a process further modified by the environmental influences it must contend with. The HPT axis is inhibited in response to energy deficits or chronic stress. However, animals submitted to energy-demanding situations, such as chronic cold or exercise, have altered HPT axis activity and may present high or normal circulating levels of TH, although *Trh* expression in the PVN and serum TSH levels may be differentially affected (Table 1).

HPT responses to acute stimuli

Several neuromodulators regulate the excitability of TRHergic neurons of the PVN; NPY inhibits neuronal activity whereas α-MSH and leptin stimulates it, effects consistent with the known responses on TSH or T_4 release (Ghamari-Langroudi *et al*. 2010). GCs rapidly suppress glutamatergic excitatory inputs onto various parvicellular neurons of the PVN, including those expressing TRH; the effect is mediated through membrane receptors, whose activation causes the release of endocannabinoids that act at the presynaptic side to inhibit glutamate release (Di *et al*. 2003). TRH neurons in the PVN receive afferents from the suprachiasmatic nucleus that modulate TRH expression in a circadian manner (Kalsbeek *et al*. 2000, Zoeller *et al*. 1990).

Acute stress inhibits the HPT axis at different levels; 1 h restraint or 2 h immobilization decrease *Trh* mRNA expression in the rat PVN together with TRH and TSH release during the following hour; the response to immobilization is higher in female rats (Cizza *et al*. 1996, Gutiérrez-Mariscal *et al*. 2012). However, a stressful condition, such as the defensive burying test that involves increased physical activity (burying), augments the levels of *Trh* mRNA in the PVN but not TSH release (Gutiérrez-Mariscal *et al*. 2008). Situations of energy demand, such as physical activity or cold exposure, cause an immediate response of the HPT and the HPA axes. TH and GCs are rapidly released; the latter mobilize glucose for fast responses and the former activate fuel distribution to oxidizing tissues (Klieverik *et al*. 2009, Rose & Herzig 2013). This is coupled with changes in TRH biosynthesis. For example, physical activity in the open field increases the expression of *Trh* in the PVN accompanied by enhanced TRH and TSH release, but only if animals are tested during the dark period. The stress response is stronger in animals tested during the light hours when the HPT response is blunted (Gutiérrez-Mariscal *et al*. 2012). Another type of exercise, such as swimming in a water maze, activates the HPT, increasing the expression of *Trh* mRNA in the PVN and the release of TSH; the response of the HPT is opposite to the degree of stress induced by the test (Aguilar-Valles *et al*. 2005, 2007).

In response to a cold stress, catecholaminergic afferents from the brain stem onto the PVN rapidly contribute to increase *Crh* and *Trh* mRNA levels in the PVN, concomitant with the release of TRH, TSH, and corticosterone (Zoeller *et al*. 1990, 1995, Rondeel *et al*. 1991, Uribe *et al*. 1993). The changes in *Trh* mRNA are transient, with increases at 1 h and normalizing by 2 h, even when animals are kept in the cold for long periods; *Trh* mRNA levels rise again at 6 h but only if animals are exposed during the light period (Uribe *et al*. 1993, Zoeller *et al*. 1995). Acute administration of some drugs alters *Trh* expression and these changes do not always coincide with changes in TSH or TH serum concentrations (Table 1). Although ethanol increases *Trh* mRNA levels in the PVN, it blocks increases induced by cold-exposure (Zoeller *et al*. 1995). Cold increases *Trh* mRNA expression independent of high-TH serum levels (Zoeller *et al*. 1990), but a mild stress 2 h before being submitted to cold suppresses the expected increase in *Trh* mRNA levels in the PVN but not in serum TSH in male rats (Uribe *et al*. 2011).

Cold exposure activates the HPT axis more in non-lactating female rats than in males or lactating females (Sánchez *et al*. 2001). Ovariectomized rats with increased body weight and high leptin serum concentration respond to cold stimulation augmenting *Trh* mRNA levels in the PVN, but the effect is suppressed by high doses of 17β-estradiol, while serum TSH and corticosterone levels are similar to those found in the ovariectomized animals (Uribe *et al*. 2009). In contrast, food-restricted female rats display greater increases in *Trh* mRNA levels in the PVN than controls after cold exposure, but TSH response is blunted (Jaimes-Hoy *et al*. 2008). These results exemplify situations in which the response to a life-threatening situation such as cold exposure is altered by stress, corticosterone, sex, and other as yet unidentified effectors.

As mentioned, TRH is involved in prolactin release during lactation (Galas *et al*. 2009), and the levels of *TRH* mRNA increase in mid-PVN 30 min after initiation of suckling (Sánchez *et al*. 2001). TRH hypophysiotropic neurons co-express CART, which is also induced after 1 h of cold exposure (Sánchez *et al*. 2007) but not after suckling. Since CART inhibits prolactin release *in vitro* and cold exposure does not induce the release of prolactin, CART may serve as a co-modulator of TRH in this physiological circumstance, stimulating TRH and TSH release while blocking prolactin release (Sánchez *et al*. 2001, 2007, Raptis *et al*. 2004).

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Because GCs affect not only the electrical activity of PVN–TRH neurons, but potentially the expression of *Trh* in rodents and humans, it is relevant to understand how they interact with the stimuli that control the HPT axis. The timing of GR activation is of utmost importance; GR is released from a cytosolic multi-protein complex upon ligand binding, phosphorylated, and translocated to the nucleus within minutes, where it acts as a transcription factor. The dynamics of GR transport and its half life depend on the presence of ligand, DNA binding, and activity of several kinases (Ratman *et al*. 2013, de Kloet 2014). The response of PVN *Crh* expression to restraint is blunted if animals receive corticosterone 1 h but not 3 h previously, supporting the relevance of timing of GR activation; in contrast, the increase in c-FOS due to restraint is not diminished by corticosterone pretreatment (Osterlund & Spencer 2011). *Trh* mRNA levels in the PVN correlate negatively with corticosterone serum concentrations in animals under several circumstances; therefore, we studied the effect of a peripheral corticosterone injection on cold-induced *Trh* expression in the PVN. In male rats kept at room temperature, *Trh* mRNA expression is enhanced (greater than threefold) 2 h after corticosterone injection in the mid- and caudal-PVN, where TRHergic neurons co-express GR (Cintra *et al*. 1990). Rats that received injections of vehicle responded to cold as expected, increasing *Trh* mRNA levels in the three zones of the PVN and TSH serum concentration; however, in corticosterone pre-treated rats, *Trh* expression does not increase further after cold exposure and the response of TSH is suppressed (Sotelo-Rivera *et al*. 2014). GCs may inhibit TRH or TSH release directly at the median eminence or the pituitary respectively (van Haasteren *et al*. 1995, John *et al*. 2003). TRH synthesis and release might be uncoupled in those circumstances when TSH is not increased within an hour of exposure.

The interference of corticosterone with the cold-induced activation of *Trh* synthesis may be due to a cross talk between activated GR and elements of the PKA pathway, as demonstrated *in vitro*. Hypothalamic cells co-stimulated with dexamethasone and forskolin do not display increased *Trh* mRNA levels as are detected when they are incubated separately with each drug (Pérez-Martínez *et al*. 1998, Cote-Vélez *et al*. 2005), nor the recruitment of pCREB, GR, or Pol II to the *Trh* promoter (Díaz-Gallardo *et al*. 2010*b*). The effect is probably due to GR–PKA protein–protein interaction with PKA impeding CREB phosphorylation and GR binding to DNA (Sotelo-Rivera *et al*. 2012). Similar cross talk between the PKA and GR intracellular pathways is detected also in cell lines, supporting the hypothesis that the effect is an intracellular event, and not due to the average of differential responses of TRHergic cells from different hypothalamic nuclei present in primary cultures (Cote-Vélez *et al*. 2005). The interference of GCs with the cAMP-stimulatory effect on *Trh* expression is avoided with inhibitors of the ERK/MAPK pathways (Cote-Vélez *et al*. 2008). These results, although obtained *in vitro*, support the possibility of fast cross talk between signaling pathways that may alter an immediate response (Fig. 2).

As mentioned in the previous section, long-term energy-demanding situations do not seem to keep the HPT axis activated, but the response to acute stimuli may prevail or be modified. One may envisage situations when TRH neurons are activated, TRH synthesis increases and simultaneously processed TRH is released, TSH secretion follows, causing TH release that modulates different metabolic reactions at target organs. The feedback effect includes not only TH at TRH or TSH synthesis, but molecules of the target tissues, GCs if HPA is co-stimulated and TH acting on other brain areas involved in the required circuit. TH affects metabolism acting at various hypothalamic nuclei increasing, for example, glucose production, modulating central autonomic outflow, and eating behavior or stimulating the dorso-medial hypothalamus (DMH) which activates the sympathetic-BAT response involved in thermogenesis (Fliers *et al*. 2014).

TRHergic neurons may thus respond transiently to energy-demanding situations and maintain energy homeostasis. However, stress could impede a fast and efficient response of the HPT axis in which, for example, in cases such as cold exposure or increased physical activity, the lack of opportune lipolysis needed for fuel supply may oblige the organism to display alternative responses, such as increased food intake and decreased activity due to fatigue, thus contributing to the metabolic syndrome.

Hypothalamic TRH neuronal populations

In mammals, various brain circuits contribute to maintenance of energy balance (Schneeberger *et al*. 2014). Central administration of TRH or TRH agonists consistently reduces food intake in normal rodents and hungry rats, and in models of stress-induced feeding increases thermogenesis and arousal (Lechan & Fekete 2006, Akieda-Asai *et al*. 2014). These effects may involve various hypothalamic targets because, for example, local injection of TRH into medial and lateral hypothalamus (LH) reduces feeding in rats (Suzuki *et al*. 1982) whereas administration into the preoptic area, dorsomedial, or ventromedial

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hypothalamus (VMH) increases BAT temperature in hamsters (Shintani *et al*. 2005). ISH of *ppTRH* mRNA, its receptors (TRH-R1 and TRH-R2), and inactivating enzyme (PPII) together with immunostaining of *Trh* and of TRH, as well as autoradiography of TRH binding sites, has been used to generate maps of brain TRHergic neurons, receptors, and inactivation sites (Vargas *et al*. 1987, Hökfelt *et al*. 1989, Lechan & Segerson 1989, Heuer *et al*. 2000). However, the circuits in which they are involved are currently poorly understood as few of the projection fields of the TRH neurons have been identified with anterograde and retrograde techniques (Simmons & Swanson 2009, Wittmann *et al*. 2009*a*,*b*, Fekete & Lechan 2014). Understanding the role of hypothalamic TRHergic neurons (Fig. 1) requires consideration of their distribution because various regions, such as the LH, PVN, preoptic, suprachiasmatic, and dorsomedial nuclei express *Trh* mRNA (Fig. 1). The physiological state of TRH neurons has been evaluated by few electrophysiological studies on identified neurons and by biochemical markers of activation, such as detection of immediate early genes, or of TRH biosynthesis, but the output of TRH neurons is difficult to obtain *in vivo* because of sampling or sensitivity issues.

Even in a single nucleus, TRH neurons are not homogeneous. In the rat PVN, TRH-expressing neurons are distributed along the rostro-caudal part of the parvocellular and some in the magnocellular subdivisions (Fekete & Lechan 2014). Almost half of the total PVN–TRHergic cells in the parvocellular part of the PVN are non-neuroendocrine or preautonomic, concentrated in the rostral or anterior PVN (aPVN) in the rat, with only few present in the mid-PVN and even fewer in the caudal. As mentioned, the hypophysiotropic or endocrine cells are concentrated in mid- and caudal-PVN (Simmons & Swanson 2009, Fekete & Lechan 2014). In mouse PVN, 69% of TRHergic endocrine cells project to the median eminence, while 17% project to the neurohypophysis, and the rest are non-endocrine cells localized in the anterior zone (Ghamari-Langroudi *et al*. 2010, Kádár *et al*. 2010). PVN–TRHergic neurons differ not only in their afferents, projections, and receptor expression (Fekete & Lechan 2014) but also according to time of birth; rat non-neuroendocrine neurons peak at embryonic days 11–12 and neuroendocrine neurons at days 12–14 (Markakis & Swanson 1997).

The non-neuroendocrine TRHergic neurons of the rat aPVN receive a robust innervation from NPY/AgRP and α-MSH/CART neurons from the ARC (Fekete & Lechan 2014) and from adrenergic and noradrenergic fibers from the brain stem (Füzesi *et al*. 2009). The α-MSH input seems to be functional since central administration of α-MSH enhances the phosphorylation of CREB in *Trh* neurons (Sarkar *et al*. 2002) and mediates the effect of i.c.v. leptin injection, which rapidly increases pCREB in aPVN–TRH neurons (Perello *et al*. 2006). The adrenergic input may be relevant to thermogenesis because cold induces a fast increase in *Trh* mRNA levels in the aPVN as it does in the hypophysiotropic neurons (Sánchez *et al*. 2001). TRH neurons of the aPVN send projections to the ARC, dorsomedial, ventral-premammilary nuclei, and medial-preoptic region, and to several additional limbic regions such as various amygdaline nuclei, the bed nucleus of the stria terminalis, and lateral septum (Wittmann *et al*. 2009*a*). TRH neurons of the aPVN do not express GR (Cintra *et al*. 1990), but a corticosterone injection increases *Trh* mRNA levels, probably through direct stimulation of GC membrane receptors, and the cold-stimulatory effect is additive (Myers *et al*. 2014, Sotelo-Rivera *et al*. 2014). *Trh* expression in aPVN is not modulated after suckling or food restriction, whereas it is stimulated after dehydration-induced anorexia (DIA; Sánchez *et al*. 2001, Alvarez-Salas *et al*. 2012). As regulation of biosynthesis is often coupled with changes in peptide release, the results indicate that aPVN–TRH neurons transfer multiple modalities of metabolically relevant information to postsynaptic targets; confirmation of their role will require experimental testing, but it is interesting to note that the intestinal administration of long-chain fatty acids enhances the activity of aPVN neurons (Randich *et al*. 2004), and adiponectin stimulates only non-neuroendocrine PVN–TRH cells (Hoyda *et al*. 2009).

The ARC expresses both *Trh-R1* and *Trh-R2* receptor mRNAs (Heuer *et al*. 2000, Ebling *et al*. 2008), its dorsomedial part receives a dense TRHergic innervation (Lyons *et al*. 2010) arising, at least in part, from the aPVN and some from the perifornical area (Wittmann *et al*. 2009*a*,*b*). Cell-specific neuron-mapping techniques in mice demonstrate a strong excitatory drive emanating from subsets of neurons from the PVN expressing TRH and pituitary–adenylate cyclase-activating polypeptide that contact and activate AgRP neurons, inducing intense feeding (Krashes *et al*. 2014). The functional role of TRH in this circuit was not tested, but TRH does not regulate the electrical activity of α-MSH or NPY neurons in slices of rat ARC (Zhang & van den Pol 2012), instead TRH regulates that of tuberoinfundibular dopaminergic neurons present in the dorsal ARC which are surrounded by TRH-ir terminals; TRH causes a transition from phasic to tonic firing which probably decreases dopamine output (Lyons *et al*. 2010).

The DMH has an important role in energy homeostasis (Schneeberger *et al*. 2014). A significant population

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of TRH neurons is detected in this nucleus (Hökfelt *et al.* 1989, Horjales-Araujo *et al.* 2014), which receive afferents from the subparaventricular zone, an output region from the suprachiasmatic nucleus (SCN); the DMH sends a glutamate–TRH projection to the LH area (Chou *et al.* 2003) which expresses both TRH receptors, predominantly TRH-R1 (Heuer *et al.* 2000). TRH receptors are probably present in orexin neurons as they respond to TRH with robust increases in their action potential firing rate, an effect that persists under conditions of synaptic isolation (González *et al.* 2009). TRH also increases the firing activity of presynaptic GABAergic interneurons (Hara *et al.* 2009). The DMH–TRHergic neuronal projection onto or near orexin neurons may be part of a circuit required for the circadian activation of behavioral and endocrine functions, including the circadian control of awareness (González *et al.* 2009). In agreement with this possibility, the effect of TRH on locomotor activity is reduced in orexin-ablated mice (Hara *et al.* 2009). TRH also inhibits the activity of melanin-concentrating hormone (MCH) neurons of LH indirectly through the excitation of GABA neurons, a result consistent with the detection of TRH axons terminating on or near GABA neurons (Zhang & van den Pol 2012). Interestingly, *Trh* mRNA levels in the DMH are increased after 2 weeks of moderate exercise compared with the pair-fed group (Uribe *et al.* 2014). The LH contains another large group of TRH cells throughout the rostrocaudal axis of the hypothalamus (Hökfelt *et al.* 1989, Lechan & Segerson 1989, Heuer *et al.* 2000, Horjales-Araujo *et al.* 2014) and in the perifornical region (Wittmann *et al.* 2009*a*,*b*). These neurons do not coexpress orexin, MCH, or neurotensin (Horjales-Araujo *et al.* 2014). In the juxtaparaventricular area, a small group of TRH-immunoreactive cells stains for enkephalin and urocortin 3, and their projections partially overlap those of the aPVN (Wittmann *et al.* 2009*b*, Horjales-Araujo *et al.* 2014). AgRP and α-MSH populations of terminals form close appositions onto TRH cells in the LH; the TRH-expressing cell population of the LH may link metabolic signals and the generation of arousal (Horjales-Araujo *et al.* 2014). Increased expression of *Trh* is detected in LH of male rats after 5 days of DIA and in the pair-fed group (de Gortari *et al.* 2009) while *Crh* mRNA levels increase only after DIA (Watts *et al.* 1999). DIA and pair-fed rats eat by the fifth day only 20% of the intake of control animals; the increase in TRH expression may be related to increased arousal as increased anxiety is produced by these conditions (Jaimes-Hoy *et al.* 2008).

TRH neurons heavily innervate histaminergic (HA) neurons in all subdivisions of the rat tuberomammillary nucleus (TMN) (Sárvári *et al.* 2012) where TRH-R2 is present (Gotoh *et al.* 2007). A TRH microinjection above the TMN activates HA TMN neurons and histamine turnover in PVN and VMH projections (Gotoh *et al.* 2007). As hypothalamic neuronal histamine is involved in the regulation of body weight and acts as an anorexic agent (Schneider *et al.* 2014), it is indeed possible that a HA projection contributes to the anorectic effect of TRH.

Are changes in the expression of neuropeptides and TRH in total hypothalamus indicative of a metabolic alteration?

Owing to the crucial involvement of TH thermogenesis and metabolism (Klieverik *et al.* 2009, Silva 2011, Mullur *et al.* 2014), a more active HPT would in principle reflect a higher metabolism. However, a cause–effect relationship between gene expression of ARC peptides and that of TRH in total PVN is difficult to identify (Table 1 and Supplementary Table 1). If, for example, there is an opposite response of *Trh* expression in aPVN versus mid–caudal PVN, the results of the whole PVN may overshadow the endocrine response. Even more if levels of expression are measured in total hypothalamus since TRH in several nuclei may have important roles in energy homeostasis. For example, comparison between lean and fat rats (Lou/C versus Wistar (Veyrat-Durebex *et al.* 2013)) or selectively bred chickens (Byerly *et al.* 2009) show increased *Trh* mRNA but also increased *Npy* and *Agrp* together with decreased *Pomc* in the lean animals which would seem to contradict the effects of these peptides on TRH expression in the PVN (Fekete & Lechan 2014). Although it is not possible to delineate the hypothalamic regions where these reported changes occur, coincident changes in these two species include the enhanced expression of brain-derived neurotrophic factor (BDNF) which *in vitro* increases *Trh* mRNA levels (Ubieta *et al.* 2007). BDNF and its receptor TRKB are expressed in several hypothalamic nuclei including TRHergic cells of the PVN (Ubieta *et al.* 2007). BDNF regulates neurogenesis, neuronal plasticity, and support during development and throughout the animal's life-span (Jeanneteau & Chao 2013). In the hypothalamus, BDNF plays an important part in energy homeostasis (Levin 2007, Rothman *et al.* 2012). Support for this role stems from the effects of transferring the *Bdnf* gene into hypothalami of adult mice, which display decreased body weight and body fat and increased expression of *Mc4r*, *Lepr*, *Trh*, and *Crh* compared with wild type mice despite having strangely greater than tenfold induction of the orexigenic peptides NPY and AgRP. Under HFD, they gained less weight and little fat

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compared with normal mice and displayed increased *Mc4r*, *Lepr*, *Trh*, *Crh*, *Cart* (*Cartpt*), and *Pomc* expression, as well as expression of the metabolic genes of target tissues such as WAT and liver (Cao *et al*. 2009). These results, although not defining the particular hypothalamic nuclei involved, reveals the importance of additional participants such as BDNF in re-setting the response of several hypothalamic–peptidergic systems and their relationship with body weight and adipose tissue.

Another metabolic relevant brain area, the hindbrain

The brain stem is an important part of the circuitry involved in sensing and conveying information regarding the nutritional and energy conditions of the organism (Schneeberger *et al*. 2014). *Trh* is synthetized in the raphe pallidus (Rpa), raphe obscurus (Rob), and parapyramidal regions; these neurons project to neurons of the dorsal motor nucleus of the vagus, the nucleus tractus solitarius, and the ventrolateral medulla. They contain TRH-R1 and leptin receptors involved in coordinating vagal and sympathetic outflows (Taché *et al*. 2006). As in mid-PVN, *Trh* expression in these nuclei is downregulated in hyperthyroidism (Yuan & Yang 1999) and upregulated by cold exposure (Taché *et al*. 2006). Some TRH neurons in the caudal raphe receive orexin afferents and project to premotor neurons that innervate BAT increasing thermogenesis; others project to the vagal circuit involved in hepatic, pancreatic, and gastrointestinal function (Taché *et al*. 2006). In contrast to the inhibitory effects of fasting on HPT activity, hindbrain *Trh* mRNA levels are elevated in rats fasted for 24 or 48 h, and refeeding restores them to normal levels (Ao *et al*. 2006). Studies of the regulation of TRH expression in these neurons contribute to the understanding of the role of brain TRH in metabolism and thermogenesis.

Neuroendocrinology revisited

Recognition of the effects of environment on metabolism and epigenetic changes has led to questions with regard to what are the adequate controls in experimental research with rodents? Although laboratory rodents have been domesticated for hundreds of generations, their 'standard' living conditions are sedentary with no other activity but to eat *ad libitum* and develop obesity (Martin *et al*. 2010). This is evident comparing pair-fed with naïve rats; calorie restriction even as low as 18% of naïve food intake and loss of 8% body weight increased their corticosterone serum concentrations (Uribe *et al*. 2014). In the case of 300 g male rats, normal cages do not allow standing, reducing even further their possibility of physical activity (RSPCA 2011). Stressful conditions include differences in individual versus group housing depending on the age and sex of the animal, whether animals are transported or isolated within 1–3 h previous to the experiment, etc. (Koolhaas *et al*. 2011, Uribe *et al*. 2011).

Postnatal development, lactation, and adolescence are periods of high sensitivity to stress when epigenetic changes may program the individual to having low stress resilience, changing the expression of key molecules such as BDNF and GR in a region-specific manner; in hippocampus their inhibited expression alters the inhibitory feedback on the activity of the HPA leading to hyperactive stress responses and reduced learning (Meaney & Szyf 2005, Pervanidou & Chrousos 2012, Bath *et al*. 2013). The nutrition or stress experience of mothers during gestation as well as litter size has long-lasting effects on metabolism and stress response (Breton 2013). In the case of the HPT axis and TRH expression in particular, reports are scarce. Dexamethasone injections during the last week of gestation diminish *Trh* mRNA levels in the PVN in female offspring and TRH fibers and T_4 in both sexes during the second week; only *Trh* expression remained low in adult females, accompanied by low body temperature during estrous and diestrous (Carbone *et al*. 2012*a*). Low levels of immunoreactive TRH are also detected in the fibers of the LH (Carbone *et al*. 2012*b*). This treatment blunts stress-induced *Bdnf* expression in male rats or HFD-induced increases in females (Carbone & Handa 2013).

Neonatal stress produced by maternal separation causes epigenetic changes in hippocampal *Gr* and *Bdnf* promoters, their reduced expression contributes to the altered HPA-response to stressors in adulthood (Meaney & Szyf 2005), *Bdnf* expression is altered in a strain-, sex-, and region-specific manner (Bath *et al*. 2013). Adequate programing of activity and response of the HPT axis is altered under various conditions. Maternal separation increases *ppTrh* mRNA in the aPVN and circulating T_4 serum concentrations of adult rats, and attenuates the response to exercise or cold exposure but that to fasting is normal (Jaimes-Hoy *et al*. 2013). Offspring of mothers that experienced a 40% food restriction, during gestation and lactation were hypothyroid (low FT_4 and high TSH) and had a blunted response to 24 h cold exposure (Ayala-Moreno *et al*. 2013). In contrast, post-natal overnutrition during lactation, which predisposes the offspring to being overweight, leads to leptin resistance and overexpression

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of orexigenic pathways (Breton 2013), alters the HPT axis in rats in contrast to the situation opposite to what is observed after HFD because *Trh* expression in the PVN and T_4 serum concentration are decreased and the response to fasting is suppressed (Rodrigues *et al*. 2009, Aréchiga-Ceballos *et al*. 2014) compared with HFD-induced overweight, which display increased *Trh* mRNA and TH serum levels, but without an altered fasting response (Perello *et al*. 2010).

The opposite scenarios apply to mice grown in enriched environments; their body weight is reduced, BDNF expression is increased in ARC and DMH/VMH, *Trh* in the PVN and in the DMH/VMH, despite decreased expression of *Pomc*; animals with access to wheels for running for several weeks maintain lower body weight and reduced adiposity, but *Trh* mRNA levels tend to decrease and *Crh* and *Crh*-receptors increase in contrast to the effects of the enriched environment (Cao *et al*. 2011). The contradictory results observed between the effects of neonatal stress and those of growing in an enriched environment on the relationship of GR and BDNF (Jeanneteau & Chao 2013) include the responses of the HPA, HPT, and SNA that affect energy balance. Improvements and standardized housing conditions seem required to define adequate controls and obtain reproducible data between different laboratories, together with adequate reports of the animals' age and body weight, as some correspond to the adolescent period (McCutcheon & Marinelli 2009). Another recently discovered but very important problem is the materials used in animal houses such as water bottles made of polycarbonate that release chemicals that act as endocrine disruptors causing deleterious effects, particularly during development (Zoeller 2010), and the differential leakage that occurs depending on the state and treatment of the bottle (Guart *et al*. 2013).

Conclusion

This review summarizes what is known about the regulation of the TRHergic neurons involved in energy homeostasis and the interfering effects of stress. In spite of the limitations of the *in vitro* systems or transgenic mice models, evidence indicates demonstrate an inhibitory role of T_3 and a stimulatory one of pCREB, SP1/KPL, GR-cJun, and STAT3 on *Trh* transcription; interaction of the transduction pathways involved may explain some of the interfering effects of stress on neuronal stimulations observed in various *in vivo* situations, but they have not yet been fully placed in a sub-type of TRH neuron context. *Trh* expression levels in the PVN, detected during chronic 'static' situations, are difficult to relate to changes in other known modulators as they represent a 'snap shot' that might not readily correspond to the activity of the target organs involved. TRH neurons can rapidly respond to energy demands for thermogenesis or physical activity; these responses may be modified due to nutritional status, and stress history. We propose that combined approaches of chronic situations with acute responses might lead to a better understanding of TRH physiology in the PVN and in other relevant metabolic hypothalamic nuclei.

Supplementary data

This is linked to the online version of the paper at http://dx.doi.org/10.1530/JOE-14-0593.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the review.

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