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Review article

# The forgotten effects of thyrotropin-releasing hormone: Metabolic functions and medical applications

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ABSTRACT

Thyrotropin-releasing hormone (TRH) causes a variety of thyroidal and non-thyroidal effects, the best known being the feedback regulation of thyroid hormone levels. This was employed in the TRH stimulation test, which is currently little used. The role of TRH as a cancer biomarker is minor, but exaggerated responses to TSH and prolactin levels in breast cancer led to the hypothesis of a potential role for TRH in the pathogenesis of this disease. TRH is a rapidly degraded peptide with multiple targets, limiting its suitability as a biomarker and drug candidate. Although some studies reported efficacy in neural diseases (depression, spinal cord injury, amyotrophic lateral sclerosis, etc.), therapeutic use of TRH is presently restricted to spinocerebellar degenerative disease. Regulation of TRH production in the hypothalamus, patterns of expression of TRH and its receptor in the body, its role in energy metabolism and in prolactin secretion are addressed in this review.

## 1. Introduction

Thyrotropin-releasing hormone (TRH), also termed thyroliberin, was the first hypothalamic releasing factor to be identified, but the peculiar N- (pyroGlu) and C-terminal (amide) residues delayed solving its TRH structure (Joseph-Bravo et al., 2015). Initially, its action as a hormone was unidentified and it was termed thyrotropin releasing "factor" instead of "hormone". Problems included difficulties in isolating TRH, non-reactivity with ninhydrin due to the blocked $NH_2$ terminus, and variations in bioactivity of extracts obtained from animals of different thyroid status. Finally, in 1969 the final structure of TRH was identified (Boler et al., 1969). In addition, quantification of the peptide was challenging because specific antibodies for detection by radioimmunoassay (RIA) and adequate protocols for pre-treatment of the blood sample had to be developed (Duntas et al., 1991).

Most of the body's TRH is produced by nuclei of the hypothalamus, which is the key regulator of arousal, metabolism and energy level. Food intake, temperature, fluid, endocrine and reproductive functions, sleep and wakefulness, emotion, stress circadian rhythm, visceral function, reward and punishment are all regulated by hypothalamic nuclei. The hypothalamus can be divided into three parts, the anterior, middle/tuberal, and posterior part. The anterior part is mainly involved in circadian rhythm and thermoregulation (Saper and Lowell, 2014). Eating, blood pressure and heart rate, satiety and gastrointestinal tract stimulation are regulated by the middle part and nuclei of the posterior part play roles in blood pressure, energy balance, feeding, sleep, arousal, memory, and learning. Influence on cognitive functions is achieved by interaction with nuclei of the thalamus. The endocrine system is organized in feedback regulatory loops, involving the hypothalamus, pituitary gland as first targets and endocrine glands as second targets, and various tissues in the body as ultimate targets (Fig. 1). Hypothalamic hormones regulate hormone secretion by the pituitary, which in turn regulates hormone secretion from the adrenal cortex, thyroid, ovaries and testes or activity of the ultimate targets (various peripheral tissues) (ElSayed and Bhimji, 2017; Tweed et al., 2012).

This review aims to explain the multifaceted action of TRH by invoking the existence of various forms of TRH and TRH-like peptides, and their interactions with PRL secretion. Further, use of TRH in diagnostic and therapeutic applications is discussed. Whenever possible, sex differences are pointed out. In most studies the number of enrolled patients was too low so that no stratification according to sex could be made.

## 2. TRH and the hypothalamic-pituitary axis

### 2.1. Hypothalamus

Hormone-producing cells of the hypothalamus include neurons of the preoptic area, medial basal region, suprachiasmatic nucleus and

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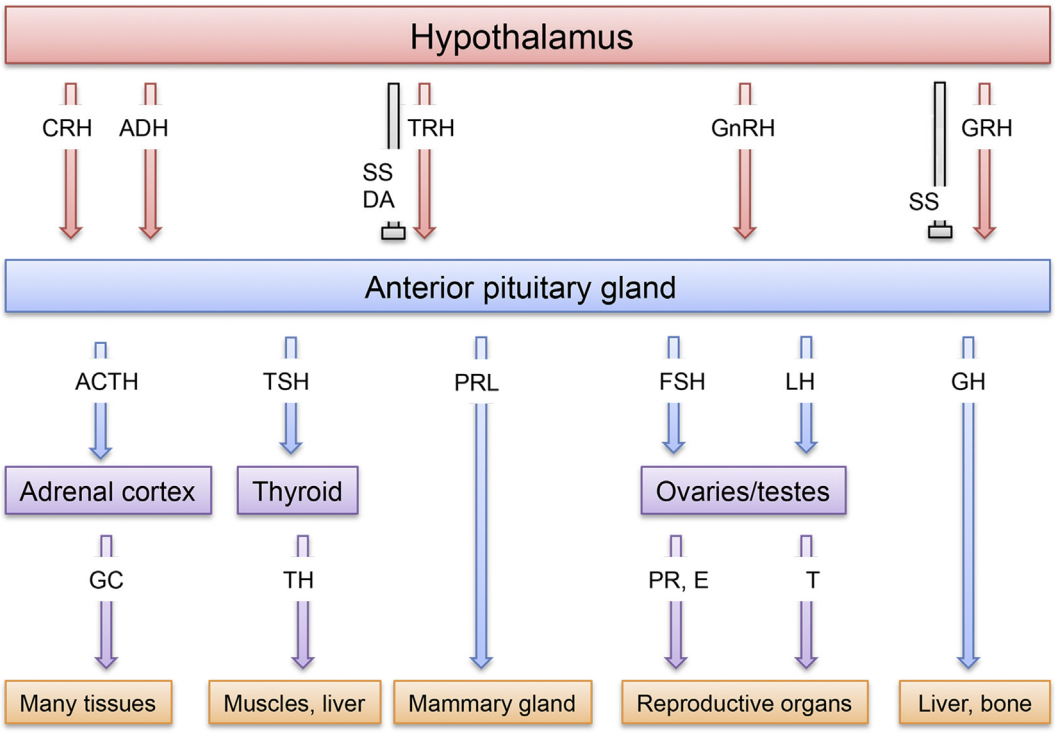


**Fig. 1.** Hierarchic regulation of endocrine hormone production (adapted from (Tweed et al., 2012)). Stimulation of the pituitary gland by corticotropin-releasing hormone (CRH), antidiuretic hormone (ADH) thyrotropin-releasing hormone (TRH), gonadotropin-releasing hormone (GnRH) and growth hormone (GH), and inhibition by dopamine (DA) and somatostatin (SS) from the hypothalamus. Stimulation of primary target organs with adrenocorticotropic hormone (ACTH), thyroid-stimulating hormone (TSH), prolactin (PRL), follicle-stimulating hormone (FSH), luteinizing hormone (LH), growth hormone (GH, somatotropin) released by the anterior pituitary gland. Glucocorticoids (GC, cortisol, corticosterone and aldosterone) from the adrenal cortex, thyroid hormone (TH, triiodothyroinine and thyroxine) from the thyroid gland, and progesterone, estradiol (PR, E), testosterone (T) from ovaries and testes, respectively, stimulate the secondary target organs.

arcuate nucleus (ARC) for the production of gonadotropin-releasing hormone (GnRH), parvocellular neurons in the dorsomedial portion of the paraventricular nucleus (PVN) for TRH and CRH, ARC neurons for DA and GnRH, and neurons of the periventricular nucleus for somatostatin (Aguilera and Liu, 2012) (Fig. 2). Antidiuretic hormone (ADH, vasopressin) and oxytocin are produced by the supraoptic nucleus (SON) and PVN by a specific population of neurons, the magnocellular neurons. These hormones are then transported by axons to the posterior part of the pituitary gland.

### 2.2. Pituitary gland

Hypothalamic hormones reach the anterior lobe of the pituitary gland by the portal system at the median eminence at the upper part of the gland (above the infundibulum). The pituitary gland, also termed the hypophysis, is divided into an anterior part (adenohypophysis) and a posterior part (neurohypophysis). The anterior part consists of the pars tuberalis, intermedia, and distalis, and the posterior part of the infundibulum and pars nervosa (Fig. 2). The intermediate part, located between the anterior and posterior parts of the pituitary gland, is derived from the anterior part and produces melanocyte-stimulating hormone (MSH). The posterior part of the pituitary gland receives hypothalamic hormones through axonal transport (Hinson and Raven, 2015).

Cells of the distal part of the anterior pituitary gland secrete adrenocorticotropic hormone (ACTH), thyroid-stimulating hormone (TSH), prolactin (PRL), follicle-stimulating hormone (FSH), luteinizing hormone (LH), and growth hormone (GH, somatotropin). In the tuberal part of the gland, gonadotropic hormones are produced but also TSH. This tuberalis-derived TSH does not stimulate the thyroid gland and is under control of melatonin and not influenced by TRH (Ikegami et al., 2014). This particular TSH product has a longer half-life, which may be

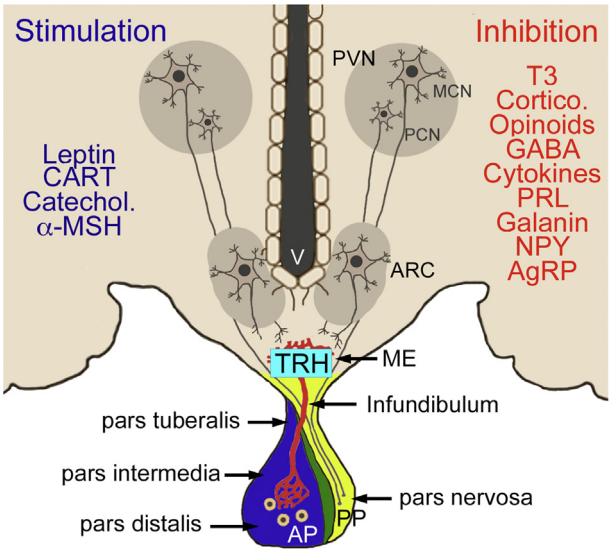


**Fig. 2.** TRH secretion by the hypothalamus. Magnocellular neurons (MCN) of the paraventricular nucleus (PVN) produce peptides that are transported to the posterior part of the pituitary gland by axonal transport. Thyrotropin-releasing hormone (TRH) secreted by parvocellular neurons (PCN) and neurons of the arcuate nucleus (ARC) reach the pituitary gland by the portal system at the median eminence. Important regulators of TRH secretion are listed. Abbreviations: AgRP, Agouti-related peptide/protein; AP, anterior part of the pituitary gland; ARC, arcuate nucleus; CART, cocaine and amphetamine-regulated transcript; catechol., catecholamines; cortico., corticosteroids; GABA, γ-aminobutyric acid; ME, median eminence; α-MSH, melanocyte-stimulating hormone; NPY, neuropeptide Y; PP, posterior part of the pituitary gland; PRL, prolactin; T3, triiodothyronine; V, ventricle.

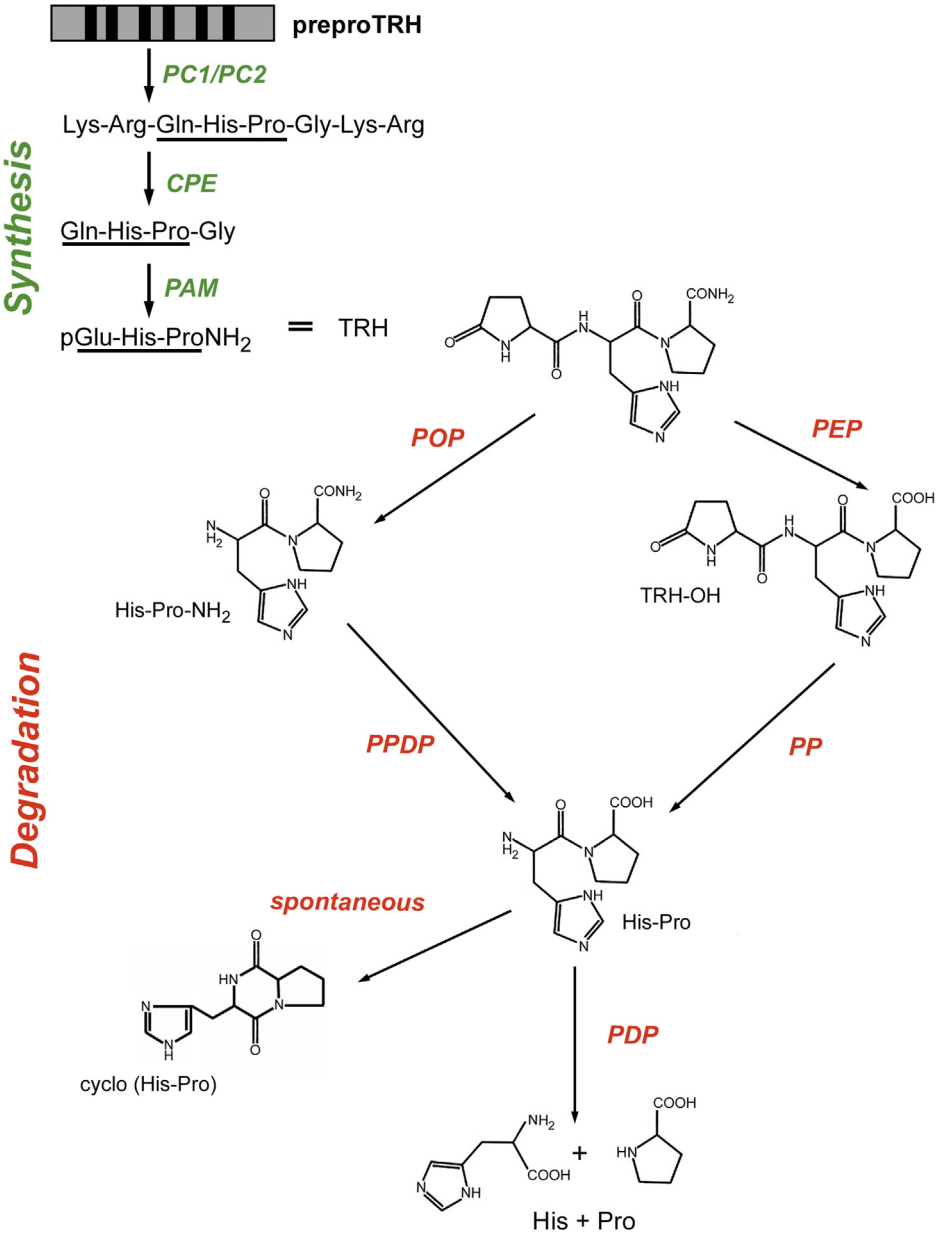


**Fig. 3.** Overview of synthesis and degradation of thyrotropin-releasing hormone (TRH). PreproTRH contains six copies of the TRH progenitor sequence (black boxes), which are converted to mature TRH. Abbreviations: CPE, carboxylpeptidase E; PC1, PC2, prohormone convertase 1 and 2; PDP, proline dipeptidase; PAM, α-amidating monooxygenase; POP, prolyloligopeptidase; PP, pyroglutamylaminopeptidase; PPDP, postproline dipeptidyl aminopeptidase.

due to different post-translational modification (sialylated multi-branched N-glycans) and to albumin binding. In seasonally breeding animals, tuberal TSH stimulates GnRH secretion (Nakayama and Yoshimura, 2018).

## 3. TRH: cellular expression, synthesis, degradation and plasma levels

### 3.1. Cellular/tissue expression

Expression of central nervous system (CNS) TRH starts 2–3 weeks postpartum (Daimon et al., 2013). There is a slight age-dependency of TRH production in humans with lower levels at older age (65–80 years), when expression of its receptor is also decreased (Stan and Morris, 2005). mRNA expression of TRH has been detected in addition to the PVN in neurons of the suprachiasmatic nucleus, perifornical area and lateral hypothalamus (Guldenaar et al., 1996). Production of TRH in the hippocampus has not been demonstrated beyond doubt (Daimon et al., 2013). Extrahypothalamic tissues such as the thyroid, brain, duodenum, endometrium, testis, salivary gland, pancreas, heart, thymus, and ovary express TRH (Fagerberg et al., 2014). TRH is also expressed in hair follicles and induces hair shaft elongation, hair growth and prevention of keratinocyte apoptosis (Gaspar et al., 2010).

The TRH protein expression pattern, in general, matches mRNA expression. Highest TRH levels were detected in the brain. Cytoplasm of neuronal cells, neuronal projections and glial cells of the hypothalamus, glandular cells of the thyroid gland, exocrine glandular cells of the pancreas, renal tubule cells, and glandular cells of endometrium and cervix contain TRH protein (proteinatlas). Staining of smooth muscle, bronchial epithelial cells, bile duct and gall bladder for TRH is moderate, suggesting lower content. Cells of the gastrointestinal tract, placenta and the immune system were also shown to contain TRH protein (Quintanar and Guzman-Soto, 2013). Expression of TRH is particularly high in the male reproductive tract, with the highest expression being in

the epidymis, prostate and testis. These tissues contain mainly so-called TRH-like peptides and a small amount (25%) of authentic TRH (Gkonos et al., 1993). In contrast, Bilek et al. did not detect any authentic TRH in the mature rat prostate (Bilek, 2000). TRH-like-peptides are characterized by substitution of the basic amino acid histidine with neutral or acidic amino acids such as Glu, Phe, Gln, Tyr, Leu, Val, Asp or Asn. Substitutions by Leu or Val were identified in the brain and by Asp or Asn in testis (Bilek et al., 2011). These peptides appear to form a paracrine network possibly regulating prostatic growth and normal growth and function of the gonads. The peptides can cause negative feedback on LH and testosterone secretion and it was proposed to name them “gonadins” (Bilek et al., 2011).

Internal and external effects (e.g. disease or chemicals) can alter TRH levels. In the euthyroid sick syndrome, characterized among other things by decreased T4 and T3 levels, usually with elevated reverse T3, normal basal TSH and a blunted TSH response to TRH, levels of proTRH mRNA in the hypothalamus are low and degradation of TRH in the blood is decreased (Duntas et al., 1999). This indicates compromised regulation of thyroid hormones under physiological stress, such as carbohydrate deficiency, surgical intervention, liver or kidney failure, and intensive care. Exposure of rats to the plasticizer di-2-ethylhexylphthalate resulted in disruption of signaling pathways. TRHR in the hypothalamus is down-regulated, protein and mRNA of TRH receptor (TRHR) in the pituitary is up-regulated, and RNA of TSH-receptor in the thyroid is down-regulated (Sun et al., 2018).

### 3.2. Synthesis

The trh gene is present on chromosome 3 and is encoded by an exon for the 5′-untranslated region and two exons for the full 29-KDa preproTRH. Human preproTRH contains six copies of the TRH progenitor sequence, which are cleaved and further modified to yield mature TRH (Fig. 3). PreproTRH is produced at the rough endoplasmic reticulum (rER). This is then transported to the ER, where partial folding of proTRH occurs. In the trans-Golgi network (TGN), cleavage by the converting enzymes PC1/3 and carboxypeptidase takes place and two different types of peptides of 9 kDa and 5.4 kDa exit the TGN. These peptides from the N-terminal and C-terminal proTRH molecules are sorted into different populations of secretory vesicles, which are released at the axon terminal (Perello et al., 2008). Peptides derived from the N-terminal part are secreted under basal conditions and upon depolarisation of neurons, while peptides derived from the C-terminus are secreted mainly upon stimulation. The peptides are transformed to active TRH by two enzymatic steps (Fig. 3). Thyrotrophs of the pituitary gland can react directly to preproTRH because they possess the respective receptors. PreproTRH has partly similar (e.g. antidepressant) effects to TRH (Redei et al., 1999), inhibition of ACTH secretion by corticotrophs was seen only in animals (Perras et al., 2007). In addition to TRH, TRH-like peptides have been identified. These peptides bind to TRH receptors with different potency and mediate a variety of effects, including negative feedback of TRH secretion, positive effects on fertility, regulation of the thyroid state, proliferation of prostate cells and inhibition of insulin release.

### 3.3. Degradation

Released TRH is a tripeptide (pGlu-His-Pro-$NH_2$), which is rapidly degraded with a half-life of 4–5 min. TRH may be metabolized by different pathways in order to generate the dipeptide His-Pro, which is then cleaved by proline dipeptidase (PDP) to His and Pro. TRH may be deamidated to TRH-OH by prolyl-oligopeptidase (alternative names: prolyl endopeptidase or post-proline cleaving enzyme) (Bauer, 1980). TRH-OH lacks biological activity (Sanchez et al., 2013). Subsequently, pGlu is hydrolysed by pyroglutamyl peptidases (PP), also termed pyroglutamyl aminopeptidase. Alternatively, His-Pro-$NH_2$ is formed by hydrolysis at the pGlu-His bond by PP and deamination by postproline dipeptidyl aminopeptidase (PPDP). His-Pro-$NH_2$ may also rapidly form cyclo(His-Pro) (His-Pro-diketopiperazine, DKP) (Brabant et al., 1981; Peterkofsky et al., 1982) (Fig. 3). DKP reaches a concentration about three times higher than that of TRH and has biological activity. This suggests that metabolization is not a simple inactivation step and that biosynthesis by alternative pathways may contribute. The cyclized metabolite is more stable than TRH, not included in synaptosomes like TRH but bound to carrier, acts on PRL secretion, thermoregulation and appetite, and can display contrary effects to TRH (Lamberton et al., 1984).

PP exists in different forms: PP I (EC 3.4.19.3) is a cytosolic cysteine peptidase with broad substrate specificity, which possesses a variety of other names. PPII (EC 3.4.19.6) is an integral membrane metallopeptidase with a large extracellular domain to degrade TRH present in the extracellular space. PP II has a substrate specificity restricted to TRH and TRH-like peptides and, therefore, has also been termed thyroliberinase or TRH-degrading (ecto-)enzyme. PPII is present in neuronal cells and cells of the anterior pituitary gland (Charli et al., 1998). TRH specifically inhibits PPII activity in TRH-responsive lactotrophic cells (Cruz et al., 2008; Vargas et al., 1994). Studies on circadian levels of TSH indicate lower TRH levels during the day and higher levels at night. In the brain, region-specific circadian variations were measured (Pekary et al., 2006). Plasma levels during the night showed few fluctuations and ranged from 10.3 to 11.7 pg/ml (Azukizawa et al., 1980). Higher levels at night than during the day have also been reported for PRL (Roelfsema et al., 2012). PPII activity was transiently increased in β-tanycytes and later in the median eminence (ME) by fasting, while drugs inhibiting thyroid function, like methimazol, decreased PPII activity in tanycytes (Lazcano et al., 2015). This suggests that regulation of TRH degradation is involved in the decreased TRH levels during fasting.

### 3.4. TRH plasma levels

Normal plasma levels in healthy human subjects ranges between 24 and 138 pg/ml with no effect of thyroid status (Mallik et al., 1982). Circulating TRH is probably derived from extrahypothalamic tissues, predominantly the pancreas. Levels are higher in fetal and neonatal blood due to TRH produced by the placenta in combination with less degradation in fetal blood. Levels in adults are lower and decline only at > 65 years of age (Stan and Morris, 2005). TRH levels per se can be used to indicate TRH-secreting tumors, but this is not frequently done. Measurement of TRH was also under evaluation for use as biomarker. It was found that TRH-like peptide pGlu-Glu-Pro$NH_2$ is secreted by carcinoid tumors (Klootwijk et al., 1996).

## 4. TRH receptor

Cellular action of TRH is mediated by TRHR ligation, which has been detected in the brain (mainly hypothalamus and pituitary gland), uterus, ovary and testis, intestinal epithelial cells, retina, lymphoid tissue and bone marrow (Fukusumi et al., 1995). Follicular and C-cells of the thyroid express TRHR (De Miguel et al., 2005). Humans possess only one type of TRHR, TRHR1, through which TRH exerts all its cellular effects. Rodents and several other species also express a second form, designated TRHR2. Both receptor types consist of seven-transmembrane receptors activated by phosphorylation after binding of TRH. Activation leads to stimulation of phospholipase Cβ, which hydrolyses phosphatidylinositol 4,5-biphosphate (PIP) to inositol 1,4,5-triphosphosphate (IP3) and 1,2-diacylglycerol (DAG). Intracellular calcium levels increase and protein kinase C (PKC) is activated. TRHR1 signaling also stimulates calcium/calmodulin-dependent protein kinase and mitogen-activated protein kinase (MAPK) (Hinkle et al., 2012). Binding of TRH to its receptor induces desensitization, which is important as TRH is secreted in a rhythmic pattern. Continuous application of TRH leads to a transient burst in IP3 within seconds and

subsequent decrease within a minute. Intermittent application decreases the IP3 response with each successive stimulation. Main mechanisms of desensitization include receptor phosphorylation, arrestin binding and internalization (Hinkle et al., 2012).

The overlapping pattern of TRH mRNA and TRHR expression in brain, thyroid, intestine, uterus, ovary, testis and lymphoid tissue suggests a local (paracrine) action of TRH but mechanisms are largely unknown. After activation of TRHR1 in islet cells of the pancreas insulin is secreted (Luo and Yano, 2004). TRH secretion in the fetal period by the pancreas is high but it is not clear whether it can substitute hypothalamic action on the pituitary gland (Engler et al., 1981). Studies in rats suggest that TRH is produced in pancreatic islets (Aratan-Spire et al., 1990). TRH levels in human fetal pancreas increased from 15th to 34th week of pregnancy and then decreased at term until one year infants. In contrast, insulin levels increased during pregnancy until one year postnatal (Martino et al., 1986). During pregnancy low amounts of maternal TRH reach the fetus because the peptide is enzymatically cleaved (Bajoria et al., 1996). When TRH is administered to pregnant women between 19th and 35th of gestation, fetal TSH secretion but not PRL secretion increases (Bajoria et al., 1998). When TRH is administered later, TSH and PRL levels increase in the fetal circulation (Ballard et al., 1992a). TRH in the systemic circulation of neonatal rats is mainly derived from the pancreas and gastrointestinal tract. At postnatal day 5, levels in the pancreas were 289 ± 35 pg/mg compared to 13 ± 3 pg/mg in the hypothalamus. Pancreas TRH levels decreased until day 10 to very low levels. No TRH was detected in liver, spleen, kidney, or heart (Engler et al., 1981).

It is hypothesized that TRH plays a crucial role in metabolic regulation as it is able to reverse diabetes mellitus in animal models (Luo and Jackson, 2007). The mechanisms responsible for many TRH effects are still unclear. This includes neuromodulatory action on synthesis of classic neurotransmitters, on cardiovascular and respiratory function, and in the induction of hyperthermia. TRH is expressed in rat myocardium and overexpression leads to myocyte hyperplasia (Schuman et al., 2014). It appears that alterations are restricted to the left ventricle and that fibroblasts in the myocardium are the source of TRH.

## 5. Negative feedback regulation of TRH

### 5.1. Hypothalamic-pituitary-thyroid axis

The main role of TRH in the healthy organism is regulation of TSH expression in the thyrotrophs of the anterior pituitary gland, which in turn induces the synthesis and release of thyroid hormones by the follicular cells of the thyroid gland. Thyroxine (T4) is the predominant secretory product of the thyroid gland, whereas only 20% of the circulating T3 are secreted directly by the thyroid. In the periphery T4 is deiodinated supplying roughly 80% of the circulating triiodothyronine (T3). Reciprocally, synthesis and release of T4 and T3 are inhibited when hormone levels in plasma exceed a pre-set level. To a large extent, TRH defines this pituitary-thyroid axis set point and contributes to the maintenance of homeostasis of the plasma hormone levels (Fig. 4). In contrast to invertebrates, other releasing hormones such as CRH play no role in the regulation of thyroid hormones (De Groef et al., 2006).

In the pituitary TRH regulates not only the secretion of TSH but also its bioactivity required for correct receptor binding. Administration of TRH to patients with central hypothyroidism increases receptor binding and bioactivity (Beck-Peccoz et al., 1985). Normal feedback regulation of TRH secretion does not occur via TSH levels. Under rare conditions a special form of inappropriately elevated TSH can be observed, in which in parallel to normal TSH macro-TSH circulates in plasma. It may be responsible for laboratory interference leading to spuriously high TSH levels in TSH assays. These circulating macro-TSH levels are increased by TRH stimulation. Macro-TSH is a complex of TSH with anti-TSH antibodies, having low bioavailability and a different circadian rhythm than free TSH (Kadoya et al., 2017). Complexes of TSH and albumin as well as with naturally occurring antibodies have also been observed (Ikegami et al., 2014). “Macro”-forms of hypophyseal releasing hormones have been described for PRL, LH and FSH (Vieira et al., 2003; Webster et al., 2010) with macro-PRL being the most common. Macro-PRL shares with macro-TSH the characteristic low bioactivity and reaction to TRH (Richa et al., 2010). Hypothetical reasons for the different biological action of free and macro forms include the large size, which prevents passage of the complex through the endothelium to the target cells, and the binding of the antibody complexes to the receptor. The generally higher stability of macro forms appears to be caused by post-translational modifications. A fraction of macro-PRL amounting to 30–60% can still be regarded as normal, while the prevalence of macro-TSH (0.6%) is much lower and the prevalence of macro-LH and macro-FSH is rare (Mills et al., 2013).

Rarely, complete resistance to TRH due to a mutation in the TRH receptor gene results in inhibition of the signaling cascade whose net effect is the production of more TSH. Symptoms of hypothyroidism are detected in this syndrome, but not severe enough to cause cognitive or neurologic deficits. Fertility and lactation were reported as not impaired (Bonomi et al., 2009). These data suggest that loss of regulation by TRH can be compensated without dramatic physiological effects.

Released thyroid hormones are transported in the blood bound to thyroid hormone-binding plasma proteins: The principal binding-proteins are thyroxine-binding globulin (TBG), Transthyretin (TTR) and serum albumin. These binding proteins differ in affinities and release rates for T4 and T3 (Refetoff, 2015). The biological effects of thyroid hormones are displayed only by the small fraction of non-plasma protein-bound thyroid hormones, the so-called free fraction (approximately 0.03% of T4 and 0.3% of T3). Only these free hormones enter the cell and are subject to homeostatic control by the hypothalamic-pituitary thyroid axis. Synthesis of TTR not only occurs in liver but also -and even more intense- in the choroid plexus. This favours T4 transport from blood via the cerebrospinal fluid to brain providing more substrate for conversion of the biologically less active T4 into the biologically more active T3 by deiodinases in the brain (Schreiber, 2002).

### 5.2. Molecular mechanism of the thyroidal feedback regulation

Hypothalamic TRH levels are regulated by a variety of signals, such as thyroid disorders. Through thyroid hormone feedback regulation, playing a crucial role, thyroid metabolism has a marked influence on TRH levels.

Hypothyroidism increases TRH mRNA, TRH release, synthesis of TSH, TRH receptor (TRHR) and thyroid hormone uptake transporters in plasma membranes of target cells and circulating TSH levels. Hyperthyroidism decreases TRH synthesis, TRH processing and TRH concentration in the median eminence of the pituitary.

In particular, circulating free T4 and T3 are taken up through the blood brain barrier by thyroid hormone uptake transporters (e.g. OATP1C1 and the monocarboxylate anion transporters (MCT) 8 and 10) into target cells [35]. Due to the hydrophobicity of the molecule, diffusion also appears possible.

Three types of iodothyronine deiodinases (DOI) with different tissue distribution and functions can activate or inactivate thyroid hormones [41] and their activity can substantially alter thyroid hormone signaling. A given cell type will express only one type of deiodinase at a given time, though some tissues express none (Bianco and Kim, 2006). High activity of DIO1 is expressed in liver, kidney and thyroid and is essential to facilitate T3 release into the circulation. DIO2 serves to convert a variable rate of intracellular T4 to T3 independent of circulating levels. The cytoplasmic pool of T3 includes therefore both T3 from the plasma and T3 generated by DOI2. The activity of this endoplasmic reticulum enzyme can be regulated by ubiquitination and is influenced by the thyroid state (increased in hypothyroidism and decreased in hyperthyroidism) in addition to nutritional factors, fasting and weight gain. DOI2 allows the pituitary and hypothalamus to

monitor both plasma T3 and T4 independently and plays therefore a critical role in the feedback regulation of thyroid hormone secretion (Larsen, 1982). DIO3 catabolize thyroid hormones and terminates T3 action and is also influenced by thyroid state (decrease in hypothyroidism and increase in hyperthyroidism). Circulating T3 levels in feedback regulation result from metabolization by DIO1 and DIO2 (Fig. 4).

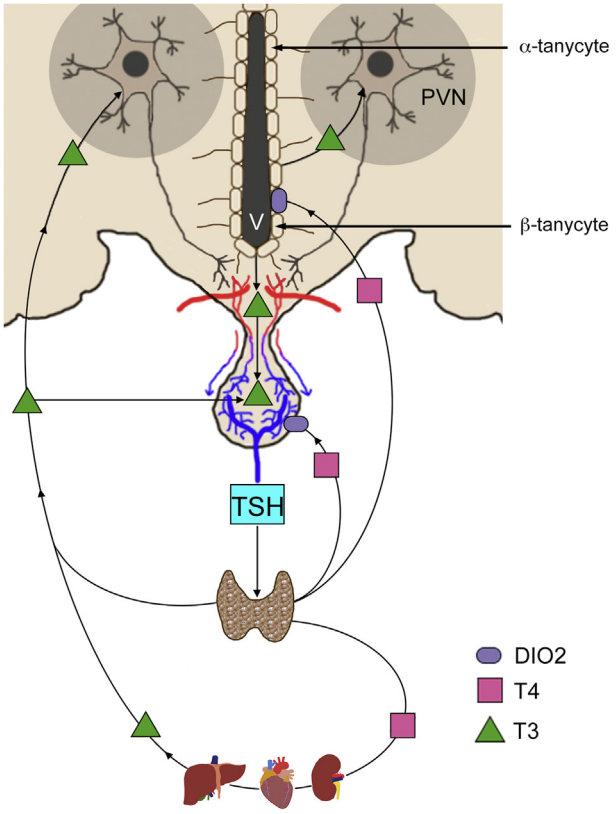


**Fig. 4.** Regulation of thyrotropin-releasing hormone (TRH) by the thyroid-hypothalamus-axis. TRH secreted by the neurons of the paraventricular nucleus (PVN) stimulates thyroid-stimulating hormone (TSH) release by the anterior part of the pituitary gland. TSH increases production of thyroxine (T4) and triiodothyronine (T3) by the thyroid gland. T3 levels act as main feedback mechanism and reduce secretion of TRH. T4 is converted either in the periphery or in the tanycytes of the brain to T3. Abbreviations: DIO2, deiodinase type 2; V, ventricle.

In brain DIO2 is expressed in tanycytes, glial cells, astrocytes. Tanycytes are the main regulators in converting T4 to T3. They are a subtype of ependymal cells and line the third ventricle and the floor of the fourth ventricle of the cerebrospinal ventricular system. When localized in the dorsal part of the third ventricle near the dorsomedial and ventromedial hypothalamic nuclei, they are termed alpha 1 and 2 tanycytes. Cells in the more ventral part close to ARC and median eminence of the pituitary gland are termed beta 1 and 2 tanycytes. They possess microvilli but no cilia unlike conventional ependymal cells and have processes that project into the brain parenchyma (Bolborea and Dale, 2013).

Tanycytes can modulate their cellular T3 level by means of DOI2. This deiodinase converts variable rates of T4 to T3 and thus adapt the hypophysiotropic cellular T3 level to the metabolic requirement. DOI2 in tanycytes is increased in hyperthyroidism.

Locally produced T3 represents the primary source of T3 in the feedback regulation and can either be transported via axons to TRH neurons of the PVN or reach them by diffusion (Fig. 2). Hypophysiotropic neurons do not express DOI2 (Fekete and Lechan, 2007).

Thyroid hormones, especially T3, once inside the cell can initiate genomic and non-genomic effects, the former by binding of the thyroid receptor (TR)-T3 complex as a heterodimer with the retinoid X receptor (RXR) to thyroid response elements (TRE), which activates gene transcription. There are two types of TR, TR alpha (TRα) 1 and 2 and TR beta (TRβ) 1 and 2. TRα is widely expressed with high expression in cardiac and skeletal muscles. TR-β1 is predominately expressed in brain, liver and kidney and TR-β2 in hypothalamus and pituitary (Flamant et al., 2006). The TRβ2 is the only isoform that is not ubiquitously expressed in the brain. Non-genomic effects are initiated by binding to surface receptors (integrin αv/β3 receptors). Thyroid hormones can also bind to cytosolic sites, mitochondria and microsomes, and enhance oxidative phosphorylation. Other targets are ion channels, second messengers and protein kinases (Brent, 2012). The main effects of thyroid hormones are to increase basal metabolic rate and thermogenesis by stimulation of anabolic and catabolic actions on glucose, proteins and lipids. More recently, effects on central control of homeostasis and on rigidity of the plasma membrane have also been postulated (Hulbert, 2000; Warner and Mittag, 2012).

Findings based on the use of siRNA against the TRβ2 and of experiments with TRβ2-null mice showed that T3 repression of TRH production needs functional TRβ2 receptors (Chiamolera and Wondisford, 2009). Binding of T3 to the TRβ2 receptor decreases expression of preproTRH.

### 5.3. Molecular mechanism of glucose level regulation

In addition to its major action on thyroid hormone secretion, TRH affects blood glucose levels and energy metabolism. These effects are caused by local as well as by central actions. TRH injected into the ventricular system acts on insulin producing β-cells of the pancreas by inducing hyperinsulinemia. This effect is caused by direct action in contrast to hyperglycemia, which could be prevented by adrenalectomy, and appears to be mediated by action on the adrenal gland (Marubashi et al., 1988). TRH and TRH-like peptides were identified as key regulators of pancreatic development and ß-cell maturation and may modulate insulin secretion directly. The peptides were identified in pancreas cells. This indicates that TRH in addition to regulation by the hypothalamus may also act in a paracrine manner (Luo et al., 2014). Murine β-TC-6 cells, which secrete insulin in response to glucose, were used to study the effect of TRH on pancreatic β-cells. The authors found that TRHR1 modulated epidermal growth factor (EGF) signaling (Luo et al., 2006). TRH activation of G-protein-coupled TRHR1 induced dissociation of the αβγ complex with activation of cellular und sarcoma (Src) kinase by the βγ subunit. The subsequent effects were mediated by Src and include activation of metalloproteinase 3 (MMP-3) to cleave heparin-binding EGF to EGFR, and changes to EGFR phosphorylation. Inhibition of PKC results in decreased phosphorylation of threonine, and phosphorylation of tyrosine EGFR. Pancreatic TRH secretion is stimulated by glucose and suppressed by insulin. Experimental data in rodents suggest that TRH can mediate various effects in β-cells, including stimulation of proliferation, prevention of apoptosis, and promotion of islet cell development by stimulating migration of stem and progenitor cells from bone marrow (Luo et al., 2018). It is, however, not clear if similar effects may occur in humans.

The finding that obese adults and obese children show increased TSH release in response to TRH supported the link of TRH to metabolism, obesity, and diabetes. The exaggerated response to TRH was not linked to Body Mass Index (BMI), body weight and inflammatory processes (Rijks et al., 2016).

The adipocyte-derived polypeptide hormone leptin regulates adaptive processes to food deprivation in mammals. Leptin mediates its action on the thyroid axes by regulating TRH gene expression in PVH. Low leptin levels induced by fasting decrease TRH secretion. Increased degradation of TRH by PPII during fasting can enhance this effect (Lazcano et al., 2015). Furthermore, decreased POMC levels and

increased AgRP and NPY levels were noted in fasted animals. Decreased TRH levels increase food intake and decrease TSH levels.

### 5.4. Molecular mechanism of the non-thyroidal TRH regulation

TRH levels are also regulated by a variety of other signals (Fig. 2). Since the hypothalamus is involved in the maintenance of a constant interior milieu, opposing stimuli (for instance low and high temperature) may affect secretion of TRH in a similar way. This complex regulation may explain why, depending on the experimental setting or the employed model, different effects on TRH were reported for the same substances. One example is the effect of glucocorticoids (GC), which were shown to increase or decrease TRH transcription (Nadolnik, 2012). As stimulators of trh transcription, cold temperature, depression, norepinephrine (NE), alpha melanocyte-stimulating hormone (α-MSH), cocaine and amphetamine-regulated transcript (CART), leptin, and glucocorticoids have all been suggested (Ben-Slomo and Melmed, 2011). As inhibitors, high temperature, physical activity, starvation, somatostatin, DA, neuropeptide Y (NPY) and GC are indicated. Other publications mention aside from low thyroid hormone levels (T3, T4), estrogens, cold exposure, heat, exercise (aerobic state), forskolin, low level laser therapy for autoimmune thyroiditis, lithium, valproate, ketamine, and electroconvulsive therapy as stimulators of TRH release, and aside from high thyroid hormone levels, stress, cortisol, inflammation, orexin, NPY, ghrelin, leptin-resistance, fasting/starvation, chemotherapy as inhibitors of TRH secretion (selfhacked).

Fig. 5 illustrates the action of the main regulators of trh transcription in PVN neurons (Fig. 5). α-MSH binds to the melanocortin 4 receptor (MC4R) and acts on adenylate cyclase (AC) via protein-activated kinase and extracellular-regulated kinases (ERK) to phosphorylate the cAMP response element-binding protein (CREB) and specificity protein 1 (SP-1) (Glas et al., 2016). These transcription factors bind to the promoter region of the trh gene, stimulating transcription. GC induce a similar effect through binding of the glucocorticoid receptor to the promotor region (Joseph-Bravo et al., 2015). Leptin increases trh gene transcription by binding to the Leptin/Obese (ObRb) receptor and activation of Janus tyrosine kinase 2 (JAK) and signal transducer of activated transcription 3 (STAT3). Binding of STAT3 increases production of preproTRH. After binding of T3 to the thyroid hormone receptor beta2 (TRβ2) the T3-TRβ2 complex inhibits transcription of the trh gene. Inhibition of TRH transcription by NPY takes place through binding to the Y-1,5 receptor and inhibition of cAMP formation (Cyr et al., 2013). Phosphorylation of CREB is prevented and transcription of the trh gene cannot be increased. Together with α-MSH, CART stimulates TRH transcription and potentiates the action of NE (Ben-Slomo and Melmed, 2011). Agouti-related peptide/protein (AgRP) prevents binding of α-MSH to its receptor and activation by α-MSH signaling does not occur (Nillni, 2010). TRH production can be controlled through the regulation of enzymes such as PC1 and PC2 and carboxypeptidase E. The former two are down-regulated by T3- TRβ2 and up-regulated by leptin and NE.

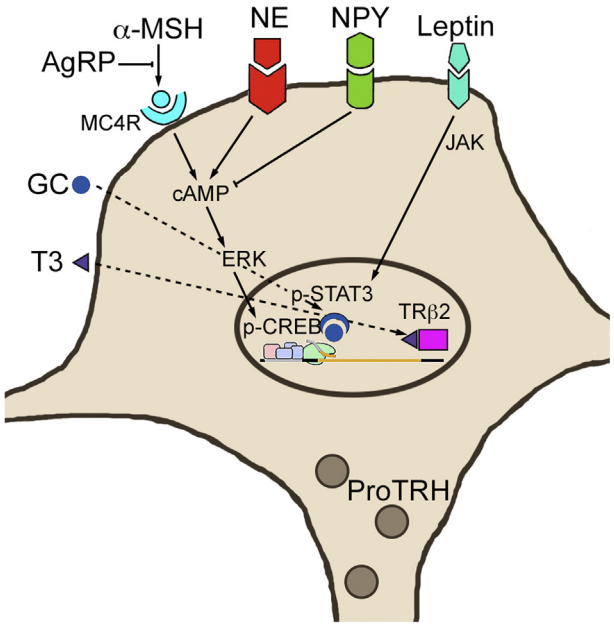


**Fig. 5.** Regulation of TRH secretion of parvocellular neurons by melanocyte-stimulating hormone (α-MSH), norepinephrine (NE), neuropeptide Y (NPY), leptin, glucocorticoids (GC), triiodothyronine (T3). Abbreviations: cAMP, cyclic adenosine monophosphate; AgRP, agouti related peptide; JAK, Janus tyrosine kinase; ERK, extracellular-regulated kinase; MC4R, melanocortin 4 receptor; α-MSH, melanocyte-stimulating hormone; p-CREB, cAMP response elements-binding protein; p-STAT, phosphorylated signal transducer of activated transcription; T3, triiodothyronine, TRβ2, thyroid hormone receptor beta2; TRH, thyrotropin releasing hormone.

Not all effectors act directly on the TRH-producing neurons of the PVN. DA, somatostatin, interleukin (IL)-6, IL-1β, and tumor-necrosis factor (TNF-α) increase TRH secretion by inhibiting the activity of the pituitary gland (Sugimoto and Mori, 2012).

Furthermore, degradation can be influenced by several other factors. Processes (end feet) of the β2 tanycytes can degrade TRH released by TRH neurons of the PVN by action of pyroglutamyl peptidase II (PPII). PPII expression is increased in hyperthyroidism.

The model by Sanchez-Jaramillo et al. proposes that TRH is degraded by membrane-bound PPII in the periportal space in response to elevated circulating levels of thyroid hormone (Sanchez et al., 2009). Upon stimulation of the TRHR by either TRH or the synthetic analogue taltirelin, the Gα subunit activates phospholipase C (PLC) and stimulates inositol-1,4,5-trisphosphate ($IP_3$) production and intracellular calcium release (Muller-Fielitz et al., 2017). This causes increase of the β2 tanycyte endfeet size, on the one hand, and increase of PPII activity, on the other. The combination of both effects decreases TRH release into the pituitary vessels.

Neurons of ARC are the key regulators of TRH secretion in the PVN and crucial for the action of insulin, glucose and leptin in the regulation of energy balance. ARC is seen as the “feeding center” of the brain. The nucleus is well positioned to sense peripheral hormone levels because it is located in a brain region where the blood-brain-barrier is leaky. The nucleus contains pro-opiomelanocortin (POMC) and CART secreting neurons, all decreasing food intake. POMC can be cleaved to several peptides including ACTH, β-endorphin, and α-MSH. Effects of CART are similar to POMC but have been less well studied. Orexogenic neurons in the ARC primarily release NPY, AgRP and GABA, which act synergistically. Loss of one transmitter by knockout has only small effects, suggesting that compensation mechanisms are in place (Gali Ramamoorthy et al., 2015). ARC neurons are the targets for three signaling substances, glucose, leptin, and insulin. Central application of leptin induces an increase in TRH mRNA levels in hypophysiotropic neurons, while AgRP/NPY-synthesizing neurons are inhibitory to hypophysiotropic TRH neurons and CART- and a-MSH-synthesizing neurons are stimulatory (Fekete et al., 2006). Neurons in the ARC express leptin and insulin receptors and are glucose-sensitive. NPY-producing neurons are inhibited, while α-MSH producing neurons are activated by insulin, leptin and glucose. Although insulin and leptin are both decreased under fasting conditions, exert anorexic effects and increase energy expenditure, only central application of leptin affects central mRNA TRH levels.

TRH further appears to play also a role in weight gain in pregnancy as transcription levels in mice are increased in mid pregnancy compared to virgin controls (Pazos et al., 2013).

## 6. The hypophysiotropic effect of TRH on prolactin

### 6.1. Action of TRH and thyroid status on prolactin

Although the main targets of TRH are TSH-secreting cells (thyrotrophs) of the distal pituitary gland, TRH may also act on PRL secretion because lactotropic cells (lactotrophs, PRL-secreting cells) also possess TRHR. Clinical data support such a link. Subclinical hypothyroidism with increased TRH, high TSH levels and normal thyroid hormone levels is accomanied by increased PRL secretion (Hollowell et al., 2002). Furthermore, hyperprolactinemia has been detected in about 20% of patients with subclinical hypothyroidism and in 39–57% in overt hypothyroidism (Bahar et al., 2011). Average TSH levels were higher in hyperprolactinemic women but there was no correlation of TSH levels to PRL levels (Ahmed et al., 2017; Longcope, 1996; Thomas and Reid, 1987). Increased production of PRL in hyperthyroid women, on the other hand, may be due to increased clearence of PRL in hyperthyroidism (Cooper et al., 1979). Autoimmune processes like autoimmune thyroiditis may also increase PRL levels (Onishi et al., 1975). Hyperthyoird women with low (< 0.5 mU/l) TSH levels had higher (23.7 ng/ml) PRL levels than normothyroid women (16 ng/ml) (Sanjari et al., 2016). The level of total estrogens did not play a role but it is possible that the fraction of free estrogens was reduced due to increased levels of sex hormone binding protein in hyperthyroidism.

Studies of infertile women showed that hypothyroidal women had significantly higher PRL levels (Binita et al., 2009). High PRL levels were associated with abnormal menstrual pattern. Prevalence of hyperprolactinemia in hypothyroidal women ranged between 16.6 and 57%, depending on the study, and the severity of menstrual abnormalities was associated with high TSH levels (Goswami et al., 2009). Hypothyroidism, not hyperthyroidism, was linked to infertility although hyperthyroidism also causes menstrual irregularities. These irregularities, however, rarely lead to infertility. This may suggest that hyperprolactinemia as a consequence of high TRH levels is the reason for infertility. Earlier studies suggested a link between TRH and development of prolactinoma but more recent studies did not identify a role of TRH in lactotroph differentiation or proliferation (Courvoisier, 1999).

### 6.2. Molecular regulation of PRL levels

PRL transcription and secretion by lactotrophs is regulated by various receptors, particularly those for DA and vasoactive intestinal peptide (VIP) (Fig. 6). The existence of a hypothetical unique PRL-releasing hormone has not been demonstrated thus far. While TRH and VIP are produced in the PVN, DA is secreted by ARC neurons, which tonically suppress PRL secretion. All neurotransmitters reach the lactotrophs of the pituitary gland by the portal system. Estrogens act via nuclear receptors on the lactotrophs, VIP, DA, TRH, growth factors and cytokines by surface receptors (Fig. 7). Higher PRL levels in premenopausal than in postmenopausal women suggest an influence of estrogens on PRL secretion. DA causes a decrease, VIP an increase of cyclic adenosine monophosphate (cAMP). TRH acts via phosphatidylinositol 4-phosphate (PIP)- PKC and calcium signaling. Somatostatin (SS) acts through G-protein-coupled somatostatin receptors (SSTRs), which inhibit adenyl cyclase and L-type $Ca^{2+}$ and $K^{+}$ channels (Peverelli et al., 2015). The lower inhibition of SS on lactotrophs than on growth hormone-producing pituitary cells is due to the lower receptor density (Enjalbert et al., 1986). Fibroblast growth factor (FGF)-2 and EGF are able to modulate the response of lactotrophic cells to TRH and DA. Furthermore, effects of TRH and DA on PRL secretion were dependent on estrogen levels. FGF-2 increased PRL secretion induced by TRH and reduced DA inhibition. EGF increased TRH effects without modifying the inhibitory responses to DA (Spuch et al., 2006).

PRL is produced as a preprohormone. After the signal peptide of preproprolactin has been removed, glycosylation of the peptide occurs in the Golgi apparatus. An acid protease cleaves the PRL peptide, which already has biological activity. Phosphorylation and deamidation take place in secretory granules and modulate the bioactivity of PRL. Secretion occurs by calcium-dependent exocytosis (Sarapure, 1997). Similar to TRH, secretion of PRL has circadian rhythmicity with low levels during the day and higher levels at night. The upper limits of PRL levels are 20 ng/ml in women and 10 ng/ml in men (Langan et al., 2010). Secreted peptides have different molecular weights and different biological actions. They are termed “little”, “big” and “bigbig” PRL (Tutunculer et al., 2006). Little (23 kDa) PRL is involved in feedback regulation in the hypothalamus, whereas big (50–60 kDa) and bigbig (> 100 kDa) PRL have antagonistic effects on vessel formation. Although pituitary tumors showed splice variants of the TRHR there was no link between TRH signaling and prolactinoma.

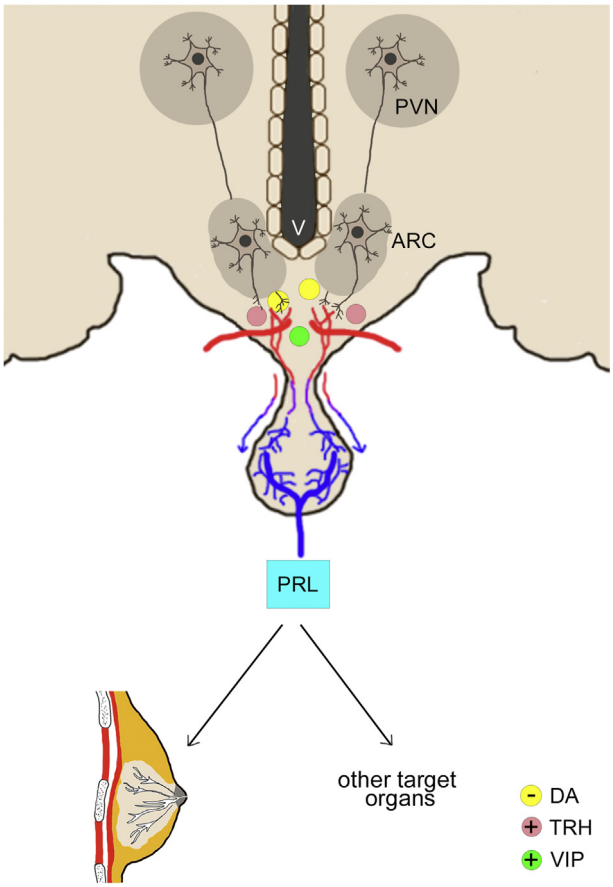


**Fig. 6.** Overview of the main regulators of prolactin (PRL) secretion. PRL secretion is tonically inhibited by dopamine (DA) released by neurons of the arcuate nucleus (ARC). Inhibitory action of somatostatin (SS) secreted by ventromedial nucleus of the hypothalamus is not indicated. TRH and VIP secreted by neurons of the PVN stimulate PRL release. Abbreviations: ARC, arcuate nucleus; DA, dopamine; PVN, paraventricular nucleus; TRH, thyrotropin-releasing hormone; V, ventricle; VIP, vasoactive intestinal peptide.

### 6.3. Action of PRL

Binding sites for PRL have been identified in the hypothalamus and substantia nigra. Upon application of TRH, turnover of DA is increased, the opoidergic system is stimulated, and immune responses, corticoid secretion and learning are improved, while testosterone levels, body temperature, and libido are decreased (Sobrinho, 1993). Some of these effects have also been considered as TRH effects. Breast swelling, tiredness, headache, nausea and dizziness are reported as symptoms of hyperprolactinemia (Marinaki, 2016) but also as effects of high TRH doses in the stimulation TRH test (see section “Diagnostic use”). In

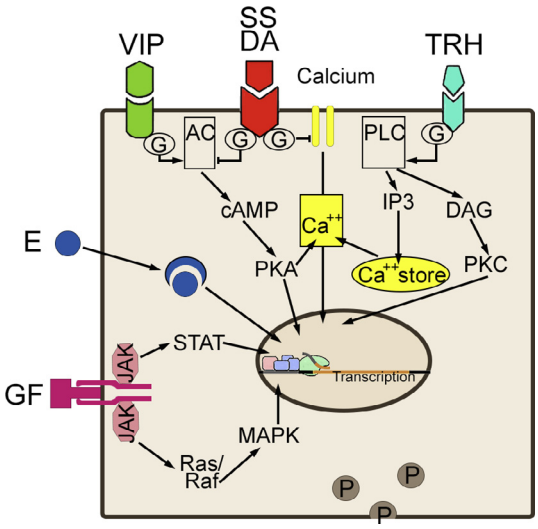


**Fig. 7.** Regulation of prolactin secretion by lactotroph cells. Stimulation of prolactin (P) secretion by thyrotropin-releasing hormone (TRH), vasoactive intestinal peptide (VIP), various growth factors (GF), and estrogens (E) bound to its cytoplasmic receptor as well as inhibiting action of dopamine (DA) and somatotropin (SS) is indicated. Abbreviations: AC, adenylate cyclase; cAMP, cyclic adenosine monophosphate; DAG, 1,2-diacylglycerol; G, G-protein; IP3, inositol 1,4,5-triphosphosphate; JAK, Janus tyrosine kinase; MAPK, mitogen-activated protein kinase; PKA, protein kinase C; PLC, phospholipase C; P, prolactin; Ras/Raf, Rat sarcoma/Rapidly accelerated fibrosarcoma; STAT, signal transducer of activated transcription.

addition to being a moderate immune stimulant, PRL increases insulin secretion and glucose sensitivity, leptin resistance, and fat cell production (Triebel et al., 2015). The spectrum of PRL actions is extended by the effects of specific by-products of PRL (vasoinhibins). Vasoinhibins are a family of N-terminal antiangiogenic PRL fragments with 14–18 kDa molecular masses generated in the hypothalamus (Zamorano et al., 2014). They appear to counteract PRL effects and cause depression and anxiety, inhibit vessel growth, vasopermeability and vasodilation. PRL is expressed in almost all tissues and plays an important role not only in reproduction but also in angiogenesis, osmoregulation, immune responses, metabolism and behavior. More information on PRL synthesis, function, and regulation is available in reviews focused on this topic (see for instance (Freeman et al., 2000; Ignacak et al., 2012; Torner, 2016)).

### 6.4. *Potential role of TRH in breast cancer*

TRH-like peptides have been detected in the mammary gland at levels about four-fold higher in breast cancer, suggesting a potential role of these peptides in tumor development (Ghilchik et al., 2000). Consistent with such a role of TRH, breast cancer patients have elevated TSH levels and excessive TSH responses to TRH stimulation (Rose and Davis, 1978). Other data support a link between PRL levels and breast cancer. TRH-induced increases in PRL were higher in breast cancer patients than in normal subjects (Barni et al., 1986) and basal and TRH-stimulated PRL levels were increased in breast cancer patients. Survival and mean disease-free interval were slightly shorter in patients with either exaggerated TSH or PRL response to TRH (Aldinger et al., 1978). The TRH stimulation test, on the other hand, was not helpful in the identification of gynecological tumors because basal TSH levels and reaction to TRH stimulation in breast cancer patients and controls were similar (Evans et al., 1986; Kohler et al., 1981). TRH levels are regulated by thyroid hormones and the link between TRH and breast cancer may be part of the association of thyroid pathologies with breast cancer. Increased risks of breast cancer for patients with hypothyroidism, hyperthyroidism, goiter or thyroid autoimmune diseases have been published, but other studies did not report a link between thyroid pathologies and breast cancer. These studies differed in quality, not all were cohort studies, and some did not distinguish between hypo- and hyperthyroidism. A nationwide population-based cohort study on breast cancer in Denmark identified a weak correlation with thyroid hormone status (Sogaard et al., 2016). Patients with hyperthyroidism had a slightly higher risk with standardized incidence ratio (SIR): 1.11, 95% confidence interval (CI): 1.07–1.16) for development of breast cancer than women with normal thyroid function. Hypothyroidism, by contrast, was accompanied by a slightly lower risk for breast cancer (SIR: 0.94, 95% CI: 0.88–1.00). Laboratory data, levels of T3, T4, and TSH were not available in that study and no differentiation into clinical and subclinical thyroid disease at the time of breast cancer diagnosis was possible. TRH influences PRL levels. PRL secretion, on the other hand, has been suspected to play a role in breast cancer because it is higher in breast cancer patients with recurrent disease than in primary tumors and the risk of developing breast cancer was reported to be higher in women who had PRL levels > 9.7 ng/ml (Emiliano and Fudge, 2004; Miyazaki et al., 1979; Ohgo et al., 1976). Furthermore, elevated PRL levels (> 12.6 ng/ml) were correlated with poor survival in patients after mastectomy, and metastasis was seen in patients with levels > 32 ng/ml. There was, however, no relation between pituitary function and endometrial or ovarian tumors (Ylikorkala et al., 1979). According to a meta-analysis based on seven studies, plasma PRL is associated with breast cancer risk for estrogen receptor (ER)-positive/progesterone receptor (PR)-positive tumors, not for ER-negative/PR-negative tumors (Wang et al., 2016). There was no correlation with invasiveness and lymph node positivity in postmenopausal women. Furthermore, the prolactin receptor may be a marker for metastasis (Shemanko, 2016).

The influence of circulating thyroid hormones on breast cancer risk could be greater than the effect of increased TRH and PRL levels. It is also possible that hormone receptor status of tumors may hide the correlation of hyperprolactinemia and cancer development. Although ER-positive/PR-positive tumors represent the majority of breast cancer cases (63% in one study (Dunnwald et al., 2007)), such a bias cannot be excluded. Prospective studies identified an association of high T4 and breast cancer incidence (Brandt et al., 2015; Tosovic et al., 2012). Elevated T3 levels were also correlated with a higher incidence of breast cancer as well as shorter survival time (HR 2.80) (Tosovic et al., 2010, 2013). In this situation, both TRH and TSH levels are expected to be low due to feedback regulation, which argues against a role of high TRH in disease pathogenesis. Other factors may be anti-thyroid antibodies which are relatively frequent in thyroid pathologies and also play a role in breast cancer (Fröhlich and Wahl, 2017). A meta-analysis of 8 studies reported higher levels of anti-thyroid peroxidase (TPO) antibodies, anti-thyroglobulin (Tg) antibodies, T3, and T4 in breast cancer patients than in controls (Shi et al., 2014). Anti-thyroid antibodies appear to promote breast cancer development but not tumor propagation. Studies on patients with newly diagnosed breast cancer reported that anti-TPO antibodies were linked to lower incidence of metastasis and less lymph node involvement (Farahati et al., 2012; Kemal et al., 2015; Özmen et al., 2015).

## 7. Non-hypophysiotropic effects of TRH

Besides the above-mentioned regulations TRH shows also other multifaceted effects. It stimulates proliferation of immune cells (thymocytes and splenocytes), inhibits monocyte activity and suppresses cell-mediated cytotoxicity in natural killer cells (Quintanar and Guzman-Soto, 2013). Oral application of TRH produces a unique immune regulation pattern but patterns of TRH, CRH, and GnRH are similar. Although action of the different releasing hormones on pituitary cells in general is specific, similar effects of TRH and GnRH antagonists on PRL secretion have also been observed (Chantilis et al., 1995). As effects of PRL are pleitropic it cannot be excluded that those of TRH are

in part caused indirectly by PRL. Identification of TRH effects is further complicated by its action on thyroid hormone levels. Thus, it is not clear whether effects are caused directly by TRH or through thyroid hormones.

Cellular studies have been applied to identify direct TRH effects. It was found that TRH has relaxant effects on human myometrium and umbilical cord (Potter et al., 2004). Furthermore, TRH acted independently of TSH, negatively on iodine metabolism of isolated thyrocytes by reducing organification and conversion of iodine (Kallee et al., 1993; Wahl et al., 1992). These effects may be masked by compensatory mechanisms *in vivo*.

TRH production by the pancreas inhibited amylase secretion and increased glucagon secretion. TRH injection combats elevated glucose blood levels in hyperglycemic mice by helping in the regeneration of β-cells (Luo et al., 2008).

The role of TRH-like peptides in the prostate (see also Section 3.1), however, is not completely clear, but a role in proliferation and normal growth and function of the gonads has been proposed (Bilek et al., 2011). TRH and TRH-like peptides follow a circadian cycle and are influenced by androgen, PRL, and thyroid hormone levels (Bilek, 2000).

Thalamic TRH probably acts as an intrinsic regulator of the thalamocortical network activity as demonstrated in tissue slices. This may suggest an effect of TRH on selective attention (Broberger and McCormick, 2005).

While the molecular action of TRH on lactotrophs is well characterized on the cellular level (see Section 6.2), other effects of TRH in nonendocrine disorders are more complex and, therefore, more difficult to explain. They include regulation of arousal, autonomic function, circadian rhythmicity, endotoxic and hemorrhagic shock, mood, pain perception, seizure activity, spinal motor function, regulation of blood pressure, heart rate, food intake, and gastric motility.

## 8. Diagnostic use of TRH

### 8.1. TRH overexpression

Increased TRH mRNA expression relative to normal tissue is seen in breast cancer and endometrial cancer (proteinatlas). TRH immunoreactivity was found to be expressed in 2 of 6 melanoma cell lines and in extracts of melanoma tissues (Ellerhorst et al., 2004). Patients with TRH-immunoreactive dysplastic nevi were more likely to develop melanoma. The functional significance of this finding is not completely clear but patients with melanoma present with a high prevalence of hypothyroidism. The hypothesis has been proposed that melanoma cells, similar to neuroendocrine cells of the hypothalamus, can sense hypothyroidism and produce TRH in response.

### 8.2. TRH stimulation assay

Chemically synthesized TRH, termed protirelin, is structurally identical to the natural peptide and is used in the TRH stimulation test. TRH stimulation by application of 200 or 400 μg intravenously, 1 mg intranasally or 40 mg orally, however, has been used in the diagnosis of thyroid pathologies because TSH levels increase upon TRH application in normal subjects. Normal individuals respond with a doubling of their TSH levels in about 30 min after injection of synthetic TRH. The reaction of TSH to TRH administration is linear with a plateau at 400 μg (Snyder and Utiger, 1972) probably reflecting maximal stimulation of the thyrotrophs. TSH response following TRH stimulation is directly proportional to the basal TSH. TRH can induce transient changes in blood pressure (either decrease or increase) within 15 min of application. Breast enlargement, headache, convulsion, nausea, urgent need but inability to pass urine, bad taste in mouth, abdominal discomfort, and light-headedness can also occur. Some of these symptoms have also been reported in patients with hyperprolactinemia, suggesting that some TRH effects may be mediated by PRL. Sex has little influence on TRH response, but responsiveness decreases with age and circadian variations can be 11–40%. A patient with tertiary hypothyroidism will show a normal response, but the peak is delayed to 45–60 min after injection. Patients with secondary hypothyroidism will not respond and patients with primary hypothyroidism will show an exaggerated TSH response. A blunted response is seen in hyperthyroidism but also in depression, schizophrenia, and alcoholism. Several drugs can influence the response to TRH. Drugs can also influence the TRH test; attenuated TSH responses are obtained upon medication with lisuride, L-Dopa, cyproheptadine, salicylates, morphine, heroin, methysergide, fenclofenac, etiroxate, and carbamazepine. Metoclopramide, sulpiridine, chlorpromazine, biperidine, haloperidol, cimetidine, estrogens in men, iodine, iodine-containing contrast agents, amiodarone, lithium, spironolactone, and theophylline cause exaggerated responses (Loosen, 1988). Comparison between morning and evening levels of TRH levels indicated a circadian pattern in depression.

Due to the development of ultrasensitive TSH assays by the mid-1990s, performance of TRH stimulation to diagnose classic hyperthyroidism is no longer needed in the view of many endocrinologists and has contributed to the currently low usage of TRH testing. Since 2002 when Ferring Pharmaceuticals, the only supplier of TRH in the U.S., was required to remove their TRH product (Thyrel) from the market due to questions regarding their production processes, TRH is no longer available there (Rapaport et al., 2010). Lack of use in the U.S. was also caused by concerns regarding the reliability of this test in the pediatric range. In other countries protirelin is still available.

The TRH stimulation assay, however, may be indicated for the detection of hypothyroidism when hormone levels are still normal. The main use is in distinguishing between TSH-secreting pituitary tumors and the pituitary variant of thyroid hormone resistance, and in following up pituitary tumors (Faglia, 1998). In combination with measurements of TSH, T3, and T4, TRH levels can help to differentiate between primary, secondary and tertiary hypothyroidism. In primary hypothyroidism (defect at the level of the thyroid gland) the circulating levels of T3 and T4 are low, while TSH levels are high due a lack of thyroid hormone negative feedback on the anterior pituitary. In secondary hypothyroidism (defect at the level of the pituitary gland), the circulating levels of TSH, T3 and T4 are abnormally low and in tertiary hypothyroidism (defect at the level of the hypothalamus) levels of TRH, TSH, T3 and T4 are abnormally low. The TRH stimulation test is important for the diagnosis of congenital central hypothyroidism (secondary and tertiary hypothyroidism) in neonates, because appropriate therapeutic intervention can be undertaken quickly. The term central hypothyroidism is often preferred because differentiation between secondary and tertiary hypothyroidism may pose problems. Central hypothyroidism in children can be caused by a variety of pathological conditions, including pituitary hypoplasia, defects in TSH synthesis, loss-of-function mutations of the TRHR1, TSHβ subunit, and pituitary transcription factors, craniopharyngeoma, or cranial irradiation (Haugen, 2009; Wiersinga, 2014). According to recent findings, immunoglobulin superfamily member 1 (IGSF1) regulates TRH signaling in the pituitary gland. Mutations in Igsf1 have been identified in children with central hypothyroidism and Igsf1 knockout mice show reduced TRHR expression and impaired TRH stimulation of TSH secretion (Bernard et al., 2018). More information on central hypothyroidism and its causes can be found, for example, in (Gupta and Lee, 2011).

Deficient TRH secretion by the hypothalamus results in abnormal glycosylation of TSH, where immunological properties may be preserved and normal levels are measured but physiological function is lost. Variation in glycosylation pattern can also be seen in primary hypothyroidism, nonthyroidal illnesses, and in TSH-secreting pituitary adenomas (Trojan et al., 1998) and even under physiological conditions for example in healthy subjects during the nocturnal TSH surge and in normal fetuses during the last trimester of pregnancy.

TRH stimulation tests also enable a diagnostic and prognostic statement in acute and even pre-clinical cases of acromegaly.

Approximately 70–80% of acromegalic patients show an abnormal growth hormone response following TRH stimulation. Normally TRH does not cause release of growth hormone. This paradoxical effect is thought to be nonspecific, but an altered neurotransmitter milieu as underlying factor is also discussed (De Marinis et al., 1990; Kageyama et al., 2005).

While basal TSH levels in thyroidectomized thyroid cancer (TC) patients under thyroxine therapy were normal in 20/30 patients, the response to TRH was blunted in almost all patients (27/30 patients) (Castagnoli et al., 1986). It was proposed that the TRH stimulation assay may be useful for monitoring TC patients to identify optimal levothyroxine dose for TSH suppression (Gorges et al., 2002).

According to one study, TRH induced calcitonin release in medullary but not in follicular TC patients (Nakamura et al., 1987) while another study did not report increased calcitonin levels in medullary TC. TRH stimulated calcitonin secretion only by normal C-cells of the thyroid, while only pentagastrin caused calcitonin release in medullary TC (Ahuja, 1990). These studies show that the relevance of TRH assay as a cancer biomarker remains elusive.

## 9. Therapeutic use of synthetic TRH

Application of TRH in thyroid cancer in order to stimulate radioiodine uptake did not produce promising results. Oral TRH alone and in combination with lithium did not affect $^{131}I$ uptake in TC although TSH levels increased (Ang et al., 1995). Treatment with TRH in central hypothyroidism was able to increase receptor binding and bioreactivity (production of cAMP) of TSH isolated from these patients (Beck-Peccoz et al., 1985). When these patients were treated with TRH over 20–30 days they produced TSH with higher receptor binding and bioreactivity.

In addition to endocrine effects, TRH activates cerebral and sympathetic nerves, improves spinal function, has antidepressant activity, and suppresses gastric acid secretion (Quintanar and Guzman-Soto, 2013). It is therefore not surprising that effects have been studied for many applications. Metabolic effects were achieved by combination of TRH with GH-releasing peptide 2, which acted anabolic in critically ill patients (Van den Berghe et al., 1999). The application of TRH as an antagonist in ethanol intoxication yielded conflicting results with two studies showing efficacy and one enhancing ethanol toxicity. Various experiments and clinical trials addressed effects on the central nervous system.

Animal experiments with TRH demonstrated improvement of amyotrophic lateral sclerosis (ALS). Although TRHR expression in patients with ALS was decreased, therapeutic application of TRH did not have any positive effects in these patients (Gary et al., 2003). Analysis of the published studies showed that important information was lacking in some of them (Brooke, 1989). By weighting the studies according to quality, the author concluded that TRH caused a definite, acute, and transient response but that effects on the disease process were unclear. Different responses might be caused by hormone-dependent action of TRH. In mice, testosterone increased sensitivity to TRH (Miller and Warnick, 1989). Better defined studies may be needed to follow up these results. Another potential indication for TRH application is depression. A link to thyroid hormone levels has been assumed because depression is associated with low T3 levels in some patients. The blunted TRH response often recorded in depressed patients could be explained by the fact that TRHR levels are down-regulated as a consequence of chronic hypersecretion of TRH (Hage and Azar, 2012). Increased TRH levels in the cerebrospinal fluid of these individuals support this theory. Furthermore, TRHR1-knockout mice show increased anxiety and depression-like behavior, suggesting an influence of TRH independent of thyroid hormones. Based on animal data it has been hypothesized that serotonin inhibits the secretion of TRH (Kronig and Gold, 1985). Consistent with this assumption the serotonin reuptake inhibitors (SSRI) escitalopram up-regulated TRH and TRH-like peptides and increased TRH levels in various brain regions (cerebellum, medulla oblongata, striatum) (Sattin et al., 2008). Upon treatment with TRH, improvement of depression was seen within hours in some patients. However, only 42% of the studies demonstrated significant effects in depressed patients (Mason et al., 2000). Another problem was that effects were only short-lived. A link between TRH, depression and PRL may also be postulated since the effect of SSRIs was improved at elevated PRL levels (Froes Brandao et al., 2016). TRH application in cancer fatigue patients improved Visual Analog Scale-Energy, sleep disturbances and life quality (Kamath, 2012). Potential anxiolytic effects have not been followed up in clinical trials. Animal experiments showed neuroprotective effects in spinal injury. Intrathecal TRH application reduced lactate levels following spinal cord trauma. Thyroid hormone and glucose levels were not affected by the treatment (Cengiz et al., 2008). Encouraging positive effects of TRH obtained in small trials on spinal cord injuries (Pitts et al., 1995), spinal muscular atrophy (Tzeng et al., 2000), infantile spasms (Matsumoto et al., 1987), were either not pursued or could not be confirmed in subsequent trials.

Based on a link of poor TRH response and abnormal pregnancy, effects of TRH in pregnant women were studied (Kivinen et al., 1979). TRH, in contrast to TSH, is able to cross the placental barrier and its administration to the mother can therefore be used to treat the fetus. Application of TRH in addition to corticoids to mothers was studied in various trials. Although first trials suggested positive effects on respiratory distress syndrome (RDS) (Ballard et al., 1992b), later trials identified only moderate effect in pre-term infants (Crowthy, 1997). Effects of the combination of TRH and corticoids at 24–31 weeks did not improve the need for $O_2$ therapy and survival at day 28, or the incidence of RDS. A slight decrease in the risk of severe RDS and a slight increase for mortality prior to discharge were reported. Pregnant women experienced similar side effects as patients in TRH stimulation tests: nausea, vomiting, flushing and blood pressure increase.

TRH is not frequently used in therapeutic applications. One reason is the poor reproducibility of positive TRH effects in clinical trials in several applications. Another reason is the fact that TRH itself is a poor drug candidate due to its low intestinal and cerebral permeability, cardiac and endocrine side effects, and rapid degradation (Khomane et al., 2011). Application of TRH is possible via intranasal delivery (Veronesi et al., 2007). Controlled release to prolong duration of action can be achieved by subcutaneous implantation of TRH-containing poly (lactic-co-glycolic acid) (PLGA) microspheres (Heya et al., 1994). Transdermal delivery is possible with prodrugs (Moss and Bundgaard, 1992). Use of prodrugs can also improve delivery to the brain. Once absorbed, passage through the blood-brain barrier presents a major hindrance. Use of prodrugs with sufficient lipophilicity can facilitate uptake into the CNS (Prokai-Tatrai and Prokai, 2009). For oral application, TRH analogs have to be used.

Based on the anti-aging effects in mice, sublingual protirelin (Abaris™) and protirelin acetate for oral use have been commercialized as health products. Mice treated by chronic application of TRH in drinking water showed prevention of age-related kidney changes and increased testis weight and spermatogenesis (Pierpaoli, 2013). Efficacy of the treatment in humans has not been demonstrated.

### 9.1. TRH analogs

Analogs have been produced by modification of different parts of the peptide. Poor access to the brain, instability in plasma, endocrine side effects and poor receptor selectivity are other problems of these analogues. According to the use of stems in the selection of international non-proprietary names for pharmaceutical substances, TRH-related compounds have the suffix –“tirelin”. Compounds that were tested in clinical trials include montirelin, posatirelin, and taltirelin.

Compounds have been obtained by variations at the different parts of the TRH molecule (Daimon et al., 2013). The analog taltirelin, carrying a modification at pGlu and used in spinocerebellar degenerative

disease (SCD), is active at 100-fold lower concentration and with an eight-fold increased duration than natural TRH. Montirelin, effective for the treatment of seizures, recovery from anesthesia and loss of consciousness, acts at a 10-fold lower dose and for a longer time than natural TRH (Jantas, 2010). Azetirelin is 10–100 times more potent and has an 8–36 times longer analeptic effect in mice. Trials for anticonvulsant activity were positive but oral bioavailability of the compound is low. Another option is modification of the middle peptide residue. Substitution of L-His with pyridinum moieties increases CNS activity (nootropic and antiepileptic action). NP-647 (L-pGlu-2-propyl)-L-His-LPro$NH_2$ could be a candidate for oral antiepileptic action because it resists pH and enzymatic degradation, and bioavailability is neither limited by dissolution nor by solubility. According to animal studies, this higher CNS activity is caused by a higher affinity to TRHR2 than TRHR1. The dual substitution of L-His and pGlu in posatirilin was effective in improvement of learning and memory. Central action was five times more potent than natural TRH but strong anorexiogenic and hypothermic action were the reason that development was stopped. Dual substitutions at pGlu and L-Pro$NH_2$ increased proteolytic resistance but action was not devoid of endocrine hormone effects. Rovatirilin is another analog that may have an orally effective therapeutic potential in patients with SCD. A neuroprotective action has been linked to the L-Pro$NH_2$ residue. The cyclic derivate of TRH dipeptide, histidyl-proline-diketopiperazine (CHP) also acts as a neuroprotectant but mechanisms are poorly understood.

All analogs showed reduced affinity for the TRH receptor, better penetration into the CNS and resistance to degradation. TRH has been tested in clinical trials for several diseases listed in the following section.

### 9.2. Potential applications of TRH analogs

Due to their increased stability, TRH analogs can be used for oral application. Taltirelin, montirelin, rovatirelin, azetirelin, NP-647 are suitable for oral delivery; the TRH analog posatirelin can be applied by the intramuscular route (Sasaki et al., 1994). Indications for the use of TRH analogs are similar to those of TRH. In studies with patients presenting “disturbances of consciousness” the TRH analogue montirelin administered over 14 days improved ratings of global clinical state in 73% of patients (Haruhiko et al., 1996). Cancer-related fatigue could be improved by the TRH analogue taltiralin in models of colon cancer-bearing mice for radiation and chemotherapy (Dougherty et al., 2017).

The most promising application of TRH remains its therapeutic use in SCD. Improvements were noted in gait, speech, and coordination in both phase II and phase III trials. The drug appeared to slow disease progression in large phase III studies (Kinoshita et al., 1998). Based on these studies, Taltirelin (Ceredist) has been approved for therapy of SCD in Japan (Gary et al., 2003).

## 10. Conclusions

TRH has multiple roles in the human body, the most important being the regulation of thyroid hormone secretion. The multiple extrahypothalamic actions of TRH have led to the concept that it is a ubiquitous neurotransmitter that has been co-opted by the pituitary as a releasing factor (Morley, 1979). Many controversial findings have been published over the years. Nowadays, with the availability of modern sophisticated analytical and imaging techniques, it might be useful to re-investigate the role of TRH as neurotransmitter. Diagnostic use of the TRH stimulation test is currently focused on distinguishing between different forms of central hypothyroidism. Effects on PRL levels may be important because TRH effects were more pronounced at high PRL levels. The link between elevated levels of TRH, TRH-like peptides, TSH and PRL to breast cancer is not fully understood. An exclusive role of TRH levels in its pathology is unlikely as studies also reported a link between breast cancer and high levels of T3, T4, anti-thyroid antibody and PRL. Despite several promising studies, TRH is only approved for the treatment of SCD. Targets of direct TRH action are difficult to identify due to its influence on thyroid hormone and prolactin with their broad range of effects in the organism.

## Conflict of Interest

Authors declare that there is no conflict of interest.

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