HYPOTHALAMIC REGULATION OF THYROID-STIMULATING HORMONE AND PROLACTIN RELEASE: THE ROLE OF THYROTROPHIN-RELEASING HORMONE

Hypothalame regulatie van schildklier-stimulerend-hormoon en prolactine afgifte: de rol van thyrotrophin-releasing hormone (TRH)

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Up in my head,
Just over my tongue,
A little thing from my brain is hung,
To make it work there are factors new
That tell it when and how to pitu.

Murray Saffran

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CHAPTER 1

INTRODUCTION

1.1 General introduction

Thyrotrophin-releasing-hormone (TRH), a tripeptide, is produced by hypothalamic neurons and transported along their axons to the median eminence (ME). From there it is released at nerve terminals into hypophyseal portal blood. It is then transported to the anterior pituitary gland where it stimulates the function of the 11 thyrotrophs and lactotrophs, which synthesize and release thyroid-stimulating-hormone (TSH) and prolactin (PRL) respectively. TSH, in turn, stimulates the secretion of triiodothyronine (T3) and thyroxine (T4) from the thyroid gland. PRL is involved in a broad spectrum of biological activities. In this thesis its role in the initiation and maintanance of lactation will be described.

Damage to the hypothalamus or transsection of the pituitary stalk results in a hypothyroid status in rats. Circulating thyroid hormones exert powerful negative feedback control on the thyrotrophs, and to a lesser extent on the TRH-producing neurons of the hypothalamus (Fig.1). In addition to TRH, there is a variety of secondary modulators which play a role in controlling TSH secretion. Somatostatin and dopamine (DA) are important modulators in the inhibition of the TSH secretion, whereas α-adrenergic pathways are in general stimulatory. Other modulators of thyroid hormone secretion include glucocorticoids, various cytokines, and other inflammatory mediators. The net result of all these factors is the maintenance of a steady output of TSH and, thus, of thyroid hormones.

PRL plays a predominant role during lactogenesis. The neuronal control of PRL release involves both PRL-inhibiting and -releasing factors. The rapid increase in plasma PRL levels in the lactating rat may result from an increased hypothalamic secretion of PRL-releasing factors (PRFs), an enhanced sensitivity for PRFs, or from suppressed hypothalamic secretion of PRL-inhibiting factors (PIFs) into the portal system. TRH has been considered as one of the major PRFs in lactating rats, whereas DA is the main PIF.

Suckling-induced variations in plasma PRL are not in proportion to those in plasma TSH. However, the lack of a parallel increase of plasma TSH and PRL during suckling does not exclude a physiological role of TRH as a PRF, since separate control of PRL and TSH release by other factors might play a role under different physiological conditions.

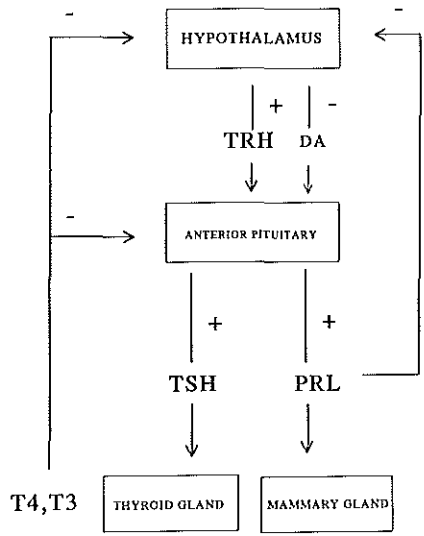

Figure 1. Schematic representation of the hypothalamic-pituitary axis leading to TSH and PRL secretion. The fundamental actions are hypothalamic stimulation of thyrotroph and lactotroph function, balanced by the powerful negative feedback inhibition exerted by thyroid hormones or PRL, respectively.

1.2. Basic aspects of TRH

1.2.1. Historical background

In "De Usu Partium" (2nd century A.D.), Galen of Pergamon was the first to describe a connection between the encephalon and the anterior pituitary gland (1). Galen described two pairs of channels for the elimination of thicker residues, the pituita, from the encephalon. According to him, one pair of channels was used under normal conditions, whereas the second pair of channels was used when there was too great a quantity of residues to be carried off by the other pair, for example when one has a cold. Vasalius supported Galen's description and illustrated the pathway along which the 'pituita' of the brain were transported on their way to the pituitary gland and from there to the nasal cavaties in more detail (2).

The belief that the pituitary is more than a conduit for pituita, was proposed by Smith et al in 1927 (3), who described a variety of disabilities caused by hypophysectomy in rats. Geoffrey Harris was the first who seriously argued that the

hypothalamus controlled the pituitary gland, if not by nervous signals, then by chemical means (4). In 1957 it has been demonstrated that the mammalian hypothalamus secretes a substance stimulating the thyrotrophic function of the pituitary (5). This observation formed the foundation for the simultaneous description of the isolation and chemical characterization of TRH, by the groups of Schally and Guillemin in 1969 (6, 7). Using immunohistochemical techniques, the presence of TRH was demonstrated mainly in the paraventricular area of the hypothalamus. These localisation studies have demonstrated that hypothalamic factors are discharged from axon terminals into a vascular network which connects the hypothalamus and the anterior pituitary. From this time on, the role of TRH as a hypothalamic hypophysiotrophic hormone releasing TSH from the anterior pituitary gland has become generally accepted (8, 9). Its role in the regulation of lactation through stimulation of PRL from the anterior pituitary is still controversial (8, 10-12).

1.2.2. TRH synthesis, processing and metabolism

Pyroglutamine-histidine-proline-amide (TRH) is synthesized following a classic peptide biosynthetic pathway, i.e. polyribosomal synthesis of a larger precursor protein which is posttranslationally processed by microsomal enzymes, and packaged into secretory granules (13). Although TRH was the first hypothalamic releasing factor to be chemically identified, the sequence of its precursor (proTRH) was the last to be elucidated (14).

The cDNA sequence of the rat TRH precursor encodes a protein of 255 amino acids, which contains five TRH progenitor sequences Gln-His-Pro-Gly flanked by pairs of basic amino acid residues Lys-Arg or Arg-Arg (Fig. 2). The remainder of the precursor consists of a signal peptide, two aminoterminal flanking sequences separated by a paired arginine sequence, four spacer peptides, and a carboxy-terminal flanking sequence.

Figure 2 also shows the processing of one TRH-progenitor sequence. First the two basic amino acid cleavage sites that are flanking the TRH progenitor sequence Gln-His-Pro-Gly are removed by a tripsin-like protease and a carboxypeptidase (15). Subsequently, Gln is cyclized to pGlu, presumably by the enzyme glutaminyl cyclase (16). Amidation of Pro by modification of Gly is the last and rate limiting step and is catalyzed by a peptidyl α-amidating monooxygenase (PAMase), requiring oxygen, copper and ascorbate as cofactors (17).

Next to TRH, processing of the prohormone produces several other proTRH-

derived neuropeptides (Fig. 2), which may in the future prove to exert important intracellular or extracellular functions (18, 19). Different processing patterns of proTRH in various brain regions and other tissues suggest tissue-specific regulating mechanisms for TRH synthesis and release (18, 20-25). Immunohistochemical studies have revealed that several intervening peptides derived from proTRH are found in terminals of the external zone of the ME (22, 26, 27), suggesting that these 'cryptic' peptides may be released in the portal blood together with TRH, and thus may reach the anterior pituitary. The biological significance of non-TRH connecting sequences is currently a matter of speculation.

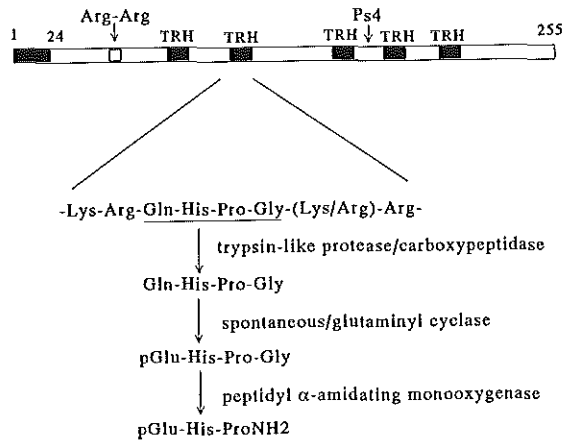

Figure 2. Schematic representation of the rat proTRH. The molecule contains 5 copies of a progenitor sequence of TRH (in black), several non-TRH sequences including two N-terminal peptides separated by an arginine-arginine residue, one C-terminal-flanking sequence, four spacer sequences and a putative signal peptide sequence (1-24). The position of proTRH-(160-169) (Ps4) is indicated. The processing of one putative proTRH moiety (underlined), flanked by paired basic amino acids, is represented.

Specific receptors for one of the cryptic peptides, proTRH-(160-169), also called spacer peptide 4 (Ps4), have been demonstrated in the pituitary gland by Valentijn et al (28). It has been demonstrated that synthetic Ps4 potentiates the TRH-induced release of TSH *in vitro* from the rat anterior pituitary (23, 29). Concurrently, Ps4 has been shown to increase the expression of TSHß subunit and PRL mRNA in primary cultures of rat pituitary cells (29, 30). However, Ps4 alone has no effect on TSH or

PRL secretion into the medium (23, 29). Therefore, Ps4 acts as a regulator of both TSH and PRL synthesis, but, unlike TRH, does not act as a secretagogue. On the other hand, further research has demonstrated that Ps4 and its receptor are widely distributed in the central nervous system (CNS) and peripheral tissues (31), suggesting that Ps4 can act as neuromodulator or neurotransmitter in the CNS. The possible biological function of the other intervening peptides still has to be revealed.

TRH is rapidly degraded in serum and tissue (32) into two main products: pGlu-His-Pro (acid TRH) and His-Pro-NH_2 (Fig.3) (33). Acid TRH results from the action of TRH-deamidating postproline cleaving enzyme and lacks biological activity. His-Pro-NH_2 is formed by hydrolysis at the pGlu-His bond by a TRH-degrading pyroglutamyl aminopeptidase (34-36). His-Pro-NH_2 is rapidly cyclized to His-Pro-diketopiperazine (DKP) which has intrinsic biological effects on PRL secretion, thermoregulation and appetite (37, 38). It has been suggested that TRH may act as a prohormone for this molecule, although recent data suggest that DKP may also arise from other pathways in several tissues (39, 40).

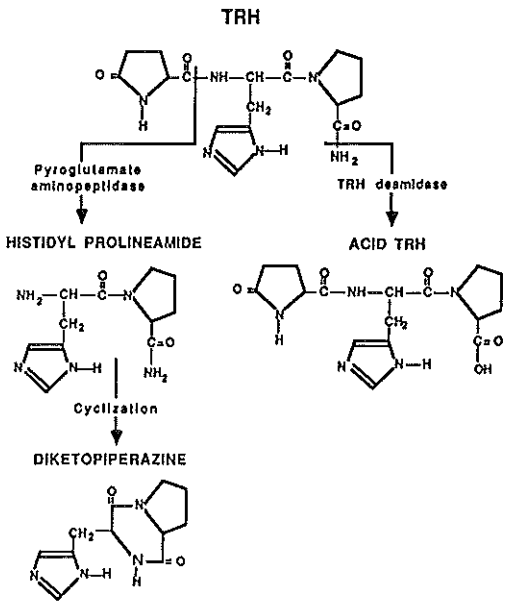

Figure 3. Metabolism of thyrotropin-releasing hormone (TRH) into two main products: pyroglutamylhystidylproline (acid TRH) and histidyl prolineamide. Most of the latter is rapidly cyclized to form histidyl proline diketopiperazine (DKP).

Due to their intracellular localization, TRH-degrading pyroglutamyl aminopeptidase and TRH-deamidating postproline enzyme cannot participate in the inactivation of extracellular TRH. This inactivation is catalyzed by a peptidase on synaptosomal and anterior pituitary membranes, which exhibits a high degree of substrate specificity, like the TRH-degrading serum enzyme (41). The ectoenzyme is localized preferentially on neuronal cells in the brain and on lactotrophic pituitary cells (42). The activity of the anterior pituitary enzyme is controlled by estradiol and thyroid hormones, whereas the activity of the brain enzyme is not (32, 42, 43). Therefore it has been postulated that this TRH-degrading ectoenzyme may serve regulatory functions by inactivating TRH after its release. In plasma the half-life of TRH is short, ranging from about 2 minutes in thyrotoxic animals to 6 minutes in hypothyroid animals. This difference reflects in part the effects of thyroid status on the serum TRH-degrading enzyme (44, 45), which suggests that this enzyme may serve a biological function as well.

1.2.3. Localization and distribution

Using immunocytochemical techniques the rat TRH precursor and TRH itself have been demonstrated to be present within the hypothalamic paraventricular nucleus (PVN) and the brainstem raphe nucleus perikarya (46, 47). A similar localization pattern for proTRH has been found by *in situ* hybridisation histochemistry, using RNA probes complementary to proTRH mRNA (96). In rats, immunoreactive TRH is found in the internal layer of the ME, the anterior horn of the spinal cord and the pituitary gland, using immunocytochemical techniques, whereas immunoreactive proTRH is not. This indicates that processing of TRH in rats occurs in the perikarya and not during axonal transport (48). In man, immunoreactive proTRH and proTRH mRNA have been demonstrated in the hypothalamus as well as in anterior pituitary tissues (49, 50). To demonstrate proTRH mRNA in human anterior pituitary reverse transcription-polymerase chain reaction has been used. Methodological differences may explain the discrepancy between proTRH gene expression in human and rat pituitary.

Immunoreactive TRH is widely distributed in the rat hypothalamus; particularly high concentrations are found in the preoptic suprachiasmatic nucleus and the periparaventricular area (46, 51-53). Nerve terminals staining for TRH in the ME of the hypothalamus are presumably derived from these cell bodies. Furthermore, networks of TRH-positive nerve fibers have been demonstrated to extend into the

posterior pituitary (46, 54-56). Lesions of the PVN reduce the level of immunoreactive TRH in the posterior pituitary glands, indicating that the hypothalamus is the probable source of TRH in this area (57). In contrast, lesions of the PVN cause an increase in TRH levels in the nucleus of the tractus solitarius, indicating that TRH fibers in this region do not arise in the hypothalamus (58).

The non-TRH peptides derived from proTRH are colocalized with TRH in cell bodies and nerve terminals in rats. In addition, they are present in brain regions where TRH is not detected (14). This suggests a differential processing of proTRH in the CNS.

In rats, TRH has been demonstrated to be present in extrahypothalamic brain areas, as well as in the spinal cord, testis, retina, gastrointestinal tract, pancreas, placenta, and other peripheral locations (55, 59-62). During the first days of postnatal life, the rat pancreas contains large amounts of TRH which are even larger than those in the hypothalamus. These levels progressively decrease over a period of a few weeks (61, 63). This finding may reflect a possible ontogenic role of TRH in pancreas development.

1.2.4. TRH receptors

The availability of a radiolabelled high affinity TRH analogue, ^{3}H-labelled [3-Me-His^{2}]TRH, has facilitated the measurement of TRH receptor binding. TRH binding sites are distributed throughout the CNS and in the anterior pituitary, but there is a wide species difference between receptor densities in various regions (64). Based on the diversity of anatomical localization and apparent physiological functions biochemical heterogeneity of these receptors was postulated. A classification of brain and anterior pituitary TRH receptors as a heterogenous group has indeed been made according to charge characteristics, differences in regulation by guanine nucleotides, and differences in amino acid sequences (65, 66).

The sequencing of a cDNA encoding mouse pituitary TRH receptor (67) revealed that this receptor is a membrane-bound protein with a seven-transmembrane-domain structure, which transduces its signal via binding to a G-protein. Zhao et al (68) isolated a full-lenght cDNA encoding a TRH receptor from GH_3 pituitary tumor cells, while Sellar et al (69) isolated a TRH receptor from a rat anterior pituitary cDNA library and successively determined its sequence and functional characteristics. In GH_3 rat anterior pituitary tumor cells two different isoforms of the TRH receptor with indistinguishable functional properties are

generated by alternative splicing (70). Also in the normal pituitary gland two TRH receptor mRNAs have been demonstrated with different C-terminal amino acid sequences (66). Both receptor forms are expressed throughout the rat pituitary gland and CNS.

Following its binding to the receptor, there are two pituitary responses to TRH: stimulation of the release of stored hormone and stimulation of gene transcription. TRH receptors can couple to the phosphoinositide or to the adenylyl cyclase pathway in GH_3 pituitary tumor cells (71-74). The adenylyl cyclase pathway leads to increased levels of cyclic AMP and the activation of protein kinase A. The phosphoinositide cascade leads to hydrolysis of phosphatidylinositol-4,5-bisphosphate which produces two intracellular second messengers - inositol 1,4,5-trisphosphate which opens calcium channels, and diacylglycerol which activates protein kinase C. The increase in intracellular free calcium is responsible for an immediate hormone release, while the activation of protein kinase C is believed to be responsible for a slower and sustained hormone secretion (74-77). It remains to be established whether the structural heterogeneity of the receptors and the different signal transmission pathways are correlated. In addition to stimulating TSH and PRL release, TRH stimulates synthesis of these hormones by promoting transcription and translation of the TSHß subunit and PRL gene (29, 78-81).

Occupancy of TRH receptors by TRH leads to a subsequent loss of TRH binding sites. This homologous down-regulation occurs in both thyrotrophs and lactotrophs (82, 83). The desensitization of the pituitary TRH receptors occurs by a decrease in number rather than a decrease in affinity of receptor sites (82). It has been suggested that the TRH-induced decrease in TRH receptor mRNA expression is not due to inhibition of mRNA synthesis alone and may present a post-transcriptional effect as well (84). For the human TRH receptor, it is believed that the carboxy-terminal domain of this seven-transmembrane-domain receptor may play an important role in receptor downregulation/internalisation (85).

The density of TRH receptors can also be modulated by other hormones, which is termed heterologous receptor regulation. Thyroid hormones exert a powerful negative control on TRH receptor binding (83, 86-89). On the other hand, experimental hypothyroidism increases TRH receptor binding and TRH receptor mRNA levels in rat pituitary (90). Estrogens increase pituitary TRH receptor levels (86, 91), which recently has been found to be regulated at the mRNA level by an increase of both the transcription rate and mRNA stability (92). Glucocorticoids also

lead to an increase of TRH receptor density on pituitary cells in culture (93). The relatively slow time course of changes in TRH receptor density suggest that glucocorticoids, like thyroid hormone and estrogen, act at the level of gene transcription.

Cellular distribution, potential functional differences, and homologous and heterologous receptor down-regulation of the different isoforms of TRH receptor are all important factors to be considered in the evaluation of the role of TRH in the regulation of the function of the anterior pituitary and CNS.

1.3. Actions of TRH

TRH exerts a number of effects at the level of the CNS and in peripheral organs. Hypothalamic TRH, transported through the portal blood to the anterior pituitary, acts at this level as a neurohormone, while extrahypothalamic TRH may act as a neuromodulator, or possibly by influencing cell-to-cell communication.

1.3.1. TRH as a neurohormone

The best defined physiological action of TRH is its role in the control of anterior pituitary TSH secretion. Various studies have demonstrated that TRH stimulates TSH secretion from the thyrotrophs and that lesions in the hypothalamus or the interruption of the hypothalamo-pituitary connection result in hypothyroidism due to impaired TSH secretion (8, 9, 94, 95). Passive immunization with anti-TRH antibodies results in decreased basal and cold-induced TSH secretion (8, 96-99)].

At the pituitary level, after binding to specific receptors on the thyrotroph membrane, TRH has a stimulatory effect in the complex system regulating TSH secretion, which also includes thyroid hormones, DA and somatostatin as inhibitory counterparts (100). In addition to stimulating TSH release, TRH also stimulates TSH synthesis by promoting transcription and translation of the TSHß subunit gene (78-81). In addition, TRH influences the glycosylation of TSH which is critical for the biological activity of TSH (101-103). In some patients with hypothalamic hypothyroidism the biological activity of secreted TSH is reduced, which is restored by prolonged TRH administration (104). In rats with hypothalamic hypothyroidism, caused by selective lesions of the PVN, carbohydrate structure of TSH is altered which may affect its bioactivity and molecular clearance rate (105).

Although first recognized because of its effects on the release of TSH, it soon

became clear that TRH also stimulates the release of PRL (see chapter 1.5) and, under particular circumstances, of some other anterior pituitary hormones (55, 106-108).

1.3.2. TRH as a neurotransmitter or neuromodulator

The extensive extrahypothalamic distribution of TRH, its localization in nerve endings, and the presence of TRH receptors in extrahypothalamic brain tissue suggest that TRH can act as a neurotransmitter or neuromodulator outside the hypothalamus. TRH has predominantly a stimulatory neuronal activity, but the mechanism by which TRH induces neural responses has still not been completely elucidated (38, 55, 109).

It has been suggested that TRH can cause changes in the synthesis and secretion of classic neurotransmitters, such as DA, noradrenaline, acetylcholine and seretonin. TRH increases the concentration of DA metabolic products, such as homovanillic acid and dihydroxyphenylacetic acid, DA release from brain slices, and activity of enzymes involved in DA synthesis, such as thyrosine hydroxylase. Furthermore, TRH increases noradrenaline and acetylcholine turnover and potentiates the effects of serotonin (38, 55, 110-113).

TRH exerts neuromodulatory effects on cardiovascular and respiratory functions in rats and cats (114-116), and is believed to act as a neurotransmitter in the human retina (62). Microinjections of TRH into the brainstem stimulate respiration, possibly by inducing the rhythmic electric activity in neurons of the nucleus tractus solitarius (117).

TRH is also involved in the physiological neural control of gastrointestinal and pancreatic functions at either the peripheral or central level (118-121). It has been reported that exogenous TRH reverses experimental hyperglycemia by stimulating insulin release through an action in the CNS. Conversely, hypothalamic TRH-containing neurons are sensitive to changes in circulating glucose and can be activated in response to such changes (122, 123).

Furthermore, TRH induces hyperthermia (124) and a beneficial psychological effect of intravenous TRH administration has been reported in depressed patients (109).

1.3.3. Paracrine action of TRH

A paracrine mechanism of action of TRH is suggested by the presence of TRH in

the ß-cells of the islets of Langerhans in neonatal rats (125). After streptozotocin (STZ) treatment, which destroys the insulin-secreting ß-cells, the content of TRH-immunoreactivity in the islets decreases markedly (126). In vitro, glucose inhibits TRH and glucagon secretion and stimulates somatostain and insulin secretion from the islets (127). Since TRH opposes the action of somatostatin, it has been postulated that TRH can play an antagonistic role in the control of islet-cell secretion (128). Exposure of cultured islets to exogenous TRH exerts a dose-dependent effect on islet secretion of insulin, somatostatin and glucagon, i.e. an inhibition of the glucose-induced insulin secretion, a blockade of the glucagon response to glucose, and a stimulation of the glucose-induced somatostatin secretion (127).

1.4. Role of TRH in the regulation of TSH secretion

To define the physiological role of TRH in the control of anterior pituitary secretion of TSH, one has to consider the effects of other modulators which interact with the hypothalamo-pituitary-thyroid axis. Some of these modulators exert their effect mainly at the level of the hypothalamus, thereby influencing TRH synthesis or release, whereas others directly affect the pituitary TSH secretion. The interactions between TRH and such secondary modulators and their separate effects at the hypothalamic and pituitary level will be discussed in this section.

1.4.1. Interactions with thyroid hormones

Thyroid hormones (T4 and T3) are involved in growth and maturation processes during fetal development. Throughout life, they play a vital role in the metabolic processes in all tissues. The thyroid gland is the only source of T4. T3, which is biologically more active than T4, is mainly produced extrathyroidally from T4. Pituitary TSH stimulates thyroid activity.

Thyroid hormones exert powerful effects on hypothalamic function, which was first demonstrated by Belchetz et al (129), who showed that injection of T3 into the hypothalamus of hypothyroid monkeys causes an acute inhibition of TSH release . Whether this effect was due to inhibition of TRH secretion or to stimulation of somatostatin or DA secretion was unknown at that time. In the succeeding years, the effect of thyroid status on hypothalamic TRH synthesis and/or release has been extensively investigated in rats. Hypothyroidism has been shown to increase TRH

mRNA in the PVN, whereas hypothyroidism suppresses the expression of TRH mRNA (130, 131). In line with these findings, hypophysectomy stimulates hypothalamic TRH synthesis and release in rats; this effect can be reversed by administration of thyroid hormones (132, 133). Deyss and Yamada showed that TRH concentrations and proTRH gene expression in the medial parvocellular division of the PVN and posterior hypothalamic nucleus, but not in any other group of TRH neurons in the brain, are specifically regulated by T3 (134, 135).

To determine whether thyroid hormone exerts these effects directly on TRH-producing neurons in the PVN, the presence of thyroid hormone receptors in these neurons has been investigated. Several nuclear thyroid hormone receptors have been identified, i.e. α and ß isoforms, that are derived from two separate genes (136, 137). Double labeling immunocytochemistry techniques revealed marked differences in staining intensity in the PVN and other regions of the brain for the specific thyroid hormone receptor isoforms (138). The distribition of thyroid hormone receptor isoforms in these regions demonstrates a selectivity in thyroid hormone sites of action. In addition, Wang et al demonstrated two binding sites for the thyroid hormone receptor on the proTRH gene (139). These results indicate that the PVN of the hypothalamus is indeed a target for thyroid hormones and that the hormones' action in this nucleus likely contributes to the regulation of TRH secretion.

In addition to their inhibitory action at the hypothalamic level, thyroid hormones exert strong effects on TSH secretion at the pituitary level. The sensitivity of pituitary thyrotrophs to circulating thyroid hormone levels has been well documented by the finding of blunted serum TSH responses to TRH following variations of thyroid hormone concentrations within the normal range through administration of minute doses of T3 or T4 (140, 141). Administration of T3 or T4 in doses sufficient to increase serum thyroid hormone concentrations above the normal range completely suppress the TSH response to TRH. Refractoriness of the thyrotrophs to TRH persists for a certain time after the withdrawal of TSH-suppressive doses of thyroid hormones (142).

Thyroid hormones exert their action at the pituitary through a direct inhibitory effect on TSHß subunit gene expression and TSH release, but they also modulate the number of TRH receptors on the thyrotrophs. In hypothyroid animals TRH binding to anterior pituitary membranes is doubled; these increased levels can be reduced by thyroid hormone replacement (86, 143). TRH itself causes a dose-

related reduction in pituitary T3 receptors and T3 responsiveness (144), yielding a further site of interaction between T3 and TRH at the pituitary level.

1.4.2. Interactions with glucocorticoids

Stress activates the hypothalamo-pituitary-adrenal axis, whereas the hypothalamo-pituitary-thyroid axis often is suppressed. As a response to stress, glucocorticoids are produced in the zona fasciculata and zona reticularis of the adrenal cortex. Glucocorticoids modulate the functions of various physiological regulatory systems. They appear to be involved in the regulation of thyroid function through interaction at the level of both the hypothalamus and pituitary.

In the studies of Brabant et al (145) and Rubello et al (146) on the effects of cortisol on TSH secretion, the unaffected TRH-stimulated TSH response suggested inhibition of TRH release by glucocorticoids. In rats, high-dose glucocorticoid treatment is followed by a reduction of proTRH mRNA in the PVN. The coexistence of glucocorticoid receptors in TRH neurons in the PVN (147) and the presence of a glucocorticoid response element in the TRH gene (148) add further credibility to this hypothesis.

Effects of glucocorticoids on pituitary TSH secretion have been described extensively (145, 146, 149-151). Brabant et al (145) demonstrated a rapid abolition of TSH pulses and a suppression of basal TSH secretion after an intravenous injection of 4 mg dexamethasone in euthyroid men. Samuels et al (151) studied the effects of 100 or 300 mg cortisol infusions over 24 h on the pulsatile secretion of pituitary glycoprotein hormones in healthy subjects. Both infusions had profound effects on plasma TSH levels. TSH pulse amplitude was decreased, the nocturnal TSH surge was abolished, while the TSH pulse frequency was unaltered. However, in a study of Rubello et al (146) infusion of a larger dose of cortisol (500 mg over 1 h), had no effect on both basal and TRH-stimulated TSH secretion. From the latter study it was concluded that only prolonged hypercortisolism interferes with pituitary TSH secretion. The underlying mechanism for acute or prolonged inhibitory effects of hypercortisolism at the pituitary level is still a matter of debate. Kokonen et al (150) have demonstrated a colocalization of glucocorticoid receptors and TSH in the anterior pituitary of rats. In contrast, only a minority of the PRL-immunoreactive cells expressed the glucocorticoid receptor. Glucocorticoids may therefore differentially regulate the secretion and/or synthesis of TSH and PRL by directly affecting the hormone producing cells of the anterior pituitary.

1.4.3. Interactions with somatostatin

Somatostatin is the major physiologic hypothalamic inhibitor of pituitary growth hormone release (152, 153). In addition, somatostatin exerts an inhibitory control on TSH secretion in both experimental animals and humans (100, 154). Many workers have tried to elucidate the potential role of somatostatin as a pituitary TSH inhibiting factor.

The periventricular and the medial parvocellular subdivisions of the rat PVN are innervated by immunoreactive somatostatin fibers originating from both PVN neurons as well as more rostal neurons in the anterior periventricular nucleus (155, 156). The dense plexus of immunoreactive somatostatin fibers in the ME originates from these two subdivisions of the hypothalamus (157-160). Immunoreactive somatostatin containing axon terminals innervate TRH synthesizing neurons in the periventricular area of the rat PVN (161, 162). In addition, immunoreactive somatostatin fibers have been found in close proximity to TRH axons in the external layer of the rat ME (48). Collectively, these observations provide anatomical basis for a neuroendocrine regulation of TRH hypophysiotrophic neurons by somatostatin.

The effect of somatostatin on TRH-producing neurons is likely to be inhibitory, since in rat hypothalamic fragments, somatostatin inhibits TRH secretion (163). Although acute cold exposure caused rapid but opposite changes of hypothalamic levels of somatostatin mRNA and TRH mRNA (164), this study could not provide evidence for a regulatory effect of somatostatin on TRH synthesis or release. Thyroid hormone has been found to stimulate somatostatin synthesis and release from rat hypothalamic fragments (165), while hypothyroidism decreases hypothalamic somatostatin content (166). It was therefore suggested that somatostatin was involved in the negative feedback effect of thyroid hormone on TRH release (167). In other studies, however, the regulation of synthesis and/or release of somatostatin by thyroid hormone could not be confirmed (168-170).

In cultured rat anterior pituitary cells (171) somatostatin inhibits basal and TRH-stimulated TSH release, an effect enhanced in the presence of low thyroid hormone levels (172). These findings led to the proposal that TSH release is regulated by the hypothalamus through a dual control system, i.e. stimulation by TRH, and inhibition by somatostatin. In studies using somatostatin antiserum in rats, increased serum TSH levels and increased serum TSH responses to both cold stress and TRH confirmed the inhibitory control of somatostatin on TSH secretion (173).

1.4.4. Interactions with tumor necrosis factor and cytokines

Host responses to infection, inflammation and injury, are characterized by changes in the immune, nervous, and endocrine systems (174, 175). Interaction between these systems is highly complex. Hormones, neurotransmitters, and neuropeptides are capable of affecting immune processes. Conversely, immune cell products such as cytokines can affect endocrine, autonomic, and central mechanisms (176, 177). Inflammation stimulates the production of a cascade of cytokines, of which, in particular, tumor necrosis factor (TNF), interleukin-1 (IL-1), and interleukin-6 (IL-6) represent key factors for communication between the immune and the endocrine systems (178-180).

In addition to its role in the coordination of host defense mechanisms, IL-1 activates the hypothalamo-pituitary-adrenal axis in rats and mice, characterized by high levels of ACTH and corticosterone in plasma (181-185). It has been suggested that during acute and chronic systemic illness, the suppression of the hypothalamo-pituitary-thyroid function is mediated by cytokines as well (186-190). The site of action of interleukins has, however, not been fully identified.

After 3 daily *iv* injections of 50, 200, and 800 µg TNF/kg BW, TRH content in the rat hypothalamus is reduced while the pituitary TSH response to TRH is preserved (189). Therefore, the authors suggested that the primary site of action of TNF is the hypothalamus. Kakucska et al (191) showed by *in situ* hybridization a reduction of proTRH mRNA in the PVN 24 h after a constant intracerebroventricular infusion of IL-1. High concentrations of IL-1 receptors mRNA are present in the hippocampus and midbrain raphe, which is thought to be an important region in the feedback regulation of the hypothalamo-pituitary-adrenal axis by affecting CRF production (192). As CRF neurons lie adjacent to TRH neurons in the PVN (193), and show increased CRF gene expression following intracerebroventricular IL-1 infusion (194), it has been suggested that CRF may mediate the effects of cytokines on TRH neurons.

In pituitary cells TNF does not affect basal TSH secretion, but it reduces TRH-stimulated TSH secretion (195). Direct action of IL-1 on pituitary cells in monolayer culture has been measured by Beach et al (196, 197) who demonstrated an increased TSH release. IL-6, the production of which is induced by IL-1 in anterior pituitary cells (198, 199), failed to cause any change in the secretion of TSH from pituitary cells (200).

In many studies the involvement of cytokines in the regulation of the pituitary

hormone secretion during systemic illness has been described, but the predominant route of modulation needs further investigation.

1.4.5. Interactions with catecholamines

An extensive network of neurons terminates on the cell bodies of the hypophysiotrophic neurons in the PVN and within the interstitial spaces of the ME, where they regulate the release of hypophysiotropic peptides into hypophyseal portal blood. In this way, the hypophysiotrophic neuronal systems that regulate TSH secretion (TRH, somatostatin, and dopamine) are influenced by networks of other neurons that project from several brain regions (201). The catecholaminergic pathway that arises from groups of nuclei located in the midbrain and projects to the hypothalamus, plays a substantial role in the regulation of the TSH secretion at the level of both the pituitary and hypothalamus. Noradrenergic and dopaminergic control on hypothalamic TRH and pituitary TSH release will be discussed in the next section.

Noradrenergic effects on the hypothalamo-pituitary-thyroid axis.

The network of noradrenergic terminals present in the hypothalamus and the preoptic area derives mostly from fibers originating from noradrenegic cell bodies in the pons and the medulla oblongata (202). The effect of noradrenaline on TRH release in the PVN seems to be stimulatory, since *in vitro* studies demonstrated that noradrenaline stimulates TRH release from hypothalamic preparations (163, 203). In addition, noradrenaline may also stimulate TRH secretion from the ME, as noradrenergic axon terminals were found adjacent to TRH axons in the external layer of the ME (204). It has been shown that noradrenaline releases TRH from both isolated ME fragments *in vitro* and push-pull cannulated ME *in vivo* (205). The effect of noradrenaline on TRH neurons may be modulated by peripheral hormonal influences, such as thyroid hormones. In hypothyroid rats increased levels of noradrenaline, in turn, stimulate TRH release. Thyroid hormone replacement can reverse this effect (206). It has been postulated that TSH may also be involved in the regulation of noradrenergic-stimulated TRH release during hypothyroidism, through a short feedback mechanism by suppressing stimulatory noradrenergic influences on TRH release to counterbalance hypersecretion of TSH (207). These observations require confirmation.

Noradrenaline and dopamine are present in rat hypophyseal portal blood at

higher concentrations than in peripheral blood and at a level that could exert physiologic action on thyrotrophs (208, 209). In rat and bovine anterior pituitary cells noradrenaline stimulates TSH release (210-212). Quantitatively, the adrenergic stimulation of TSH release is almost equivalent to that induced by TRH (210). These two agents, at maximal doses, produce additive effects on TSH release, suggesting activation of separate intracellular pathways.

Dopaminergic effects on the hypothalamo-pituitary-thyroid axis.

DA cell bodies from the nucleus arcuatus project to the external layer of the ME, where they could influence TRH secretion by contacts with TRH axons (204). It has been suggested that DA exerts direct inhibitory control on TRH secretion in rats, likely acting on TRH axons in the ME (213). However, these data are indirect and need confirmation. DA might also inhibit TRH release indirectly by stimulating hypothalamic somatostatin secretion (214-216), which in turn is inhibitory to TRH release (163). Studies on the direct effects of DA on TRH and somatostatin release using hypothalamic fragments revealed stimulatory response of both hormones (203, 216). The net result of DA control at the hypothalamic level may be mediated by modulation of both TRH and somatostatin release.

Thyroid hormones have been found to influence DA control on TRH release by directly acting on the TRH axons in the ME (206). This effect could be reversed by thyroid hormone replacement. In addition, TSH may increase inhibitory dopaminergic influences in TRH release during hypothyroidism, to counterbalance its own hypersecretion (207).

Dopamine is released directly into hypophyseal portal blood and exerts direct actions on anterior pituitary cells, particularly as the major inhibitor of PRL release, and to a lesser extent as an inhibitor of TSH release (217). In anterior pituitary cells in culture, the inhibition of TSH and PRL secretion by dopamine and dopamine-agonistic drugs is mediated via a DA receptor present upon lactotrophs and thyrotrophs, with similar characteristics on the two cell types (218). In addition to the acute inhibitory effect on TSH and PRL secretion *in vitro*, DA decreases the levels of α subunit and TSHβ subunit mRNAs in cultured pituitary cells from hypothyroid rats (81). Dopaminergic control of PRL mRNA in male pituitary has been described as well (219).

In conclusion, the existence of stimulatory noradrenergic and inhibitory dopaminergic pathways in the control of TSH secretion in rats have been

demonstrated at the hypothalamic and pituitary level.

1.5. Role of TRH in the regulation of PRL secretion during lactation

PRL is secreted by the anterior pituitary and is involved in a broad spectrum of biological actions, including nurturing of the young, control of growth and development, metabolic effects, reproductive actions, regulation of water and electrolyte balance, effects on integumentary (ectodermal) structures, and interactions with steroid hormones (220, 221). Thus, unlike most other anterior pituitary hormones, PRL does not regulate a single but a variety of functions (222).

PRL plays a predominant role in the initiation and maintenance of lactation. Suckling is the most powerful natural stimulus for PRL release in mammals, and the quantity of PRL released depends upon the intensity and duration of suckling and the time intervals between suckling episodes (223-226). In addition to the release of PRL, suckling also triggers that of oxytocin from the posterior pituitary. PRL regulates milk secretion by influencing synthesis of milk constituents and their transport from the alveolar cells into the lumen. Oxytocin regulates milk removal by causing contraction of myoepithelial cells, leading to increased intramammary pressure and milk ejection (227).

The suckling stimulus induces a classic neuroendocrine reflex. The input to the hypothalamus is neuronal (nipple initiated sensory nerve impulses that ascend in the spinal cord and pass through the midbrain into the hypothalamus), while the output from the hypothalamo-hypophysial complex is hormonal. The neuronal control of PRL release has become a central issue of PRL research, and involves both PRL-inhibiting and -releasing factors (10, 228-231).

The predominant effect of the hypothalamus on PRL secretion is that of tonic suppression by DA. Disruptions of the connections between the hypothalamus and the pituitary gland by hypothalamic lesions, pituitary stalk section or pituitary transplantation all induce hyperprolactinemia and increased PRL storage (232-235). PRL inhibits its own secretion via a short-loop feedback, by directly increasing synthesis and release of DA (236). Under resting conditions PRL release is tonically inhibited by DA. The rapid increase in plasma PRL levels in the lactating rat may result from a stimulated hypothalamic secretion of PRL-releasing factors (PRFs), an enhanced sensitivity for PRFs, or from suppressed hypothalamic secretion of PRL-inhibiting factors (PIFs) into the portal system.

Electrical stimulation of the mammary nerve to simulate suckling evokes a marked increase in plasma PRL and a transient fall in the levels of DA in portal blood (237-239). In push-pull perfusates of the ME area, suckling induces only a brief decrease in DA concentrations in conscious rats (12). DA levels in portal blood soon return to normal values despite the sustained suckling-induced PRL release, which suggests that other stimulating factors are involved in the control of PRL release (240-242).

PRF activity of several substances has been postulated, but only TRH is known to act directly on the anterior pituitary during suckling (237, 243-251). Moreover, specific membrane receptors for TRH have been characterized on lactotrophs (252, 253). Mammary nerve stimulation in anaesthetized rats increases TRH concentrations in portal blood (237), but TRH concentrations in push-pull perfusates of the ME in conscious rats decrease rather than increase during suckling (12). Although the PRL response to TRH has been found to increase during suckling (254), intravenous injection of TRH in rats does not always stimulate PRL secretion (244, 255-258). After short-term suckling TRH release has been found to increase (256, 259), while hypothalamic proTRH mRNA decreased from day 1 to day 5 in lactating rats (260). Acute suckling, after an 8 h separation of mothers and pups, has been found to increase hypothalamic proTRH mRNA briefly (261). From these data it is clear that the PRL-releasing action of TRH during suckling is still controversal, and needs further investigation.

The PVN plays a pivotal role in coordinating events associated with suckling. This has been confirmed in studies in which bilateral lesions of this area decrease suckling-induced PRL release and abolish the high amplitude, episodic pattern of PRL release in continuously lactating rats (262-265). Such lesions also induce hypothyroidism by reducing TRH content in the ME (266) and portal blood (262). Substitution with T4 completely restores PRL levels during suckling (264). Hypothalamic DA release increases in hypothyroid and in PVN-lesioned rats (262, 267, 268). This indicates that the decline in PRL secretion in PVN-lesioned lactating rats may be secondary to the increased hypothalamic secretion of DA, as well as from the decreased hypothalamic TRH release.

Several studies have shown that in lactating rats, a transient decrease in DA is necessary for effective PRL release in response to exogenous TRH. Without this reduction in dopaminergic tone, TRH does not provoke a substantial rise in PRL (237-239, 269). The sensitivity of the pituitary to TRH increases during the transient

suckling-induced DA decline. Rondeel et al (12) has estimated hypothalamic release of both TRH and DA by push-pull perfusion of the median eminence-arcuate nucleus area in conscious rats during suckling. DA secretion was transiently depressed within 15-30 minutes after the onset of suckling with a rapid return to baseline levels, which is in agreement with previous studies in anaesthetized rats during mammary nerve stimulation (237, 238, 270). However, during a 60 min suckling period TRH release did not change (12). The expected rise in TRH may not have been found in this study, due to methodological limitations of push-pull perfusion. Since the TRH concentrations in push-pull perfusates are low and vary considerably between individuals (12, 271, 272), the measurement of TRH in push-pull perfusates on the median eminence-arcuate nucleus area has been challenged (273).

The role of TRH as a PRF in lactating rats has been challenged by some investigators, because of the lack of a concomitant rise in TSH during suckling (244, 255, 258). Although the threshold dose of exogenous TRH to release PRL or TSH is the same for both hormones (274), the administration of TRH antisera causes an unequivocal decrease in levels of plasma TSH, whereas this procedure does not consistently affect the levels of plasma PRL (8, 97-99). Suckling-induced variations in plasma TSH are not in proportion to those in plasma PRL. A suckling-induced sensitization of pituitary tissue to PRFs has been demonstrated by a decrease in number of cells susceptible to inhibition by DA and an increase in those responsive to PRFs (275). Moreover, it is likely that the sensitivity of the lactotrophs and thyrotrophs to hypothalamic factors may be modulated differently by central and peripheral factors. Indeed, it has been shown that oxytocin, which is also released during suckling, blunts the TSH response to TRH in vitro (276). PRL and TSH are also differently correlated under conditions such as stress (244, 277-279) and primary hypothyroidism (280-283). Therefore, the lack of a parallel increase of plasma TSH and PRL during suckling does not exclude a physiological role of TRH as PRF. Its exact role in mediating the suckling-induced PRL release needs further elucidation.

1.6. Scope of thesis

TRH exerts control over thyroid function and lactation through the stimulation of TSH and PRL secretion from the anterior pituitary thyrotrophs and lactotrophs,

respectively. Synthesis and processing of proTRH, transport of TRH to the ME, its release into the portal blood, its binding at specific TRH receptors in the anterior pituitary and its degradation in serum and tissue, are all aspects that influence the effect of TRH on TSH and/or PRL release. In this thesis, many of these aspects will be described under different physiological conditions, in order to get further insight into the role of TRH in the control of pituitary TSH and PRL secretion.

Firstly, the role of TRH in the regulation of TSH secretion under four thyroid function-suppressing conditions - starvation, long-term food reduction, interleukin-induced systemic illness and streptozotocin (STZ)-induced diabetes mellitus - has been investigated. Starvation and food reduction cause a suppression of the metabolic rate, which is associated with low plasma levels of T3, and is therefore known as the low T3 syndrome. Interleukin-induced systemic illness and STZ-induced diabetes mellitus are examples of non-thyroidal illness, also resulting in a low T3 syndrome. With respect to the low plasma T3 levels, these four (patho-) physiological conditions are characterized by inappropriately normal or low levels of TSH. We postulated a common central mechanism to explain the generation of the low T3 syndrome and therefore investigated the role of TRH in the regulation of TSH secretion.

Secondly, the role of TRH in the secretion of PRL has been investigated during lactation. Lactation is the most powerful natural stimulus for PRL release. Although exogenous TRH has been shown to be a prominent PRL-releasing factor, the function of TRH as a PRF during suckling has been questioned, because of the lack of a concomitant rise in TSH secretion during suckling. In order to investigate the physiological role of TRH as a PRF, this thesis describes the effects of litter size throughout lactation and the effects of acute suckling after a period of separation of mothers and pups on the various aspects involved in TRH synthesis and release.

Next to TRH, various other modulators are involved in the synthesis and release of pituitary TSH and PRL. In order to analyse the contribution of glucocorticoids as such a modulator, changes in levels of this parameter have been extensively investigated under all conditions mentioned above.

Summarizing, the aim of the thesis is to define the role of TRH in the regulation of the TSH and PRL secretion under several (patho-) physiological conditions in more detail.

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CHAPTER 2

THE ROLE OF TRH IN THE REGULATION OF TSH SECRETION

STARVATION-INDUCED CHANGES IN THE HYPOTHALAMIC CONTENT OF PROTHYROTROPHIN-RELEASING HORMONE (proTRH) mRNA AND THE HYPOTHALAMIC RELEASE OF proTRH-DERIVED PEPTIDES: ROLE OF THE ADRENAL GLAND

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Starvation-induced changes in the hypothalamic content of prothyrotrophin-releasing hormone (proTRH) mRNA and the hypothalamic release of proTRH-derived peptides: role of the adrenal gland

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Abstract

The purpose of this study was to investigate the mechanisms involved in the reduced thyroid function in starved, young female rats. Food deprivation for 3 days reduced the hypothalamic content of prothyrotrophin-releasing hormone (proTRH) mRNA, the amount of proTRH-derived peptides (TRH and $proTRH_{160-169}$) in the paraventricular nucleus, the release of proTRH-derived peptides into hypophysial portal blood and the pituitary levels of TSHβ mRNA. Plasma TSH was either not affected or slightly reduced by starvation, but food deprivation induced marked increases in plasma corticosterone and decreases in plasma thyroid hormones. Refeeding after starvation normalized these parameters. Since the molar ratio of TRH and $proTRH_{160-169}$ in hypophysial portal blood was not affected by food deprivation, it seems unlikely that proTRH processing is altered by starvation. The median eminence content of pGlu-His-Pro-Gly (TRH-Gly, a presumed immediate precursor of TRH), $proTRH_{160-169}$ or TRH were not affected by food deprivation. Since median eminence TRH-Gly levels were very low compared with other proTRH-derived peptides it is unlikely that α-amidation is a rate-limiting step in hypothalamic TRH synthesis.

Possible negative effects of the increased corticosterone levels during starvation on proTRH and TSH synthesis were studied in adrenalectomized rats which were treated with corticosterone in their drinking water (0·2 mg/ml). In this way, the starvation-induced increase in plasma corticosterone could be prevented. Although plasma levels of thyroid hormones remained reduced, food deprivation no longer had negative effects on hypothalamic proTRH mRNA, pituitary TSHβ mRNA and plasma TSH in starved adrenalectomized rats. Thus, high levels of corticosteroids seem to exert negative effects on the synthesis and release of proTRH and TSH. This conclusion is corroborated by the observation that TRH release into hypophysial portal blood became reduced after administration of the synthetic glucocorticosteroid dexamethasone.

On the basis of these results, it is suggested that the reduced thyroid function during starvation is due to a reduced synthesis and release of TRH and TSH. Furthermore, the reduced TRH and TSH synthesis during food deprivation are probably caused by the starvation-induced enhanced adrenal secretion of corticosterone.

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Introduction

In the rat, thyrotrophin (TRH) is synthesized in the paraventricular nucleus as proTRH, a 255 amino acid precursor with five TRH progenitor sequences (Jackson *et al.* 1990), which is sequentially processed to yield TRH (pGlu-His-Pro-NH_2) and peptides which connect the TRH progenitor sequences (Lechan *et al.* 1986, Bulant *et al.* 1988). While proTRH-derived peptides are probably released into hypophysial portal blood (Bruhn *et al.* 1991, Valentijn *et al.* 1991), this has only been shown for TRH (de Greef & Visser 1981). Hypothalamic proTRH mRNA content and TRH release are influenced by thyroid hormones (Koller *et al.* 1987, Segerson *et al.* 1987, Rondeel *et al.* 1988, 1992*b*, Liao *et al.* 1989, Bruhn *et al.* 1991), low temperatures (Zoeller *et al.* 1990, Rondeel *et al.* 1991) and starvation (Blake *et al.* 1991, Chua *et al.* 1991, Rondeel *et al.* 1992*c*). Thus, hypothalamic TRH seems

to play a physiological role in the control of thyroid function.

During starvation, a condition with diminished thyroid function, hypothalamic TRH synthesis and release are decreased (Blake *et al.* 1991, Rondeel *et al.* 1992*c*, Shi *et al.* 1993) despite the fact that a reduced thyroid function has been found to increase TRH synthesis and release (Koller *et al.* 1987, Segerson *et al.* 1987, Rondeel *et al.* 1988, 1992*b*). Apparently, the feedback action of thyroid hormones at the hypothalamic level is disturbed during starvation. The mechanisms involved in the loss of feedback action of thyroid hormones during starvation are unknown but since corticosterone levels are increased in food-restricted and food deprived rats (Woodward *et al.* 1991, Garcia-Belenguer *et al.* 1993, Mitev *et al.* 1993) and corticosteroids have been found to decrease hypothalamic proTRH mRNA levels (Kakucska & Lechan 1991), it is possible that the enhanced adrenal secretion of corticosterone is part of this mechanism. An alternative hypothesis is that starvation affects post-translational processing of proTRH leading to an altered hypothalamic secretion of proTRH-derived connecting peptides. While the functions of these proTRH-derived peptides are not known, one of these peptides, $proTRH_{160-169}$, potentiates the TRH-induced release of thyroid stimulating hormone (TSH) (Bulant *et al.* 1990) and increases the pituitary content of TSHβ and prolactin mRNA in a dose-dependent manner (Carr *et al.* 1992). It was therefore decided to undertake the present studies to answer the following questions: (1) are the starvation-induced changes of proTRH synthesis and release reversed by refeeding; (2) is post-translational processing of proTRH affected by starvation; and (3) is corticosterone an important factor in the starvation-induced decrease in thyroid function?

Materials and Methods

Animals

Rats of a locally bred Wistar substrain (R-Amsterdam) were used, and for all experiments approval was obtained from the Animal Welfare Committee (DEC) of the Erasmus University. Since, in a previous study, it appeared that the effects of starvation on thyroid function were most apparent in young female rats (Rondeel *et al.* 1992*c*), 2-month-old female rats were used. They were housed three to four rats per cage in a temperature-regulated room (22 ± 2 °C), with a 14-h light:10-h darkness cycle (lights on 0500–1900 h) and had free access to drinking water. Their body weights were monitored daily during the experiments. In two experiments the rats were anaesthetized with urethane (ethyl carbamate; Brocades-ACF, Maarssen, The Netherlands; 1·2 g/kg, given i.p. as a 20% (w/v) solution in saline) to collect peripheral or hypophysial portal blood. Since urethane lowers the body temperature, which may affect hormone release, the urethane-anaesthetized rats were placed during blood collection on a heating pad maintained at 37 °C and their bodies were covered with a blanket. In one experiment, the rats were sham-operated or adrenalectomized 1 week before starting the experiment. Adrenalectomized rats received drinking water containing corticosterone (0·2 mg/ml (w/v); Sigma, St Louis, MO, USA) and 0·9% (w/v) NaCl, whereas sham-operated rats were given similar drinking water without corticosterone. Corticosterone was dissolved in ethanol, and this solution was added to water yielding a final concentration of 4% (v/v) ethanol. Since the rats drink between 15 and 25 ml water/day, they received about 3–5 mg corticosterone daily. Because water consumption follows a circadian rhythm, this way of administering corticosterone ensures diurnal corticosterone levels in adrenalectomized rats. In a pilot experiment, adrenalectomized rats substituted with corticosterone in the drinking water were found to have similar plasma levels of adrenocorticotrophin (119 ± 27 ng/l, n=8) as sham-operated rats (101 ± 15 ng/l, n=8). Moreover, thyroid function was identical in corticosterone-treated adrenalectomized (0·95 ± 0·10 μg TSH/l, 59·3 ± 1·7 nmol thyroxine (T_4)/l, 1·02 ± 0·02 nmol tri-iodothyronine (T_3)/l) and sham-operated (0·92 ± 0·14 μg TSH/l, 54·6 ± 4·2 nmol T_4/l, 0·95 ± 0·06 nmol T_3/l) rats.

Experimental design

In the first experiment, the hypothalamic levels of proTRH mRNA and proTRH-derived peptides, the pituitary content of TSHβ mRNA and the plasma concentrations of TSH, thyroid hormones and corticosterone were measured in female rats after food deprivation and after subsequent refeeding. The rats were randomly divided into three groups of 30 animals each. Control rats had free access to food, whereas other rats were starved for 3 days, or refed for 2 days after a 3-day starvation period. The mean body weights (±S.E.M.) of the three groups were, at the beginning of the experiment, 156·4 ± 2·9, 152·9 ± 3·2 and 151·2 ± 4·4 g respectively. The rats were decapitated between 1000 and 1200 h, and trunk blood was collected to determine plasma hormone levels. The skull was opened and the brain removed, and the hypophysial stalk was grasped with forceps and lifted from the brain. The protruding tissue fragment, comprising hypophysial stalk and median eminence but referred to as median eminence, was cut from the brain and placed in 2 ml methanol for later estimation of proTRH-derived peptides. Then, from 23 rats of each group the rest of the hypothalamus was isolated (limits: posterior border of the chiasma opticum, anterior border of the mamillary bodies, and lateral hypothalamic border; height about 3 mm), snap frozen in liquid nitrogen, and kept at −80 °C until determination of proTRH mRNA. From seven rats of each group, a 2 mm coronal slice of the brain between the

chiasma opticum and the origin of the hypophysial stalk was cut with a razor blade. Then, the area around the third ventricle, containing the entire paraventricular nucleus, was removed from this slice of brain tissue and placed in 2 ml methanol for later determination of proTRH-derived peptides; this piece of tissue is referred to as paraventricular area. From five rats of each group, the anterior pituitary gland was isolated and snap frozen in liquid nitrogen, and kept at −80 °C until determination of TSHβ mRNA. Tissues collected into methanol were homogenized with a glass grinder, and subsequently dried under a stream of nitrogen at 40 °C. Residues were dissolved in phosphate buffer (pH 7·4), and stored at −20 °C until assayed for proTRH-derived peptides (TRH, $proTRH_{160\text{-}169}$ and TRH-Gly).

In the second experiment, proTRH-derived peptides were measured in hypophysial portal blood of young female rats (body weight at the start of the experiment: 142·8 ± 2·7 g). Normally fed rats (n=11), rats starved for 3 days (n=11), and rats starved for 3 days and then refed for 2 days (n=6) were anaesthetized with urethane (1·2 g/kg). A cannula (0·96 mm outer diameter, 0·58 mm inner diameter) was inserted into the right femoral artery, and the hypophysial stalk was exposed (Porter & Smith 1967, de Greef & Visser 1981). Then, 500 IU heparin (Organon, Oss, The Netherlands) were given via the arterial cannula and, after 5 min, a peripheral blood sample was taken from this cannula to evaluate thyroid function. After cutting the hypophysial stalk, hypophysial portal blood was collected for 60 min into methanol to prevent degradation of TRH (de Greef & Visser 1981). Methanolic extracts of hypophysial portal blood samples were processed as above, dissolved in 1 ml phosphate buffer (pH 7·4) and stored at −20 °C until analysis of TRH and $proTRH_{160\text{-}169}$. Residues of these samples were dried and weighed to estimate the volume of blood collected (de Greef & Visser 1981).

In the third experiment, the effects of a 2-day or a 3-day starvation period on thyroid function in sham-operated or corticosterone-substituted adrenalectomized female rats were studied (body weight at the start of the experiment: 158·7 ± 4·6 g). Normally fed sham-operated rats were used as controls. The rats were decapitated between 1000 and 1200 h, and trunk blood was collected to measure plasma hormone levels. The median eminence was isolated and placed in 2 ml methanol. The anterior pituitary gland and the remainder of the hypothalamus were collected and stored at −80 °C until isolation of RNA.

In the fourth experiment, the effects of the synthetic glucocorticoid dexamethasone or the vehicle (saline) on the release of TRH and TSH were studied. Rats were anaesthetized with urethane and implanted with an indwelling cannula into the right femoral artery as described above. Dexamethasone sodium phosphate solution (Merck, Sharp & Dohme, Haarlem, The Netherlands; 2 mg/kg s.c.) or saline was injected 2 h later, and blood samples of about 0·5 ml were taken from the arterial cannula just before, and at 30, 60 and 90 min after the injection to estimate the plasma levels of TSH. From another group of urethane-anaesthetized rats, TRH was determined in hypophysial portal blood. Hypophysial portal blood was sampled for four consecutive periods of 30 min, and dexamethasone or saline was given after the first 30-min period. Methanolic blood samples were processed as above, dissolved in 1 ml phosphate buffer (pH 7·4), and stored at −20 °C until assayed for TRH.

Determination of proTRH mRNA and TSHβ mRNA

Hypothalamic proTRH mRNA and pituitary TSHβ mRNA were determined by Northern blotting (Sambrook *et al.* 1989). Total RNA was isolated by acid guanidinium thiocyanate–phenol–chloroform extraction (Chomczynski & Sacchi 1987), and the amount and purity of the isolated RNA was determined by absorbance at 260/280 nm. From each sample, 10 μg RNA was subjected to denaturing agarose gel electrophoresis and blotted onto Hybond N^+ filter (Amersham International, Amersham, Bucks, UK). For measurement of proTRH mRNA, the filters were hybridized at 42 °C with a ^{32}P-labelled 1322 bp EcoR1-Pst1 fragment of rat pro-TRH cDNA (Lechan *et al.* 1986, Lee *et al.* 1988), whereas for estimation of TSHβ mRNA the filters were hybridized at 42 °C with a ^{32}P-labelled rat 420 bp fragment of TSHβ cDNA (Chin *et al.* 1985, van Haasteren *et al.* 1994). After hybridization, the filters were washed and autoradiographed (Sambrook *et al.* 1989). Variation in loading was accounted for by normalizing to the β-actin mRNA content in each lane, which was measured by hybridization at 42 °C with a ^{32}P-labelled hamster actin cDNA probe (Dodemont *et al.* 1982). Autoradiographs were quantified densitometrically with a model 620 video densitometer using 2D Analyst II software (Bio-Rad Laboratories, Richmond, CA, USA). Then, the ratios between the integrated optical densities of proTRH and β-actin mRNA, or TSHβ and β-actin mRNA were calculated. Since the results may differ between various Northern blots, the variation between individual Northern blots was accounted for by the inclusion of at least three control samples on each gel. Furthermore, if sufficient RNA had been isolated from a sample, the sample was assayed twice using different gels. Results in this paper are expressed as the percentage of the mean of the control rats.

Since the proTRH mRNA content in the hypothalamus is increased in hypothyroid rats, and particularly in the paraventricular nucleus (Koller *et al.* 1987, Segerson *et al.* 1987), this experimental paradigm was used to validate our methods for the isolation and measurement of hypothalamic proTRH mRNA. Hypothalamic proTRH mRNA content was determined in six control female rats and in six female rats made hypothyroid by treatment for 4 weeks with 0·1% (w/v) methimazole in their drinking water

(Rondeel *et al.* 1992*b*). ProTRH mRNA content was higher in hypothyroid (175 ± 9%) than in control rats (100 ± 10%). In addition, pituitary TSHβ mRNA had increased in the hypothyroid rats (828 ± 63%, controls: 100 ± 4%).

Hormone determinations

Plasma levels of TSH were measured by RIA using materials and protocols supplied by the NIADDK, with TSH-RP-2 as a standard. Levels of T_3 and T_4 were estimated by established RIAs in unextracted plasma. The plasma T_4 dialysable fraction was measured by equilibrium dialysis (Sterling & Brenner 1966), and plasma free T_4 (FT_4) was calculated as the product of total T_4 and the dialysable fraction. A similar procedure was followed to measure plasma free T_3 (FT_3). Corticosterone was estimated by RIA (Marzouk *et al.* 1991). Detection limits were 0·2 μg RP-2 TSH/l, 2 nmol T_4/l, 0·1 nmol T_3/l and 1 nmol corticosterone/l. Intra- and interassay coefficients of variation for these assays varied between 3 and 12%.

The RIA of $proTRH_{160-169}$ was carried out as previously described (Bulant *et al.* 1988), and the detection limit was 2–3 fmol/tube. The RIA for TRH (pGlu-His-Pro-NH_2) was usually performed with antiserum 4319 (final dilution 1:10 000) as reported previously (Visser *et al.* 1977). This assay has a high sensitivity but a low specificity for the histidine residue in TRH. Therefore, most samples were also assayed with an RIA using antiserum 8880 (final dilution 1:40 000), which was recently produced by methods similar to that used to raise antiserum 4319. The RIA employing antiserum 8880 has a somewhat lower sensitivity (5–8 fmol/tube) than that utilizing antiserum 4319 (3–5 fmol/tube), but shows much less cross-reactivity with TRH analogues that have histidine replaced by other amino acids (Rondeel *et al.* 1995). Intra- and interassay coefficients of variation for the $proTRH_{160-169}$ and TRH assays varied between 5 and 15%.

TRH-Gly was measured by RIA using an antiserum raised against TRH-Gly coupled to BSA. TRH-Gly (8·5 mg) was coupled with 1,5-difluoro-2,4-dinitrobenzene (Sigma) to 30 mg BSA essentially according to the method of Tager (1976). In total, 12 male New Zealand White rabbits were immunized subcutaneously with 0·5 mg conjugate in 1 ml of a 1:1 suspension of water and Freund's complete adjuvant at 5- to 10-week intervals. Five rabbits responded with significant antibody production, and antiserum 9884 obtained 9 weeks after the fourth immunization from one of these animals was selected for the RIA. TRH-Gly was labelled with ^{125}I using chloramine-T, and ^{125}I-labelled TRH-Gly was separated from unlabelled peptide by HPLC on a Chromspher C8 column (Chrompack, Middelburg, The Netherlands) with a gradient elution of 8 to 40% acetonitrile in 20 mM KH_2PO_4 and 0·1% (w/v) 1-hexanesulphonic acid (Janssen Biochimica, Beerse, Belgium), pH 2·5. The RIA of TRH-Gly was conducted essentially as previously described for TRH (Visser *et al.* 1977) using antiserum 9884 at a final dilution of 1:10 000. The sensitivity of the method amounted to 12 fmol peptide per tube. The specificity of this RIA was determined by analysis of the dose–response curves of a variety of analogous peptides, and all analogues, including TRH, showed less than 1% cross-reactivity (Fig. 1).

Peptides

Peptides (the one-letter codes for the amino acids are defined as E, Glu; <E, pGlu; F, Phe; G, Gly; H, His; K, Lys; P, Pro; Q, Gln; R, Arg; Ac stands for Nα-acetyl) were obtained from several sources: <EHP-NH_2 (TRH) and <EHP were purchased from Cambridge Research Biochemicals (Northwich, UK), <EHPG (TRH-Gly), <EHPG-NH_2, <EHPGK, <EFP-NH_2 and KRQH PGKR were from Peninsula Laboratories (Belmont, CA, USA), while Bissendorf Biochemicals (Hannover, Germany) supplied KRQHPG and <EEP-NH_2. Another batch of <EHPG was synthesized by Dr W G J Schielen (Laboratory of Organic Chemistry, University of Nijmegen, The Netherlands). The peptide AcQHPG was synthesized by convential methods of peptide synthesis in solution.

Statistical analysis

Results are presented as means ± S.E.M. Analysis of variance was used to analyse the data. Provided significant overall effects were obtained by this analysis, *a posteriori* comparisons between groups were made by Duncan's new multiple range test. Differences were considered to be significant at $P \leq 0{\cdot}05$.

Results

Effect of starvation on hypothalamic proTRH mRNA and proTRH peptides, pituitary TSHβ mRNA and plasma hormone levels

The results of this experiment are presented in Table 1. Food deprivation for 3 days significantly reduced hypothalamic proTRH mRNA, whereas refeeding normalized this parameter. Levels of proTRH-derived peptides were estimated in the paraventricular area and the median eminence in starved and refed rats. Levels of TRH were similar when measured with RIAs using antisera 4319 or 8880 (data not shown). Experimental conditions had no significant effect on median eminence levels of proTRH-derived peptides, but in the paraventricular area a significant decrease of TRH and $proTRH_{160-169}$ was observed during starvation, which normalized after refeeding. The

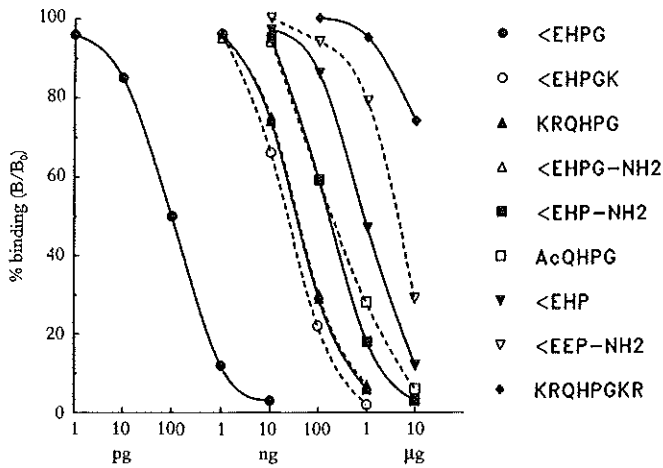

FIGURE 1. Competition for binding of ^{125}I-labelled TRH-Gly to antiserum 9884 (final dilution 1:10 000) with increasing amounts of TRH-Gly or analogous peptides added per tube.

TABLE 1. Effect of a 3-day starvation (d3) and subsequent refeeding for 2 days (d3+2) on body weight, hypothalamic proTRH mRNA, proTRH-derived peptides in the paraventricular area (PVA) and median eminence (ME), pituitary TSHβ mRNA and plasma hormone levels. Normally fed rats were used as controls (d0). Values are means ± S.E.M.

	n	d0	d3	d3+2
Body weight (g)	25	171 ± 2	136 ± 3*	154 ± 3*
proTRH mRNA*	22	100·0 ± 7·4	69·4 ± 5·8*	94·0 ± 7·2
TRH (pmol/PVA)	7	1·83 ± 0·23	1·09 ± 0·13*	2·10 ± 0·05
$proTRH_{160-169}$ (pmol/PVA)	7	0·31 ± 0·04	0·20 ± 0·03*	0·33 ± 0·01
TRH (pmol/ME)	25	3·78 ± 0·40	4·02 ± 0·34	3·41 ± 0·31
$proTRH_{160-169}$ (pmol/ME)	25	0·70 ± 0·08	0·75 ± 0·07	0·65 ± 0·06
TRH-Gly (pmol/ME)	18	0·05 ± 0·01	0·05 ± 0·01	0·07 ± 0·01
TSHβ mRNA*	5	100·0 ± 8·9	61·2 ± 7·5*	106·2 ± 12·2
TSH (µg/l)	25	0·47 ± 0·05	0·39 ± 0·03	0·49 ± 0·04
T_4 (nmol/l)	25	36·4 ± 2·4	18·2 ± 1·4*	35·4 ± 1·3
FT_4 (pmol/l)	5	10·38 ± 2·4	4·62 ± 0·55*	9·07 ± 0·76
T_3 (nmol/l)	25	1·29 ± 0·06	0·55 ± 0·06*	1·23 ± 0·06
FT_3 (pmol/l)	5	4·29 ± 0·25	2·44 ± 0·18*	4·87 ± 0·19
Corticosterone (nmol/l)	5	162 ± 21	488 ± 32*	148 ± 33

*Relative to β-actin mRNA, expressed as percentage of the mean of the controls (d0).
*$P \leq 0.05$ compared with the controls (d0).

amount of TRH-Gly in the median eminence was negligible irrespective of nutritional status when compared with the content of TRH and $proTRH_{160-169}$, and was not altered by starvation. The mean molar ratio between TRH and $proTRH_{160-169}$ varied between 5·3 and 6·4 in the paraventricular area and in the median eminence, and these ratios were not affected by starvation. Starvation also reduced the amount of TSHβ mRNA in the anterior pituitary gland and the plasma levels of thyroid hormones, and increased plasma corticosterone concentrations. Refeeding normalized these parameters. Peripheral TSH levels in the three groups were not significantly different.

Effect of starvation on hypothalamic release of proTRH-derived peptides

Effects of starvation and subsequent refeeding on TRH and $proTRH_{160-169}$ levels in hypophysial portal blood

TABLE 2. Hypothalamic release of TRH and proTRH$_{160-169}$ into hypophysial portal blood of control (d0, $n=11$), starved (d3, $n=11$) or starved and refed (d3+2, $n=6$) urethane-anaesthetized rats. Blood for TSH, T_4 and T_3 determination was taken from the right femoral artery just before the hypophysial stalk was cut, and hypophysial portal blood was collected for 60 min. Values are means ± S.E.M.

	d0	d3	d3+2
TRH (pmol/h)	2·66 ± 0·19	1·84 ± 0·04*	2·15 ± 0·18
TRH (nmol/l)	4·23 ± 0·27	2·76 ± 0·19*	3·56 ± 0·29
proTRH$_{160-169}$ (pmol/h)	0·42 ± 0·10	0·27 ± 0·09*	0·40 ± 0·06
proTRH$_{160-169}$ (nmol/l)	0·66 ± 0·05	0·40 ± 0·04*	0·67 ± 0·09
TSH (μg/l)	0·61 ± 0·05	0·45 ± 0·04*	0·54 ± 0·02
T_4 (nmol/l)	34·0 ± 2·4	18·2 ± 2·2*	28·7 ± 1·2
T_3 (nmol/l)	1·29 ± 0·11	0·70 ± 0·09*	1·29 ± 0·07

*$P \leq 0{\cdot}05$ compared with d0.

are given in Table 2. Hypothalamic release of TRH and proTRH$_{160-169}$ was reduced in starved rats, and normalized after refeeding. The volume of hypophysial stalk blood collected in 60 min was similar in the three groups of rats (634 ± 65, 668 ± 18 and 624 ± 60 μl respectively). The mean molar ratio between TRH and proTRH$_{160-169}$ in hypophysial portal blood varied between 5·4 and 6·9, and was not affected significantly by the experimental conditions.

Effect of starvation on hypothalamic proTRH mRNA, pituitary TSHβ mRNA and plasma hormone levels in corticosterone-substituted adrenalectomized rats

The effects of a 2-day or 3-day starvation period were examined in sham-operated and in corticosterone-treated adrenalectomized female rats. Normally fed sham-operated female rats were used as controls. Results are presented in Figs 2, 3 and 4. When compared with values in control rats, hypothalamic levels of proTRH mRNA were not different after a 2-day starvation period, but had decreased after 3 days of food deprivation. Food deprivation had no significant effect on the levels of TRH in the median eminence, but significantly reduced pituitary TSHβ mRNA, decreased plasma levels of TSH and thyroid hormones, and increased plasma corticosterone in starved sham-operated female rats. Prevention of the starvation-induced increase in corticosterone by adrenalectomy followed by substitution of corticosterone in drinking water to maintain basal plasma concentrations of corticosterone (Fig. 4), had no significant effect on plasma

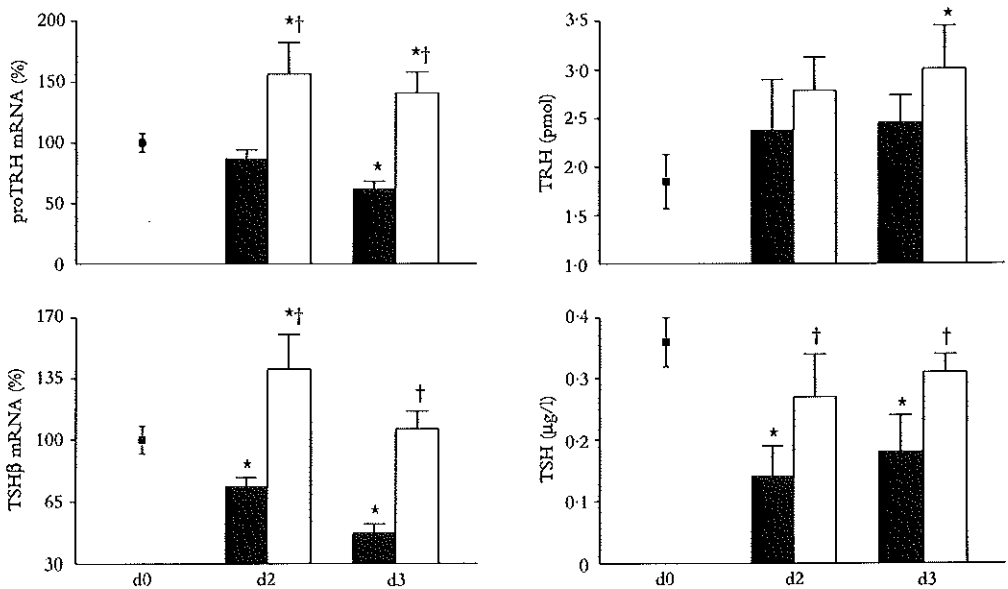

FIGURE 2. Effect of a 2-day (d2) or 3-day (d3) starvation on hypothalamic proTRH mRNA, median eminence TRH, pituitary TSHβ mRNA and plasma TSH in sham-operated (solid bars) and corticosterone-treated adrenalectomized (open bars) female rats. Normally fed sham-operated rats served as controls (d0). Results are presented as the mean ± S.E.M. of 7–15 rats. ProTRH mRNA and TSHβ mRNA are relative to β-actin mRNA, and are expressed as percentage of the mean of the controls. *$P \leq 0{\cdot}05$ compared with d0, †$P \leq 0{\cdot}05$ compared with sham-operated food deprived rats.

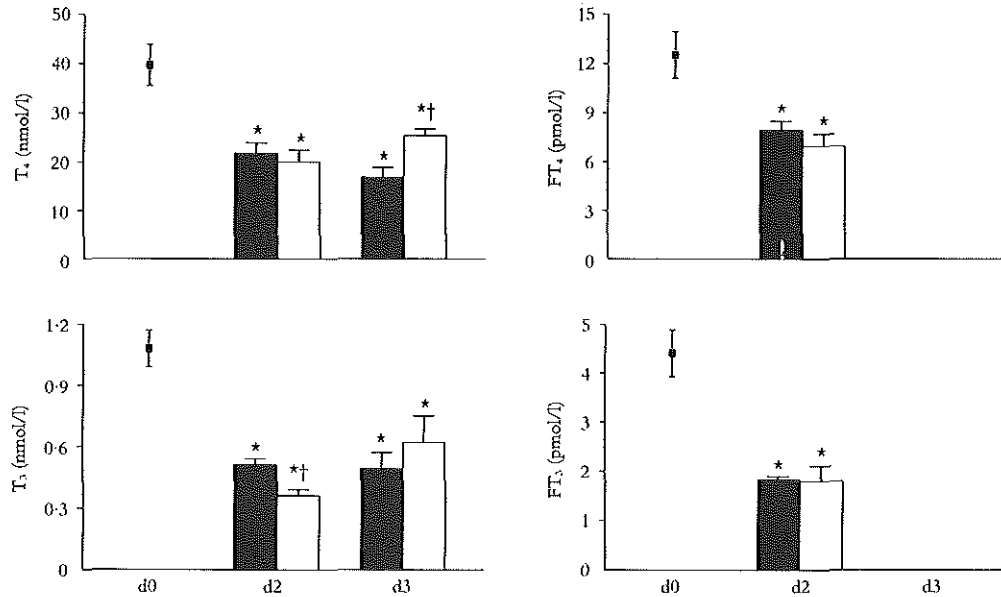

FIGURE 3. Effect of a 2-day (d2) or 3-day (d3) starvation on plasma thyroid hormone levels in sham-operated (solid bars) and corticosterone-treated adrenalectomized (open bars) female rats. Normally fed sham-operated rats served as controls (d0). Results are presented as the mean ± S.E.M. of 7–15 rats. Plasma levels of FT_4 and FT_3 were not determined on day 3 of starvation. *$P \leq 0.05$ compared with d0, †$P \leq 0.05$ compared with sham-operated food deprived rats.

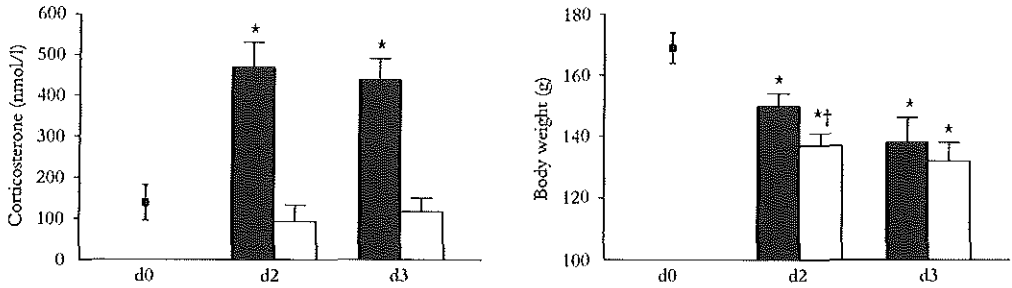

FIGURE 4. Effect of a 2-day (d2) or 3-day (d3) starvation on plasma corticosterone and body weight in sham-operated (solid bars) and corticosterone-treated adrenalectomized (open bars) female rats. Normally fed sham-operated rats served as controls (d0). Results are presented as the mean ± S.E.M. of 7–15 rats. *$P \leq 0.05$ compared with d0, †$P \leq 0.05$ compared with sham-operated food deprived rats.

thyroid hormone levels when compared with levels in starved sham-operated rats (Fig. 3). However, starved corticosterone-treated adrenalectomized rats had more hypothalamic proTRH mRNA and pituitary TSHβ mRNA and higher plasma concentrations of TSH than food deprived sham-operated rats (Fig. 2).

Dexamethasone and hypothalamic release of TRH

Results of this experiment are summarized in Fig. 5. Treatment with dexamethasone (2 mg/kg s.c.) caused a decrease in plasma TSH in urethane-anaesthetized rats within 30–60 min, whereas saline had no effect on plasma

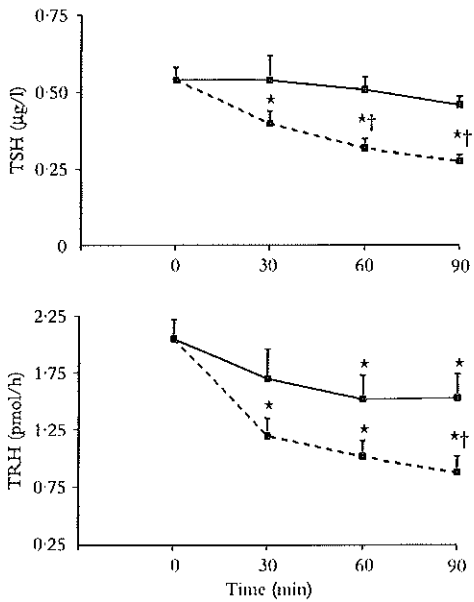

FIGURE 5. Effect of dexamethasone (broken line, n=8) or saline (solid line, n=8) on plasma TSH and on hypothalamic TRH release into hypophysial portal blood in urethane-anaesthetized rats. Peripheral blood was taken from an arterial cannula (n=7). Hypophysial portal blood was collected for four consecutive 30-min periods, and the times indicated represent the end of each period. Dexamethasone (2 mg/kg s.c.) or saline were injected after the first collection period. Values are means ± S.E.M. *$P \leq 0{\cdot}05$ compared with values observed at 0 min, †$P \leq 0{\cdot}05$ compared with saline-injected rats.

TSH. Plasma levels of T_3 or T_4 did not change significantly after the injection with dexamethasone or saline during the period of observation (data not shown). Dexamethasone rapidly reduced the hypothalamic TRH secretion into hypophysial portal blood, and although TRH release also decreased somewhat with time after saline the effect on TRH release was more pronounced by treatment with dexamethasone. Neither dexamethasone nor saline had an effect on the volume of hypophysial portal blood that was collected (data not shown).

Discussion

Starvation is known to induce diminished thyroid function, which is rapidly reversed by refeeding (Hugues *et al.* 1984). Recent studies have provided evidence that the hypothalamic release of TRH into hypophysial portal blood is decreased after a 2-day starvation period in female (Rondeel *et al.* 1992*c*) and male rats (Blake *et al.* 1991). Since, in the latter study, it was found that the *in situ* hybridization signal of proTRH mRNA in the paraventricular nucleus was lower in food deprived than in control male rats, it was concluded that the reduced thyroid function after food deprivation is primarily due to a decreased hypothalamic TRH synthesis and release (Blake *et al.* 1991). Furthermore, evidence has been provided that lack of protein is a major factor in the reduction of TRH and TSH synthesis during starvation (Shi *et al.* 1993). Since the mechanisms by which food deprivation influences thyroid function are not fully understood, we studied in particular the centrally mediated effects of food deprivation in more detail. Furthermore, since food deprivation is a stressful situation leading to enhanced adrenal release of corticosteroids (Woodward *et al.* 1991, Garcia-Belenguer *et al.* 1993, Mitev *et al.* 1993) and since high levels of corticosterone may decrease proTRH gene expression (Kakucska & Lechan 1991, van Haasteren *et al.* 1994), we also compared the effects of starvation on the hypothalamic-hypophysial-thyroid axis in sham-operated rats and in corticosterone-substituted adrenalectomized rats.

The effects of starvation on the hypothalamic-hypophysial-thyroid axis were examined in 2-month-old female rats. Levels of two proTRH-derived peptides, namely TRH and $proTRH_{160-169}$, had decreased in hypophysial portal blood after a 3-day starvation (Table 2). Also the hypothalamic synthesis of proTRH seems to be reduced by starvation in view of the diminished hypothalamic amount of proTRH mRNA and the reduced content in the paraventricular area of TRH and $proTRH_{160-169}$ in female rats starved for 3 days (Table 1, Fig. 2). However, hypothalamic proTRH mRNA levels were not significantly altered after a 2-day starvation period (Fig. 2), notwithstanding that it was observed previously that the hypothalamic release of TRH into hypophysial portal blood had decreased in female rats deprived of food for 2 days (Rondeel *et al.* 1992*c*). Although this lack of an effect on hypothalamic proTRH mRNA does not exclude the possibility of a decreased hypothalamic proTRH synthesis, the data suggest that the reduced hypothalamic TRH release is not secondary to a decreased TRH biosynthesis.

The observation that levels of proTRH-derived peptides are reduced in the hypophysial portal blood of female rats strongly suggests that the hypothyroid state associated with starvation is, at least in part, centrally mediated. Other neuropeptides, such as somatostatin and neuropeptide Y, may also be involved in the reduced thyroid function during starvation (Brady *et al.* 1990, Chua *et al.* 1991). Somatostatin inhibits TSH secretion by a direct action at the anterior pituitary gland, but it is not known whether neuropeptide Y directly affects pituitary function or whether it acts as a hypothalamic modulator. Since neuropeptide Y-containing neurones innervate TRH-synthesizing neurones in the paraventricular nucleus (Toni

et al. 1990), it might well be that neuropeptide Y alters thyroid function through an effect on TRH synthesis. In another situation with disturbed energy utilization, namely drug-induced diabetes, TRH release into hypophysial portal blood is also reduced (Rondeel *et al.* 1992*a*). The finding that tertiary hypothyroidism occurs in situations with disturbed energy utilization agrees with the view that the paraventricular nucleus is involved in an important way in the regulation of the energy balance. In line with this is a recent report that insulin administered into the paraventricular nucleus affects thermogenesis (Menéndez & Atrens 1991).

ProTRH-derived peptides were also measured in the median eminence since this content may provide an index for release of these peptides into hypophysial portal blood. In rats made hypothyroid with thyreostatic drugs or by thyroidectomy, a situation associated with a moderately increased hypothalamic TRH release (Rondeel *et al.* 1992*b*), the amount of proTRH-derived peptides is reduced in the median eminence (Bruhn *et al.* 1991). In this study, however, no consistent changes in the amount of proTRH-derived peptides in the median eminence were found in starved rats, although a significant reduction in the hypothalamic release of TRH and $proTRH_{160-169}$ was observed. Therefore, changes in median eminence content of proTRH-derived peptides do not seem a reliable measure for their release into the hypophysial portal vasculature.

Refeeding for 2 days after a 3-day starvation period was found to normalize the hypothalamic proTRH mRNA content, the levels of TRH and $proTRH_{160-169}$ in portal blood and the plasma concentrations of TSH, thyroid hormones and corticosterone (Table 1). Thus, starvation-induced changes are rapidly reversed by refeeding. Since the molar ratio between TRH and $proTRH_{160-169}$ in the paraventricular nucleus and in hypophysial portal blood was not found to be altered by starvation or refeeding, it is unlikely that starvation alters the processing of proTRH. In control rats, the ratio between TRH and $proTRH_{160-169}$ was found to be approximately 5–6 (Bulant *et al.* 1988, this study) which suggests that proTRH is completely processed in the paraventricular area before transportation to the median eminence and release into the hypophysial portal blood.

Carboxy-terminal amidation is essential for the biological activation of TRH, and it is thought that α-amidation of TRH-Gly, the presumed immediate precursor of TRH, is the rate-limiting step in the processing of proTRH (Pekary *et al.* 1990, Eipper *et al.* 1992). Since, in the median eminence, the content of TRH-Gly was negligible compared with the amount of $proTRH_{160-169}$ and TRH, it seems unlikely that α-amidation of TRH-Gly is a rate-limiting step in hypothalamic TRH synthesis. Since TRH-Gly levels were also very low in hypophysial stalk blood (W J de Greef, unpublished data), it seems that TRH-Gly is probably not very important for pituitary function, since high amounts of TRH-Gly are required to alter TSH and prolactin release (Mori *et al.* 1990). On the other hand, the presence of relatively high levels of $proTRH_{160-169}$ in hypophysial portal blood is probably of physiological importance since $proTRH_{160-169}$ modifies TSH release by potentiating the effect of TRH on the pituitary gland (Bulant *et al.* 1990).

Although the amount of pituitary TSHβ mRNA is reduced during starvation (Blake *et al.* 1991, this study), plasma levels of TSH are not consistently decreased in food deprived rats (Connors *et al.* 1985, Cokelaere & Kühn 1992, Rondeel *et al.* 1992*c*, this study). Since TSH is normally estimated by RIA, it could be that starvation has more effect on the bioactivity of TSH than on its immunoactivity. Evidence for this view is the altered carbohydrate structure of TSH and reduced bioactivity in hypothalamic hypothyroidism (Taylor & Weintraub 1989).

Because thyroid hormones exert a negative feedback action at the level of the hypothalamus (Koller *et al.* 1987, Segerson *et al.* 1987, Rondeel *et al.* 1988), the reduced thyroid status during starvation seems to be the result rather than the cause of the inhibition of hypothalamic proTRH synthesis and release. Our results provide evidence that the starvation-induced enhanced corticosterone secretion is part of the mechanism responsible for the reduced TRH and TSH synthesis and release during starvation: proTRH mRNA, TSHβ mRNA and plasma TSH were not reduced by starvation when corticosterone levels did not increase during starvation. Thus, the negative effect of starvation on thyroid function seems to be mediated by the negative effect of high levels of corticosteroids on the synthesis of hypothalamic proTRH and pituitary TSH. The presence of a glucocorticoid-responsive element in the promoter region of the proTRH gene (Lee *et al.* 1988) and the occurrence of glucocorticoid receptors in TRH-synthesizing cells in the paraventricular area (Ceccatelli *et al.* 1989) corroborate this conclusion. Further evidence is the observation that a high dose of the synthetic glucocorticoid dexamethasone reduced TRH and TSH release within 30–60 min after its administration (Fig. 5). The finding of synaptic relations in the paraventricular nucleus between corticotrophin-releasing hormone containing neurones and neurones with TRH (Hisano *et al.* 1993) is also important, since food deprivation has been found to stimulate neurones in the paraventricular nucleus involved in the release of corticotrophin-releasing hormone (Maeda *et al.* 1994). While prevention of the increase in corticosterone levels in food deprived rats resulted in plasma levels of TSH similar to values in control, normally fed female rats (Fig. 2), levels of thyroid hormones were only partially restored in corticosterone-treated adrenalectomized rats. The reason for the differential effect of this treatment on TSH and thyroid hormones is not clear, but indicates that the enhanced plasma levels of TSH are unable to stimulate thyroid function properly.

In conclusion, the reduced thyroid function during starvation seems to be due to decreases in hypothalamic TRH release, and pituitary TSH synthesis and secretion. Starvation-induced effects are rapidly reversed by refeeding. The decreased hypothalamic TRH release during food deprivation is probably not caused by an altered hypothalamic processing of proTRH. Furthermore, the starvation-induced decrease in hypothalamic proTRH gene expression and in pituitary TSH synthesis and release is probably caused by the starvation-induced high plasma corticosterone levels.

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EFFECTS OF LONG-TERM FOOD REDUCTION ON THE HYPOTHALAMUS-PITUITARY-THYROID AXIS IN MALE AND FEMALE RATS

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ABSTRACT

Many studies have demonstrated that secretion of TSH and thyroid hormones is strongly reduced during short-term starvation in rats. However, less is known about regulation of thyroid function during prolonged food reduction in rats, which is a better model for human malnutrition. In this study, the effects of 3 weeks of food reduction to 33% of normal (FR33) were investigated on the hypothalamus-pituitary-thyroid axis in male and female rats. This was done by measuring hypothalamic proTRH mRNA, median eminence TRH content, pituitary TSHß mRNA, TSH content and TRH receptor status, and serum TSH, T_4, T_3, free T_4 fraction (FFT_4), FFT_3, free T_4 (FT_4), FT_3 and corticosterone levels in FR33 and normally fed rats. At the end of the experimental period, body weight of both male and female FR33 rats was almost 50% lower than that of control rats. FR33 induced a significant increase in the adrenal weight/body weight ratio as well as a marked increase in serum corticosterone in both male and female rats. In both sexes, FR33 caused significant decreases in serum TSH, T_4, FT_4, T_3, FT_3 and FFT_3 but an increase in FFT_4. Electrophoretic analysis indicated that the decrease in serum FFT_3 was correlated with an increased serum TBG, while the increase in serum FFT_4 seemed primarily due to a decreased TBPA binding capacity. Pituitary TSH was strongly reduced by FR33 in both sexes, but hypothalamic proTRH mRNA, median eminence TRH, and pituitary TSHß mRNA and TRH receptor status were not affected except for an increased TSHß mRNA in female FR33 rats. Therefore, long-term food reduction results in a suppression of the hypothalamus-pituitary-thyroid axis in rats which is partially influenced by gender. In contrast to acute starvation, the mechanism whereby serum TSH is suppressed does not appear to involve decreases in proTRH and TSHß gene expression, although a decrease in hypothalamic TRH release is not excluded. Our results further support the hypothesis that TSH secretion may be lowered by increased serum corticosterone, although the mechanism of this effect may differ between acute starvation and prolonged food reduction.

INTRODUCTION

Caloric deprivation has a suppressive effect on the hypothalamus-pituitary-thyroid axis presumably in order to diminish the metabolic rate in the whole body. In rats,

acute starvation is known to induce a reduction in thyroid hormone secretion (1, 2) and, consequently, a reduction in serum total and free T_4 and T_3 concentrations (1, 3-5). Despite the reduced circulating levels of T_4 and T_3, basal serum thyrotropin (TSH) concentrations are usually decreased (1, 3-8). Previous studies have shown that hypothalamic proTRH mRNA level and TRH release are decreased during acute starvation (5, 9, 10), in contrast to primary hypothyroidism where the reduced feedback action of thyroid hormone results in an increase in these parameters (11, 12). This suggests a central inhibition of hypothalamic TRH synthesis and release during starvation.

To study the mechanisms behind the reduced metabolic rate, long-term food reduction seems a better model than acute starvation for human malnutrition. We have, therefore, determined the effects of 3 weeks of food reduction to 33% of normal on the central regulation of the thyroid function in male and female rats. This included measurements of hypothalamic proTRH mRNA, median eminence TRH content, pituitary TRH receptor status and TSHß mRNA, and serum TSH, T_4, T_3, free T_4 (FT_4) and free T_3 (FT_3) levels. In order to examine the possible contribution of stress to the suppression of the hypothalamus-pituitary-thyroid axis during prolonged food reduction serum levels of corticosterone were also determined.

MATERIALS AND METHODS:

Animals

Rats of a locally bred Wistar substrain, R-Amsterdam rats, were used. Since previous studies showed that effects of starvation on thyroid function may differ between male and female rats (5, 13), both sexes were studied. The rats were caged individually in a temperature regulated room (22±2 C), with a 14-h light, 10-h dark cycle (lights on 05:00-19:00 h) and were provided with commercial rat chow containing 22% protein, 4.8% fat, 66.8% carbohydrates, 0.35 mg/kg iodine and 0.29 mg/kg selenium (RMH-TH, Hope Farms, Woerden, The Netherlands) and tap water ad libitum. At the time of the start of the experiments rats were 10 weeks old: male rats weighed 216±8 g and female rats weighed 163±4 g. Their body weight was monitored weekly during the experiment.

Experimental design

Male and female rats were randomly divided into a control and an experimental

group of 8 animals each. In a pilot study it was found that the daily food intake was 24 g in male and 15 g in female rats. During the experiment control rats had free access to food, whereas the experimental groups received one-third of their normal daily food intake (FR33). The experiment lasted 21 days, and the health state of the rats was daily checked upon.

At the end of the experiment, the rats were killed by decapitation. The livers were cut into pieces, frozen in liquid nitrogen, and kept at -80 C until further analysis. The hypothalamus, median eminence and pituitary gland were isolated as described previously (14) for the determination of proTRH mRNA, TRH, and TSHß mRNA or TRH receptor density, respectively. After isolation, each median eminence was extracted immediately with 2 ml methanol tp prevent degradation of TRH. Hypothalami and pituitaries were snap frozen in liquid nitrogen, and kept at -80 C until further analysis.

Deiodinase assay

Liver microsomes were prepared as described previously (15). The microsomal type I deiodinase activity was determined by analysis of the production of radioiodide during incubations of 1 µM [3',5'-^{125}I]rT_3 for 20 min at 37 C with 25 µg/ml microsomal protein in 0.1 M phosphate buffer (pH 7.2), containing 2 mM EDTA and 5 mM dithiothreitol as described previously (16).

Hormone assays and analysis of serum thyroid hormone-binding proteins

Levels of TSH were measured by RIA using materials and protocols supplied by NIDDK, with rat-TSH-RP-2 as standard. The RIA for TRH was usually performed with antiserum 4319 (final dilution 1:10,000) as reported before (17). This assay is very sensitive but has a low specificity for the histidine residue in TRH. Therefore, most samples were also assayed with a RIA using antiserum 8880 (final dilution 1:40,000), an antiserum which was described previously and is much more sensitive to alterations in the His residue of TRH (18). The RIA employing antiserum 8880 has a somewhat higher detection limit (5-8 fmol/tube) than that utilizing antiserum 4319 (3-5 fmol/tube). Serum T_3 and T_4 were estimated by established RIAs in unextracted serum. The serum free T_4 fraction (FFT_4) was measured by equilibrium dialysis (19), and serum free T_4 (FT_4) was calculated as the product of total T_4 and FFT_4. A similar procedure was followed to determine the serum free T_3 fraction (FFT_3) and free T_3 (FT_3) concentration. Corticosterone was

estimated by RIA (20). Detection limits were 0.2 µg/l RP-2 TSH, 2 nmol/l T_4, 0.1 nmol/l T_3 and 1 nmol/l corticosterone. Intra- and interassay coefficients of variation for the assays varied between 3 and 17%.

Agar gel electrophoresis was performed using 0.9% Agar Noble (Difco, Detroit, MI, USA) and 0.2 M glycine, 0.13 M sodium acetate buffer (pH 8.6) as described by Docter et al (21) to determine the distribution of serum T_4 and T_3 over their binding proteins.

ProTRH mRNA and TSHß mRNA determination

Hypothalamic proTRH and pituitary TSHß mRNA were determined by Northern blotting as described previously (14). Results were calculated as the ratios of proTRH mRNA/ß-actin and TSHß mRNA/ß-actin.

TRH receptor assay

TRH receptors were assayed as previously reported by Donda et al (22) with some modifications. Pituitaries were homogenized in 500 µl 0.32 M sucrose, and homogenates of 3 pituitary glands were pooled for the binding assay. The homogenate was centrifuged for 10 min at 1,100x*g* at 4 C, and the supernatant was further centrifuged for 30 min at 30,000x*g* at 4 C. The pellet containing the crude membrane preparation was resuspended in 600 µl cold 20 mM sodium phosphate buffer (pH 7.4), and an aliquot was frozen at -20 C for protein measurement. The binding assay was performed using [^{3}H-Me-His^2]TRH as ligand (82.5 Ci/mmol; New England Nuclear, Boston, MA). The incubation mixture contained 30-60 µg membrane protein and 6 nM ^{3}H-Me-TRH in 200 µl phosphate buffer in the absence (for total binding) or in the presence (for non specific binding) of 25 µM of nonradioactive TRH (Boehringer, Mannheim, Germany). After 2 h of incubation on ice, the samples were filtered on Whatman GF/B glass fiber filters (Whatman, Clifton, NJ) which were washed four times with 2 ml cold 0.15 M NaCl. The filters were dried and then left overnight in 10 ml scintillation liquid (Opti-Fluor; Packard, Downers Grove, IL) before ^{3}H was counted. Specific binding was calculated as the difference between the total and the non specific binding. Nonspecific binding was less than 40% of total binding. All determinations were performed in duplicate.

Statistical analysis

Results are presented as means±SEM and tested statistically by analysis of

variance. Differences were considered to be significant at $p<0.05$.

RESULTS

Effect of food reduction on body, adrenal and pituitary weight

The effects of food reduction on body weight, adrenal weight and pituitary weight are presented in Table 1. Compared to fed controls, body weight was reduced by almost 50% in both male and female FR33 rats. Adrenal weight was decreased by 25% in males and by 37% in females, but relative to body weight it was increased by 40% in male and by 25% in female FR33 *vs.* control rats. Adrenal weight was significantly higher in control females than in control males, but this difference disppeared in the FR33 rats because of the more pronounced adrenal weight loss in females. Relative to body weight, adrenal weight was even further increased in female *vs.* male controls and was also significantly higher in female than in male FR33 rats. In both sexes, pituitary weight showed an insignificant, 10-12% decrease in FR33 rats compared to controls.

Parameter	Treatment	Males	Females
BW (g)	control	216±8	163±3[b]
day 1	FR33	219±5	164±3[b]
BW (g)	control	292±12	188±3[b]
day 21	FR33	157±4[a]	97±1[ab]
adrenal weight	control	44.0±2.1	53.6±2.2[b]
(mg)	FR33	32.9±0.8[a]	33.9±2.2[a]
adrenal/BW	control	0.15±0.03	0.28±0.01[b]
	FR33	0.21±0.05[a]	0.35±0.02[ab]
pituitary weight	control	10.8±0.7	9.0±0.4[b]
(mg)	FR33	9.8±0.5	8.0±0.4[b]

TABLE 1. Body, adrenal and pituitary weight in control and FR33 male and female rats. Data are presented as the mean ± SEM of 8 animals per group.
[a]P<0.05 vs. control rats
[b]P<0.05 vs. male rats

Effects of food reduction on hypothalamic proTRH mRNA, median eminence TRH content and specific binding of TRH to pituitary plasma membranes

Hypothalamic proTRH mRNA/actin mRNA ratios were similar in male and female controle rats and did not change after 3 weeks of food reduction (Fig. 1A). TRH levels in the median eminence were similar when measured with RIAs using antiserum 4319 or 8880 (data not shown), confirming the identity of the analyte as authentic TRH. Median eminence TRH content was similar in both sexes and was not affected by food reduction (Fig. 1B). The protein concentration of the pituitary membrane preparation was similar in male and female control rats and was strongly reduced by prolonged food reduction in both sexes (Table 2).

The specific binding (per mg protein) of ^{3}H-Me-TRH to pituitary membranes was higher, but not significantly, in female than in male controls; it tended to be higher in female FR33 rats and showed a significant increase in male FR33 rats compared to the respective controls (Table 2). Total pituitary TRH binding was unchanged in male FR33 rats, whereas there was a 50% reduction in female FR33 rats compared to controls.

Parameter	Treatment	Males	Females
^{3}H-MeTRH specific binding	control	49.4±9.6	68.7±8.1
(fmol/mg protein)	FR33	109.0±15.9[a]	102.5±37.6
^{3}H-MeTRH specific binding	control	21.0±3.9	29.8±3.3
(fmol/pituitary)	FR33	25.7±4.1	16.7±6.0
membrane protein	control	436±38	436±11
(µg/pituitary)	FR33	234±6[a]	163±5[ab]

TABLE 2. Membrane TRH receptors in the pituitary gland of control and FR33 male and female rats. Data are presented as receptor density (per mg membrane protein) and receptor content (per pituitary). Membrane protein concentration per pituitary is also shown. Data are presented as the mean ± SEM of 4 pools of 3 animals each per group.

[a] P<0.05 vs. control rats

[b] P<0.05 vs. male rats

Effect of food reduction on serum TSH and pituitary TSH and TSHß mRNA

Food reduction caused a significant decrease in serum TSH (Table 3) and in

pituitary TSH content (Fig. 1D) in both sexes, whereas TSHß mRNA/actin mRNA ratios increased by 40% in male FR33 rats and by 185% in female FR33 rats compared to their controls (Fig. 1C). Serum TSH, pituitary TSH and pituitary TSHß mRNA were all significantly higher in control males than in control females, and pituitary TSH was still significantly higher in male than in female FR33 rats (Table 3, Fig. 1C,D).

Parameter	Treatment	Males	Females
TSH	control	0.76±0.16	0.35±0.05[b]
(ng/ml)	FR33	0.11±0.02[a]	0.18±0.05[a]
T4	control	45.5±2.4	30.6±1.9[b]
(nmol/l)	FR33	30.4±1.9[a]	10.5±1.4[ab]
FFT4	control	0.017±0.001	0.024±0.002
(%)	FR33	0.020±0.001[a]	0.026±0.001
FT4	control	7.5±0.34	7.2±0.66
(pmol/l)	FR33	5.9±0.35[a]	2.6±0.24[a]
T3	control	1.3±0.03	1.2±0.03
(nmol/l)	FR33	1.0±0.05[a]	0.7±0.06[ab]
FFT3	control	0.51±0.02	0.43±0.01[b]
(%)	FR33	0.39±0.01[a]	0.30±0.06[ab]
FT3	control	6.7±0.27	5.3±0.17[b]
(pmol/l)	FR33	4.1±0.19[a]	2.4±0.8[ab]
FT3/FT4	control	0.90±0.06	0.76±0.04
	FR33	0.71±0.04[a]	0.98±0.42
corticosterone	control	104±16	230±89
(nmol/l)	FR33	614±36[a]	1160±75[ab]

TABLE 3. Plasma TSH, thyroid hormones and corticosterone in control and FR33 male and female rats. Data are presented as the mean ± SEM of 8 animals per group.
[a]P<0.05 vs. control rats
[b]P<0.05 vs. male rats

Effect of food reduction on serum thyroid hormones, their binding proteins and corticosterone levels

Results are presented in Table 3 and Fig. 2. Compared to levels in fed controls, serum T_4, FT_4, T_3 and FT_3 were significantly reduced in both female and male FR33 rats. Serum FFT_3 was significantly decreased by food reduction in both sexes, whereas FFT_4 showed a slight increase, which was significant only in male rats. Serum T_4 and FT_3, but not FT_4 and T_3, were significantly higher in control males than in control females. In FR33 rats, T_4, FT_4, T_3 and FT_3 were all higher in males than in females.

Serum of control and FR33 male and female rats was incubated with [^{125}I]T_4 or [^{125}I]T_3 and analysed by agar gel electrophoresis. Typical examples of radioactivity patterns obtained are shown in Fig. 2. In contrast to human serum (21), rat albumin and thyroxine-binding prealbumin (TBPA) were not separated by this method. Therefore, the first peak represents thyroxine-binding globulin (TBG) and the second peak contains both albumin and TBPA. Radioactive T_3 was bound by TBG and albumin-TPBA in a ratio which was similar in both sexes and which changed from 1:5 in control rats to 1:1 in FR33 rats. A single peak of protein-bound labeled T_4 was found in the albumin-TBPA region in control rats. In male and female FR33 rats a small radioactive peak appeared in the TBG region.

Levels of serum corticosterone were significantly increased in both sexes after prolonged food reduction (Table 3). Irrespective of nutritional status, serum corticosterone was lower in males than in females, which difference was significant in FR33 rats but not in controls.

Effect of food reduction on hepatic deiodinase activity

Liver type 1 deiodinase activity was 2.5 times higher in male than in female control rats. In both sexes, food reduction caused a significant decline of ≈50% in this activity (Fig. 3).

DISCUSSION

Changes in the hypothalamus-pituitary-thyroid axis induced by short-term, complete starvation in the rat have been reported by many authors (5, 6, 9, 13, 14, 23-25), but little is known of the effects of long-term food reduction. In this study we investigated the effects of a reduction in food intake to one-third of normal (FR33) during 3 weeks on the hypothalamus-pituitary-thyroid axis in male and female rats.

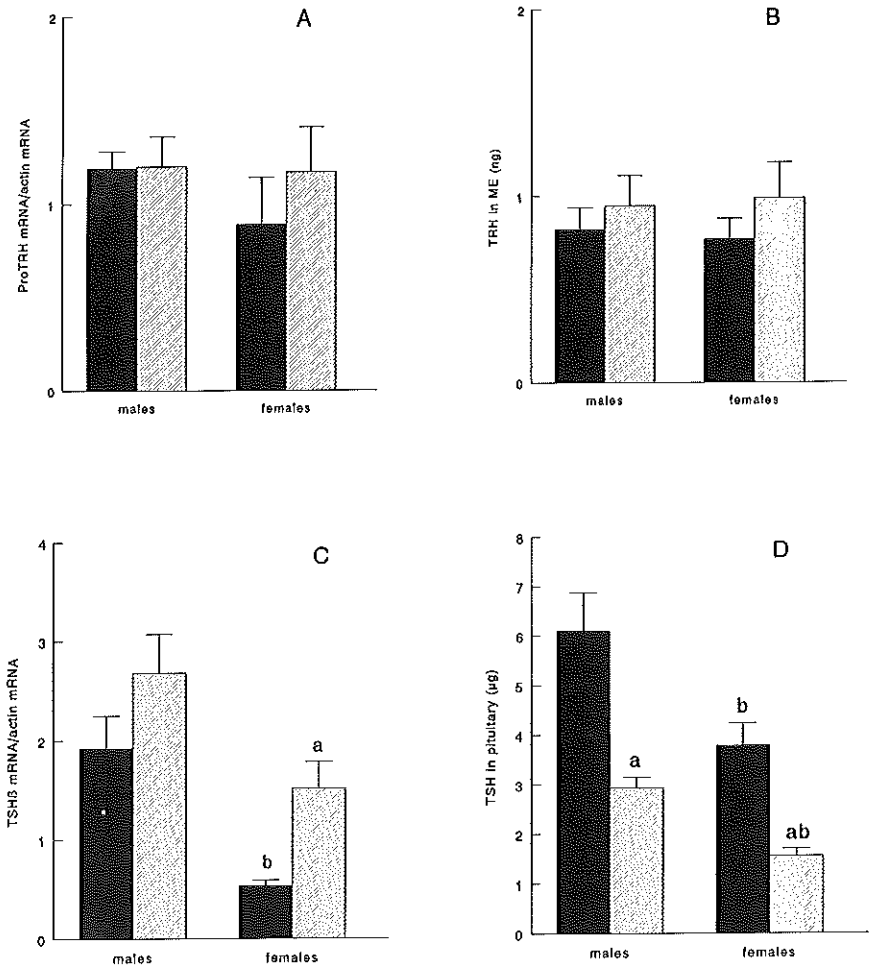

Figure 1. *Effect of 21 days food restriction (FR33; gray bars) on hypothalamic proTRH mRNA (A), median eminence TRH (B), pituitary TSHß mRNA (C) and pituitary TSH (D) in male and female rats. Normally fed rats were used as controls (black bars). Results are presented as mean±SEM of 8 rats. ProTRH mRNA and TSHß mRNA are relative to ß-actin mRNA. [a]P<0.05 compared to controls, [b]P<0.05 compared to males.*

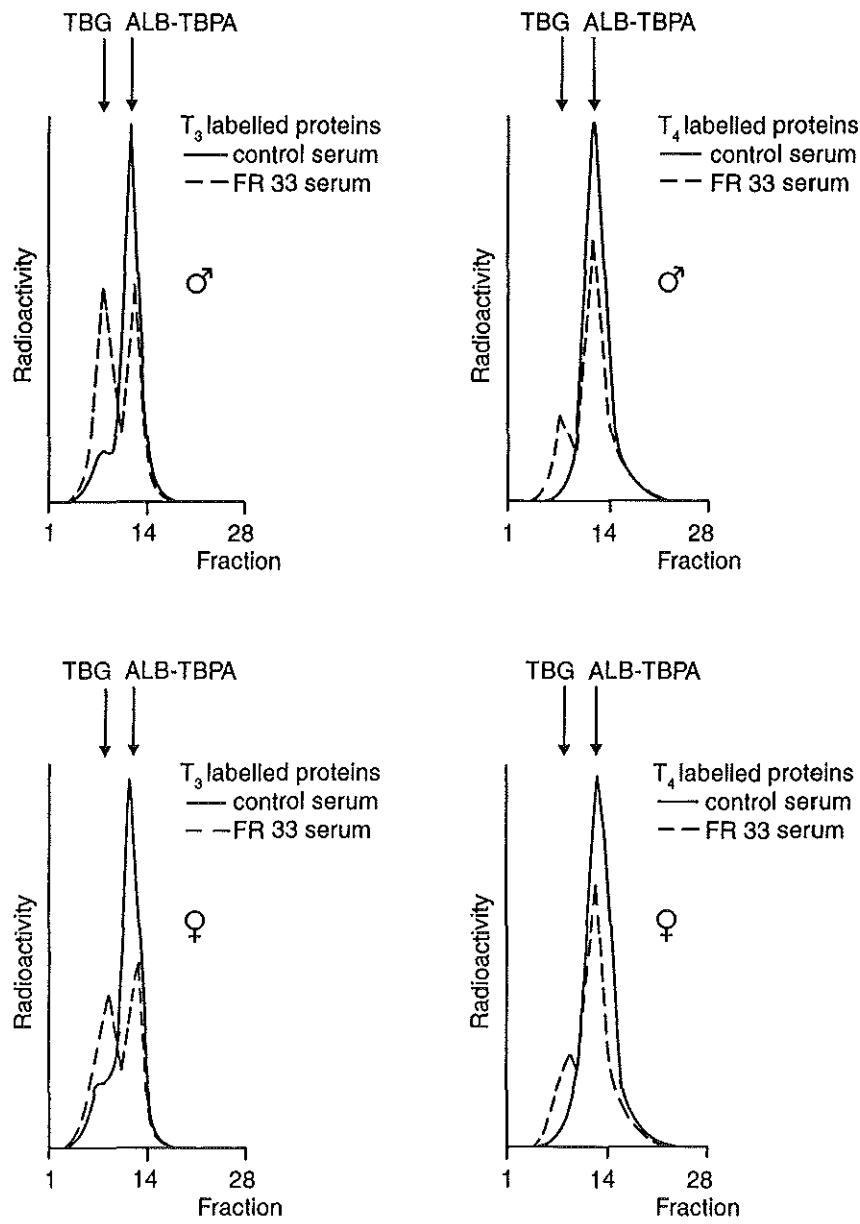

Figure 2. Representative examples of agar-gel electrophoresis of sera of control and FR33 male and female rats, pre-incubated with radioactive T3 or T4.

FR33 caused a strong reduction of serum TSH levels in both sexes. Based on data reported by Rodriguez et al (26) on TSH turnover in FR75, FR50 and FR25 rats, the decrease in serum TSH during prolonged food reduction represents a decreased TSH secretion rather than an increased TSH clearance. This decreased TSH secretion may be mediated a) at the hypothalamic level, by changes in the release of factors which regulate TSH secretion, such as TRH, somatostatin and dopamine, b) at the pituitary level, by a direct inhibition of the thyrotroph or by changes in its sensitivity to hypothalamic factors or to the feedback inhibition by thyroid hormone, and/or c) by a general effect of food reduction on protein turnover.

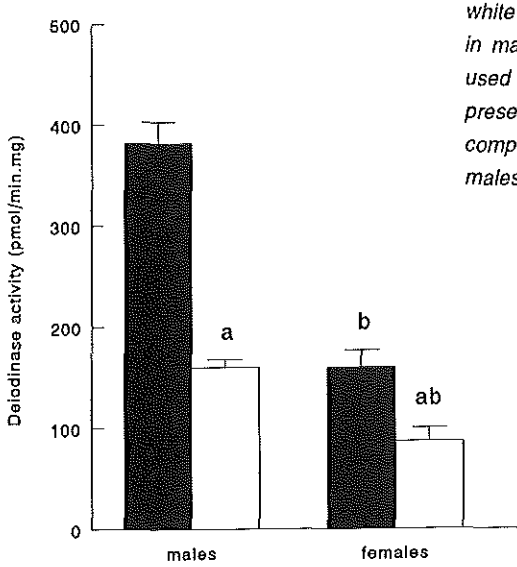

Figure 3. Effect of 21 days food restriction (FR33; white bars) on hepatic type 1 deiodinase activity in male and female rats. Normally fed rats were used as controls (black bars). Results are presented as mean±SEM of 8 rats. [a]P<0.05 compared to controls, [b]P<0.05 compared to males.

Since several findings indicate that the decrease in thyroid function during short-term starvation is associated with a lowered hypothalamic release of TRH (5-7, 27), we studied the centrally mediated effects of long-term food reduction in more detail. In this study, hypothalamic proTRH mRNA content was unaffected in male and female FR33 rats, whereas it was significantly decreased in rats starved for 2 or 3 days (9, 14). Median eminence TRH content was not affected by food reduction in both male and female rats. However, since we have not measured TRH in hypophyseal portal blood, we cannot exclude that hypothalamic TRH release was decreased despite the unaltered proTRH mRNA level and median

eminence TRH content. Our findings at least suggest that a decreased proTRH gene expression is not essential in the central mechanism mediating reduced TSH secretion during long-term food deprivation. Recent studies from our group have shown that TSH secretion may also be diminished in diabetic rats[1], in lactating rats[2] or after administration of interleukins without a concomitant change in proTRH gene expression (14, 28).

At the pituitary level, the density of TRH membrane receptors (per mg membrane protein) was significantly increased in FR33 male rats compared to controls while in FR33 female rats the increase was only a tendency, but not statistically significant. These results obtained in FR33 rats are in line with the findings by Rodriguez et al (26), who demonstrated an increase in TRH binding sites in FR75, FR50 and FR25 rats, despite a significantly decreased TSH secretion in response to TRH in both FR50 and FR25 rats. Low levels of TRH (25) and/or thyroid hormones (29, 30) may contribute to the increased density of TRH receptors, as observed also in the aged rat (22). Moreover, in the rat, there is a quantitative relationship between pituitary nuclear T_3 receptor occupancy and inhibition of TSH release (31, 32). It has been reported that starvation in rats reduces the number of pituitary nuclear T_3 receptors (26). Thus, it is unlikely that a higher sensitivity of the pituitary to the feedback inhibition by thyroid hormone during starvation is responsible for the low TSH levels. However, it should be emphasized that the changes observed for TRH and T_3 receptors in the whole pituitary may not reflect alterations in TRH and T_3 receptors at the level of the thyrotrophs.

At the pituitary level, TSHß mRNA showed a small increase in male FR33 rats and a large increase in female FR33 rats, suggesting an increased TSHß gene expression or an increased TSHß mRNA stability. However, TSH production is decreased in FR33 rats, since both TSH secretion (see above) and pituitary TSH content are decreased. The discrepancy between the decrease in TSH production and the increase in pituitary TSHß mRNA may be due to impaired translation of the latter, which could reflect a general defect in protein synthesis during prolonged and severe food reduction. In addition, the synthesis of the α-subunit may be impaired at both the transcriptional and translational level. Changes in thyroid status have been shown to affect pituitary TSHß mRNA to a greater extent than pituitary α-subunit mRNA (33, 34). However, this does not negate a selective inhibitory effect of food reduction on α-subunit production. Indeed, other pituitary hormones sharing

the same α-subunit as TSH, were found to decrease significantly during severe food reduction (7).

The results of our study confirm and extend those reported previously (8, 35), demonstrating that food restriction induces a decrease in serum T_4, FT_4, T_3 and FT_3 in both male and female rats. Serum FT_4 levels were similar in control male and female rats, while both serum total T_4 levels and serum T_4 binding were higher in males. Control serum FT_3 levels were higher in male than in female rats, while serum total T_3 levels were similar and serum T_3 binding was higher in females. Further differences in serum protein binding of T_4 and T_3 were induced by food reduction, which resulted in a slight increase in FFT_4 and a larger decrease in FFT_3 in both sexes. In adult rats, serum T_4 is primarily bound to TBPA, whereas albumin is the predominant binding protein for serum T_3 (36). Serum TBG levels and hepatic TBG gene expression are high in neonatal rats but decrease to almost undetectable levels in adults, while they increase again during senescence (37, 38). It has been shown that fasting increases serum TBG (39) but decreases serum TBPA (40) in rats. In our study the decrease in the serum FFT_3 after food restriction is correlated with an increased TBG, while the increase in the serum free T_4 fraction may be caused in part by a decrease in serum TBPA.

As a result of the significant decline in hepatic deiodinase activity in both sexes after food reduction, the peripheral conversion of T_4 into T_3 may be decreased which should be reflected in a decline in the serum FT_3/FT_4 ratio. However, after food reduction the serum FT_3/FT_4 ratio was only decreased in male rats, suggesting that the liver type I deiodinase is a more important site for peripheral T_3 production in male than in female rats. This is supported by the higher hepatic type I deiodinase activity in male than in female controls.

Concerning the sex dependence of the effects of food deprivation, Cohen et al (13) showed significantly higher levels of serum TSH in control male *vs.* female Sprague-Dawley rats, which decreased significantly in male but not in female rats after short-term starvation. However, Rondeel et al (5) found no differences in serum TSH between control male and female $(RxU)F_1$ rats, which decreased in female but not in male rats only after 4 days of starvation. In agreement with the former study, we found that control serum TSH values were significantly higher in male than in female R-Amsterdam rats, and that food reduction resulted in a more pronounced reduction in serum TSH in males than in females, although the effect was also significant in females. The reason for the discrepancy between the

previous report of Rondeel et al and the present findings may be related to duration and extent of food reduction or rat strain.

Previous studies have suggested a negative relationship between serum TSH and corticosterone in rats after induction of diabetes with streptozotocin[1], during short-term starvation (14), after administration of interleukin-1 (28), or during lactation[2]. In all these conditions, a decreased serum TSH is associated with an increased serum corticosterone. The decrease in serum TSH in starved rats is partially prevented if serum corticosterone is kept constant by adrenalectomy and corticosterone substitution (14). The present findings of sex-dependent differences in control rats as well as the effects of long-term food reduction further substantiate this negative correlation between TSH and corticosterone. In control rats, serum TSH, pituitary TSH content and pituitary TSHß mRNA were all higher in males than in females. This suggests that TSH production is higher in male than in female rats, which is associated with lower adrenal weight and corticosterone levels in males than in females. Furthermore, during prolonged food reduction serum TSH and pituitary TSH content decrease, while relative adrenal weight and serum corticosterone increase in both sexes, although the magnitudes of these changes differ. Together, these findings suggest a negative effect of corticosterone on TSH synthesis and secretion. The exact mechanism of this effect remains unknown, but chronic treatment of rats with dexamethasone has been shown to decrease hypothalamic proTRH mRNA (41), while acute dexamethasone treatment was found to lower TRH release in hypophyseal portal blood (14). However, the present findings suggests that the effect of corticosterone on TSH secretion not always involves decreases in proTRH and TSHß gene expression. It is possible that the above-mentioned posttranscriptional defect in TSH production after prolonged food reduction is mediated by the increased serum corticosterone.

In conclusion, the present study demonstrates profound effects at different levels of the hypothalamic-pituitary-thyroid axis after 3 weeks of severe food restriction in both male and female rats. In contrast to acute starvation, the mechanism whereby serum TSH is suppressed after prolonged food reduction does not involve decreases in hypothalamic proTRH or pituitary TSHß gene expression. Direct inhibition of the thyrotrophs by other regulators of TSH secretion, such as somatostatin and dopamine, as well as the precise role of corticosterone deserve further investigation.

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DIFFERENT EFFECTS OF CONTINUOUS INFUSION OF INTERLEUKIN-1 AND INTERLEUKIN-6 ON THE HYPOTHALAMIC-HYPOPHYSIAL-THYROID AXIS

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Different Effects of Continuous Infusion of Interleukin-1 and Interleukin-6 on the Hypothalamic-Hypophysial-Thyroid Axis*

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ABSTRACT

The cytokines interleukin-1 (IL-1) and IL-6 are thought to be important mediators in the suppression of thyroid function during nonthyroidal illness. In this study we compared the effects of IL-1 and IL-6 infusion on the hypothalamus-pituitary-thyroid axis in rats. Cytokines were administered by continuous ip infusion of 4 μg IL-1α/day for 1, 2, or 7 days or of 15 μg IL-6/day for 7 days. Body weight and temperature, food and water intake, and plasma TSH, T_4, free T_4 (FT_4), T_3, and corticosterone levels were measured daily, and hypothalamic pro-TRH messenger RNA (mRNA) and hypophysial TSHβ mRNA were determined after termination of the experiments. Compared with saline-treated controls, infusion of IL-1, but not of IL-6, produced a transient decrease in food and water intake, a transient increase in body temperature, and a prolonged decrease in body weight. Both cytokines caused transient decreases in plasma TSH and T_4, which were greater and more prolonged with IL-1 than with IL-6, whereas they effected similar transient increases in the plasma FT_4 fraction. Infusion with IL-1, but not IL-6, also induced transient decreases in plasma FT_4 and T_3 and a transient increase in plasma corticosterone. Hypothalamic pro-TRH mRNA was significantly decreased (−73%) after 7 days, but not after 1 or 2 days, of IL-1 infusion and was unaffected by IL-6 infusion. Hypophysial TSHβ mRNA was significantly decreased after 2 (−62%) and 7 (−62%) days, but not after 1 day, of IL-1 infusion and was unaffected by IL-6 infusion. These results are in agreement with previous findings that IL-1, more so than IL-6, directly inhibits thyroid hormone production. They also indicate that IL-1 and IL-6 both decrease plasma T_4 binding. Furthermore, both cytokines induce an acute and dramatic decrease in plasma TSH before (IL-1) or even without (IL-6) a decrease in hypothalamic pro-TRH mRNA or hypophysial TSHβ mRNA, suggesting that the acute decrease in TSH secretion is not caused by decreased pro-TRH and TSHβ gene expression. The TSH-suppressive effect of IL-6, either administered as such or induced by IL-1 infusion, may be due to a direct effect on the thyrotroph, whereas additional effects of IL-1 may involve changes in the hypothalamic release of somatostatin or TRH. As glucocorticoids are known to suppress hypothalamic TRH mRNA levels, it is speculated that the decrease in pro-TRH gene expression caused by prolonged infusion of IL-1 is mediated by the high plasma corticosterone levels. (*Endocrinology* **135**: 1336–1345, 1994)

DURING acute and chronic systemic illness, profound changes in thyroid function occur in both humans (1–3) and animals (4). In humans, the most characteristic changes are a decrease in the plasma T_3 level and an increase in the plasma level of rT_3. Plasma T_4 may also be decreased in severely ill patients (3), mainly due to reduced binding to transport proteins (5, 6), as plasma free T_4 (FT_4) usually remains within the normal range. It has been suggested that cytokines are important mediators of the changes in thyroid economy during diseases in which the immune system is activated (4, 7–11). Cytokines are polypeptides primarily produced by activated monocytes and macrophages, which play important roles not only in regulating the immune system, but also in interacting with several endocrine systems (12–17). In rats, a single injection of interleukin-1 (IL-1) lowered plasma TSH and thyroid hormone levels within 5 h (4). Continuous infusion of IL-1β induced in the rat decreases in plasma TSH, FT_4, and T_4 binding (18). It is, however, not fully understood how cytokines suppress the pituitary-thyroid function.

Inflammation stimulates the production of a cascade of cytokines, of which, in particular, tumor necrosis factor-α, IL-1, and IL-6 represent key factors for communication between the immune and neuroendocrine systems (19–21). As part of the pleiotropic effects of IL-1 is mediated by IL-6, we compared the effects of short and long term infusion of IL-1 and long-term infusion of IL-6 on the hypothalamic-pituitary-thyroid axis. To identify the sites of action of IL-1 and IL-6, their effects were measured on plasma T_4, FT_4, T_3, TSH, and corticosterone; TRH content in median eminence; hypothalamic levels of pro-TRH messenger RNA (mRNA); and pituitary levels of TSHβ mRNA. As hepatic type I deiodinase is responsible for 60–70% of peripheral T_3 production in euthyroid rats (22), we also measured the activity of this enzyme during IL infusion.

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Materials and Methods

Materials

Recombinant human IL-1α (IL-1) was kindly provided by Dr. P. Lomedico (Hoffman LaRoche, Nutley, NJ). The preparation, supplied in 50 mM potassium phosphate (pH 6.5) and 0.1 M sodium chloride, had an activity of 2×10^8 U/ml (D10 assay) and a specific activity of 3×10^8 U/mg protein. According to the specifications of the suppliers, endotoxin contamination was negligible (0.5 U/ml IL-1 solution, as detected in the limulus amoebocyte lysate assay).

Human IL-6, produced by recombinant DNA technology in *Escherichia coli*, was obtained from Sandoz (Sandoz Forschungsinstitut, Vienna, Austria). The specific activity of the preparation was 52×10^5 U/mg (by B13.29 assay). The preparation (SDZ 280-969, batch PPG9001) was supplied in 20 mM sodium phosphate (pH 6.7), and endotoxin contamination was negligible (<0.4 U/mg protein).

Both IL-1 and IL-6 were diluted in sterile pyrogen-free saline [0.9% NaCl (wt/vol) in water]. All chemicals used were of analytical grade. The concentrations of IL-1 and IL-6 used for infusion in this study were based upon the findings of a previous study performed by Hermus *et al.* (18) and a pilot study in which three concentrations of IL-6 infusion were studied in rats (data not shown).

Animals

Male albino Wistar rats (Cpb:WU) were obtained from the local breeding facility and individually housed in Plexiglass cages in an artificially lighted room (lights on at 0700 h; lights off at 1900 h). Rats were provided with commercial rat chow containing 22% protein, 4.8% fat, and 66.8% carbohydrates (RMH-TH, Hope Farms, Woerden, The Netherlands) and tap water *ad libitum*. At the time of the start of the experiments, rats were 10 weeks old and weighed 200–220 g. Animal procedures were approved by the institutional review board.

Experimental design

Long term infusion. To diminish the stress of the experimental procedure, rats were handled daily, starting at least 1 week before the insertion of an indwelling cannula into an external jugular vein. Rats were cannulated according to the method described by Steffens (23) with some minor modifications (24). After insertion, the cannula was filled with a 0.9% NaCl solution containing heparin (500 IU/ml; Organon Teknika, Boxtel, The Netherlands) and polyvinylpyrrolidone (1 g/ml; Merck, Darmstadt, Germany).

Seven to 9 days after cannulation, rats were implanted with an Alzet osmotic minipump (model 2001, Alzet Corp., Palo Alto, CA; 1 μl/h for 7 days). Rats were infused for 7 days with IL-1 (4 μg/day) or IL-6 (15 μg/day) dissolved in sterile pyrogen-free physiological saline or with saline alone. The pumps were equilibrated by immersion in physiological saline solution for 3–4 h at 37 C according to the instructions of the manufacturer and then implanted ip in ether-anesthetized animals between 1400–1600 h (day 0). The indwelling cannula and the osmotic pump were tolerated well by the rats with no obvious signs of discomfort or infection.

From the freely moving rats, blood samples of 2 ml were withdrawn from the jugular venous cannula on several days of the experiment starting 2 days before implantation of the osmotic minipumps (18). Because of the circadian rhythm in hormone release, blood was sampled at about the same time each day (between 1000–1200 h). Blood samples were collected in prechilled tubes containing 60 μl 10% (wt/vol) EDTA in saline, gently shaken, and centrifuged for 10 min at $1500 \times g$ at 4 C. After removal of the plasma, the residue containing red blood cells was resuspended in sterile physiological saline solution (1.5 ml) and returned through the jugular venous cannula to each rat. Plasma samples were aliquoted and stored at −20 C until assayed.

In all rats, body weight was measured daily between 0815–0900 h. Body temperature was measured daily between 0815–0900 h and between 1300–1430 h in conscious hand-held rats by insertion of a thermal probe into the rectum. The probe was connected to a digital temperature monitor (Digital DT100, Elbatron, Kerkdriel, The Netherlands). Mean daily temperature for each rat was determined by averaging the morning and afternoon rectal temperatures. The daily food and water intake was estimated by weighing the residual food pellets and water for individual cages.

At the end of the experiment (day 7), the rats were killed by decapitation. The livers were cut into pieces, frozen in liquid nitrogen, and kept at −80 C until the estimation of type I deiodinase activity. The skull was opened, and the brain was removed. The hypothalamus was isolated (limits, posterior border of the chiasmatic opticum, anterior border of the mamillary bodies, and lateral hypothalamic border; height, ~3 mm) for the determination of pro-TRH mRNA. Also, the pituitary gland was isolated to estimate the level of TSHβ mRNA. Both tissues were snap-frozen in liquid nitrogen and kept at −80 C until determination of pro-TRH and TSHβ mRNAs.

Short term infusion. Rats were infused with IL-1 (4 μg/day) for 1 day (osmotic minipump model 2001D; 8 μl/h for 1 day) or 2 days (osmotic minipump model 1003D; 1 μl/h for 3 days) or with saline. The pumps were implanted between 1400–1600 h. These animals had not been implanted with a cannula into the jugular vein. After 1 or 2 days of infusion, trunk blood was collected after decapitation of the rats between 1200–1500 h. The livers, hypothalami, and pituitaries were collected according to the methods described above. From animals infused for 1 day with IL-1 or saline, the median eminence was also collected. This was performed by grasping the hypophysial stalk with forceps and lifting it from the brain. The protruding tissue fragment, comprising the hypophysial stalk and the median eminence, but referred to as median eminence, was cut from the brain and placed in 2 ml methanol to determine the TRH content.

Deiodinase assay

Livers were homogenized, and type I deiodinase activity was determined in the homogenate by analysis of the production of radioiodide from outer ring-labeled rT_3 (25). Type I deiodinase activity was measured in incubations of 1 μM [^{125}I]rT_3 for 20 min at 37 C with 50 μg/ml homogenate protein in 0.1 M phosphate buffer (pH 7.2), 2 mM EDTA, and 5 mM dithiothreitol by the method described by Fekkes *et al.* (26).

Hormone assays

Levels of TSH were measured by RIA using materials and protocols supplied by the NIDDK, with TSH RP-2 as standard. The RIA for TRH was performed with antiserum 4319 (final dilution, 1:10,000), as reported previously (27). Plasma T_3 and T_4 were estimated by specific RIAs in unextracted plasma, as described by Hermus *et al.* (18). The plasma FT_4 fraction was determined by means of the SPAC FT_4 assay kit (Byk-Sangtec Diagnostica, Dietzenbach, Germany) (28), and the plasma FT_4 concentration was calculated as the product of the total T_4 level and the FT_4 fraction. Plasma corticosterone was measured by RIA, as described by Sweep *et al.* (24). Intra- and interassay coefficients of variation for the assays varied between 3–17%.

Pro-TRH mRNA determination

Pro-TRH mRNA was measured by a ribonuclease (RNase) protection assay, using a labeled antisense complementary RNA (cRNA) probe. Total hypothalamic RNA was isolated by acid guanidinium thiocyanate-phenol-chloroform extraction (29). From each sample, 10 μg hypothalamic RNA were used in a RNase protection assay, as described previously by Sambrook *et al.* (30) with a few modifications. Hybridization was carried out overnight at 55 C; for the RNase digestion, 2 U/ml RNase-T1 and 0.2 μg/ml RNase-A (both from Boehringer, Mannheim, Germany) were used. The 1322-basepair (bp) *EcoRI*/*PstI* rat pro-TRH complementary DNA (cDNA) insert in a pSP65 vector (31) was kindly provided by Dr. S. L. Lee (New England Medical Center Hospitals, Boston, MA). The cRNA probe was synthesized using fragment 981–1322 of rat pro-TRH cDNA as a template. This 351-bp *Rsa*I fragment was isolated after agarose gel electrophoresis. Variations in procedure were accounted for by normalizing to the glyceraldehyde-3-phosphate dehydrogenase gene (GAPDH) expression in each sample, using a cRNA probe transcribed from a 410-bp *Pst*I/*Sau*A1 fragment of the cDNA

inserted in pBlueScript KS(-) (Stratagene, La Jolla, CA). Under the digestion conditions used, the GAPDH signal consisted of 2 bands of about 310 and 320 nucleotides (Fig. 1). Autoradiographs were scanned densitometrically with a LKB 2222–020 UltraScan XL Laser Densitometer (Pharmacia LKB Biotechnology 1987, Bromma, Sweden). The peak areas, corresponding to the bands, were integrated by the computer. Results were calculated as the ratio between the integrated optical densities of pro-TRH and GAPDH mRNA, and expressed as a percentage of the mean of the respective control values.

Pituitary TSHβ mRNA measurement

Total pituitary RNA was isolated by acid guanidinium thiocyanate-phenol-chloroform extraction (29), and 20 μg RNA were subjected to denaturing agarose gel electrophoresis and blotted onto Hybond N^+ filter (Amersham International PLC, Aylesbury, United Kingdom). TSHβ cDNA (420 bp), inserted in the *PstI* site of a pBR322 vector, was kindly provided by Dr. W. W. Chin (Brigham and Women's Hospital, Boston, MA) (32). After electroporation in DH5 cells, DNA was isolated and digested with *PstI*. The DNA fragment was isolated after agarose gel electrophoresis. Northern blotting and random primed labeling of the TSHβ cDNA with [^{32}P]deoxy-ATP were performed according to the method of Sambrook *et al.* (30). Variations in loading were accounted for by normalizing to the β-actin mRNA content in each lane, which was measured by hybridization with a ^{32}P-labeled rat actin cDNA probe (Fig. 1). Autoradiographs were quantified densitometrically with a model 620 video densitometer using 2D Analyst II software (Bio-Rad, Richmond, CA). Results were calculated as the ratios between the integrated optical densities of TSHβ mRNA and β-actin mRNA, and expressed as a percentage of the mean of the respective control values.

Statistical analysis

Results are presented as the mean ± SEM. A nonparametric test (Wilcoxon matched pairs, signed ranks test) and analysis of variance for a repeated measures design were used to analyze the data. Provided that significant overall effects were obtained by analysis of variance, further comparisons between groups were made using Duncan's multiple range test. Differences were considered significant at $P < 0.05$.

Results

Infusion of IL-1 (4 μg/day) induced signs of physical discomfort in the animals, including piloerection and decreased physical activity, as observed on the first day after implantation of the pumps. This visually observable uneasiness gradually diminished and disappeared on day 2. Infusion of IL-6 at a dose of 15 μg/day did not induce signs of discomfort. Treatment of rats with saline did not perceptibly distress the animals.

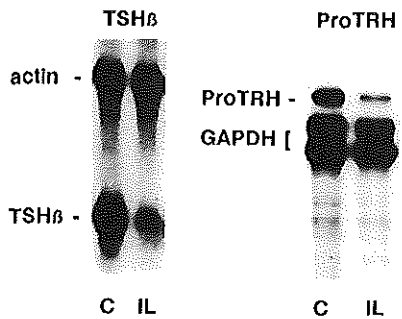

FIG. 1. Effect of continuous infusion of saline (C) or 4 μg IL-1/day (IL) for 1 week on pituitary TSHβ mRNA (*left panel*) and hypothalamic pro-TRH mRNA (*right panel*). Northern blot hybridization analysis was used to estimate TSHβ mRNA, whereas pro-TRH mRNA was determined with a RNase protection assay. Variation in loading was accounted for by normalizing to the β-actin mRNA and GAPDH mRNA contents, respectively.

Effects of IL-1 and IL-6 on rectal temperature and body weight

Saline-treated rats maintained a virtually constant mean daily rectal temperature throughout the experimental period. On the first day of infusion, IL-1 induced a significant increase in rectal temperature, which returned to normal levels between days 2–4 (Fig. 2), whereas IL-6-treated rats had no significant increase in rectal temperature compared to saline-treated rats.

There was a small decrease in body weight on the first day of saline infusion (Fig. 2). A similar weight loss was found in animals treated with IL-6 (15 μg/day), whereas rats infused with IL-1 (4 μg/day) showed a more distinct weight loss. The body weights of IL-1-treated rats reached minimal levels on the second day of infusion. Thereafter, the rate of body weight gain was slightly higher in IL-1-treated rats than in saline-treated control rats.

Effects of IL-1 and IL-6 on food and fluid intake

The effects of chronic administration of IL-1 and IL-6 on food and fluid consumption were monitored for 9 days, and results are shown in Fig. 3. There was a transient slight reduction in food consumption in saline-treated rats after implantation of the osmotic pumps. Compared to saline-treated animals, rats treated with IL-6 (15 μg/day) showed no significant change in food consumption, whereas the infusion of IL-1 (4 μg/day) caused a significant decrease in food intake compared to that in saline-treated rats during the first 5 days after starting the infusion. Chronic infusion of physiological saline, IL-1, or IL-6 into rats caused a significant decrease in total daily fluid intake on the first day of the infusion. During the following day, the fluid intake had returned to preinfusion values in all groups.

Effects of IL-1 on plasma T_4, FT_4, T_3, TSH, and corticosterone levels

Figures 4 and 5 show the effects of continuous infusion for 1 week with 4 μg IL-1/day or saline on plasma T_4, FT_4, T_3, and TSH. Infusion of 4 μg IL-1/day induced a highly significant decrease in plasma T_4, which reached minimum levels on day 2 and remained significantly suppressed throughout the experimental period. IL-1 induced a marked transient increase in the plasma FT_4 fraction (not shown), and the decline in plasma FT_4 in IL-1 rats was less pronounced and of shorter duration than that in total T_4. By the end of the infusion period, when plasma T_4 levels were still decreased, plasma FT_4 had returned to control levels. Parallel with the decrease in T_4 concentrations, plasma T_3 was significantly lower in IL-1-infused animals than in saline-treated rats. The nadir was reached on day 2 of the infusion, and plasma T_3 remained significantly lower in IL-1-treated ani-

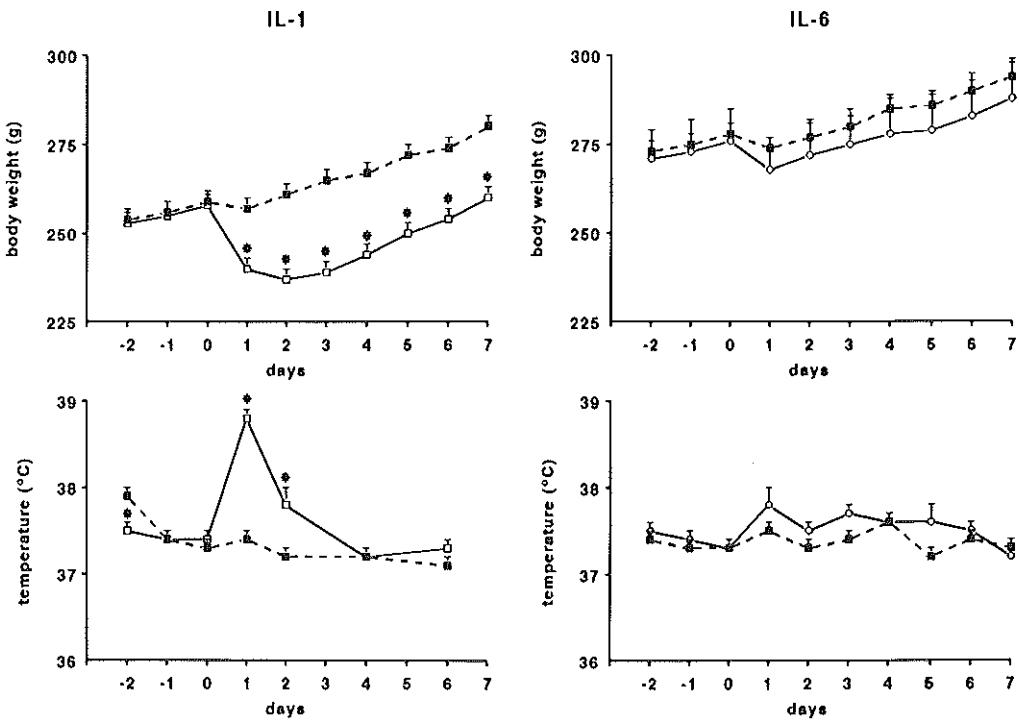

FIG. 2. Effects of continuous infusion of 4 μg IL-1/day (□), 15 μg IL-6/day (○), or saline (■) for 1 week on body weight and rectal temperature. Data are presented as the mean ± SEM of 7–17 rats. *, $P < 0.05$ compared to saline-infused rats.

mals than in saline-treated animals until the end of the experiment. Chronic administration of IL-1 induced a dramatic decline in plasma TSH. The nadir was reached on the first day of the infusion, after which plasma TSH levels in IL-1-treated rats started to increase slowly. Short term infusion with IL-1 had effects on thyroid function similar to those of long term infusion (Table 1). Levels of plasma corticosterone increased dramatically after 1 day of IL-1 infusion and were significantly elevated compared to levels in control rats for at least 4 days (Fig. 6).

Effects of IL-1 on pro-TRH mRNA, TSHβ mRNA, and type I deiodinase

In Table 2, the effects of treatment with IL-1 on hypothalamic pro-TRH mRNA, pituitary TSHβ mRNA, and liver type I deiodinase are given. During the first 2 days of infusion, the levels of hypothalamic pro-TRH mRNA in IL-1-treated rats were not significantly different from those in saline-treated rats. In addition, the TRH content in the median eminence did not change after 1 day of IL-1 infusion (Table 2). However, on day 7 of infusion, the level of hypothalamic pro-TRH mRNA was 73% lower in IL-1 rats than in controls. In the pituitary gland, the levels of TSHβ mRNA showed a significant decline after 2 days of IL-1 infusion. On days 2 and 7 of infusion, pituitary TSHβ mRNA levels were reduced to 38% of the levels in control rats. Liver type I deiodinase activity showed a significant decline due to IL-1 infusion on days 1, 2, and 7.

Effects of IL-6 on plasma T_4, FT_4, T_3, TSH, and corticosterone

Figures 4 and 5 show the effects of continuous infusion of rats for 1 week with IL-6 (15 μg/day) or saline on plasma T_4, FT_4, T_3, and TSH. Plasma T_4 was significantly lower in IL-6-infused animals than in control rats on days 2 and 3 of infusion. IL-6 produced a marked transient increase in the plasma FT_4 fraction (not shown), but plasma FT_4 in IL-6 rats did not change during the experiment. A significant decrease in plasma T_3 was found in IL-6-treated rats compared to their starting levels, but no significant effects were observed compared to saline-infused control values. Infusion of IL-6 induced a significant decline in plasma TSH. The nadir was reached on day 2 of the infusion, but plasma TSH recovered quickly, and within 4 days, the levels were again in the range found in control animals. Compared to the effects of IL-1 infusion on thyroid and pituitary function, the effects of IL-6 administration were less pronounced. This was also seen in the effects of these ILs on plasma corticosterone, because IL-6 administration did not affect the levels of plasma corticosterone, whereas IL-1 did (Fig. 6).

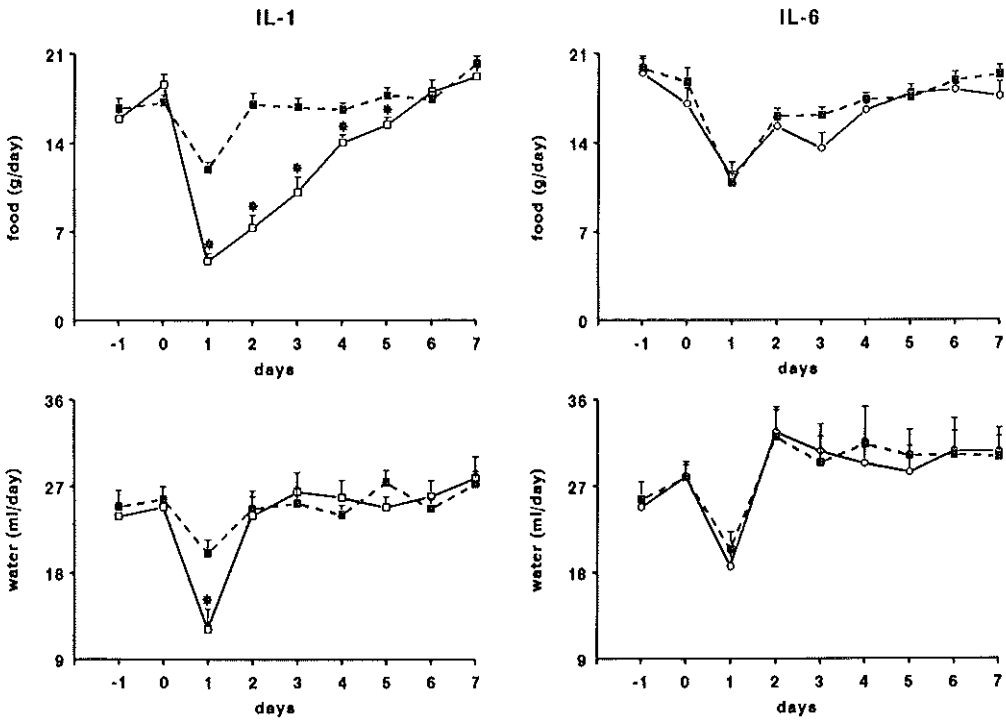

FIG. 3. Effects of continuous infusion of 4 μg IL-1/day (□), 15 μg IL-6/day (○), or saline (■) for 1 week on food and water intake. Data are presented as the mean ± SEM of 7–17 rats. *, $P < 0.05$ compared to saline-infused rats.

Effects of IL-6 on pro-TRH mRNA and TSH mRNA

In Table 3, the effects of continuous treatment with IL-6 on hypothalamic pro-TRH mRNA and pituitary TSHβ mRNA are shown. After 7 days of IL-6 administration, no effects were seen on the levels of hypothalamic pro-TRH mRNA. In the pituitary gland, the levels of TSHβ mRNA showed an insignificant decline after 7 days of infusion of IL-6.

Discussion

The suppressive effects of short term and continuous *in vivo* IL-1 administration on pituitary-thyroid function in rats have been reported in two previous studies (4, 18), in which it was shown that the reduction of food intake cannot explain the changes in thyroid hormone and TSH levels during IL-1 treatment (18). As the mechanisms of the effects of cytokine on thyroid function are not fully understood, we studied in particular the centrally mediated effects of IL-1α in more detail. Furthermore, as a number of IL-1 effects may be mediated by IL-6, we compared the effects of IL-1 and IL-6 infusions on plasma T_4, FT_4, T_3, TSH, and corticosterone; hypophysial TSHβ mRNA; median eminence content of TRH; and hypothalamic pro-TRH mRNA.

Infusion of both IL-1 and IL-6 produced a marked transient decrease in plasma T_4, which was more pronounced with IL-1 than with IL-6. Plasma FT_4 was also decreased by IL-1, but not by IL-6. These cytokines produced similar increases in the plasma FT_4 fraction (not shown), suggesting that IL-1 and IL-6 infusions both decreased plasma T_4 binding. Previous findings have shown that the decrease in plasma T_4 binding during IL-1 administration is due at least in part to a decrease in the plasma level of transthyretin, which is the principal plasma T_4-binding protein in rats (18). A decrease in transthyretin production is one of the hallmarks of the acute phase response of the liver to inflammation, which is largely mediated by IL-6 (33). As IL-1 is known to stimulate IL-6 production (33), it is likely that the effect of IL-1 on plasma T_4 binding is mediated by IL-6. However, besides the fall in plasma transthyretin a decrease in plasma albumin (33) and an increase in plasma FFA (4) may contribute to the lowered plasma T_4 binding during IL-1 and IL-6 administration.

The decrease in plasma FT_4 during IL-1 administration may be the result of a decrease in thyroidal T_4 production and/or an increase in plasma T_4 clearance. Dubuis *et al.* (4) demonstrated that plasma T_4 clearance is not affected by IL-1 administration despite a large increase in the plasma FT_4 fraction, suggesting that the metabolism of T_4 in the tissues

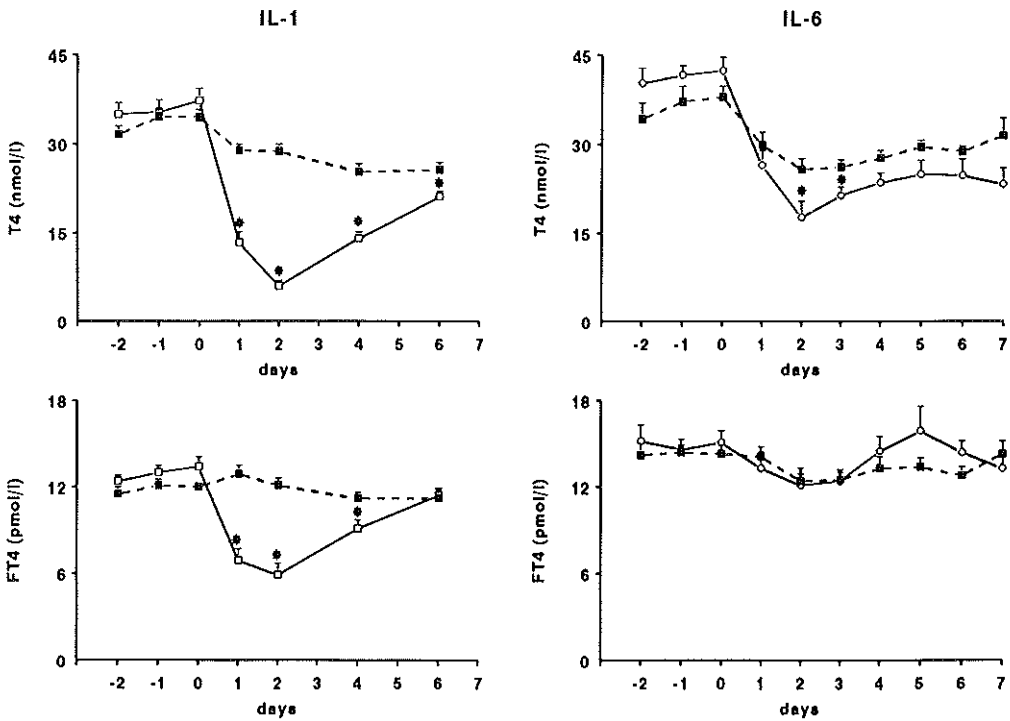

FIG. 4. Effects of continuous infusion of 4 μg IL-1/day (□), 15 μg IL-6/day (○), or saline (■) for 1 week on plasma TSH and T_3 levels. Blood samples were taken from an indwelling jugular venous cannula between 10–12 h. Data are presented as the mean ± SEM of 7–17 rats. *, $P < 0.05$ compared to saline-infused rats.

is decreased. This could be due to a decrease in tissue availability of plasma FT_4 or a decrease in the activity of T_4 metabolic pathways. Evidence has been presented that the fractional transfer rate constant for T_4 transport from plasma to liver is decreased in humans during severe illness and fasting (34, 35). Although changes in hepatic type I deiodinase activity have not been detected previously after both short and long term administration of IL-1 to rats (4, 18), significantly decreased deiodinase activities were found in the present study after 1, 2, and 7 days of IL-1 infusion. The reason for the differences in the effects of IL-1 on liver type I deiodinase between the previous (18) and the present studies could be due to the higher dose of IL-1 infused in the present study (4 *vs.* 2 μg/day). It should be stressed, however, that the decreases we observed were relatively small (~25%). It is not known to what extent these decreases were caused directly by an effect of IL-1 or IL-6 on the liver or indirectly through the IL-1-induced reduced food intake or hypothyroid state, which are both associated with a decrease in hepatic deiodinase activity (36). Surprisingly, infusion of mice with IL-1 for 3 days has been found to increase hepatic type I deiodinase activity, in contrast to the decrease found in animals with a similar reduction in food intake (7).

The reduced plasma T_4 and FT_4 levels induced by IL-1 in combination with a presumably normal plasma T_4 clearance rate, as found by others (4), suggest that IL-1 inhibits thyroidal T_4 secretion. The decrease in plasma T_3 during IL-1 administration may be due to 1) diminished T_3 secretion, 2) reduced peripheral T_3 production through a decrease in type I deiodinase activity and/or T_4 substrate availability, and/or 3) decreased plasma T_3 binding. An increased plasma FT_3 fraction was observed by Dubuis *et al.* (4) after IL-1 administration, although the effect was smaller than the increase in the plasma FT_4 fraction. IL-1 can inhibit thyroidal T_4 and T_3 secretion by a well documented direct effect on the thyrocyte, whereas IL-6 has little or no direct effect on thyroid activity (8, 37–40). However, the effects of IL-1 on thyroid function also appear to be mediated at least in part by the decrease in serum TSH.

In agreement with previous reports, IL-1 infusion resulted in a dramatic and acute decrease in serum TSH (4, 18), which was more rapid in onset and longer in duration than the decrease induced by IL-6. The latter may explain in part why, in contrast with IL-1, the decrease in serum TSH in IL-6-treated rats is not associated with a decrease in serum FT_4. Although the effects of cytokine administration on the clearance of plasma TSH have not been determined, the decreased serum TSH level probably reflects an acute decrease in

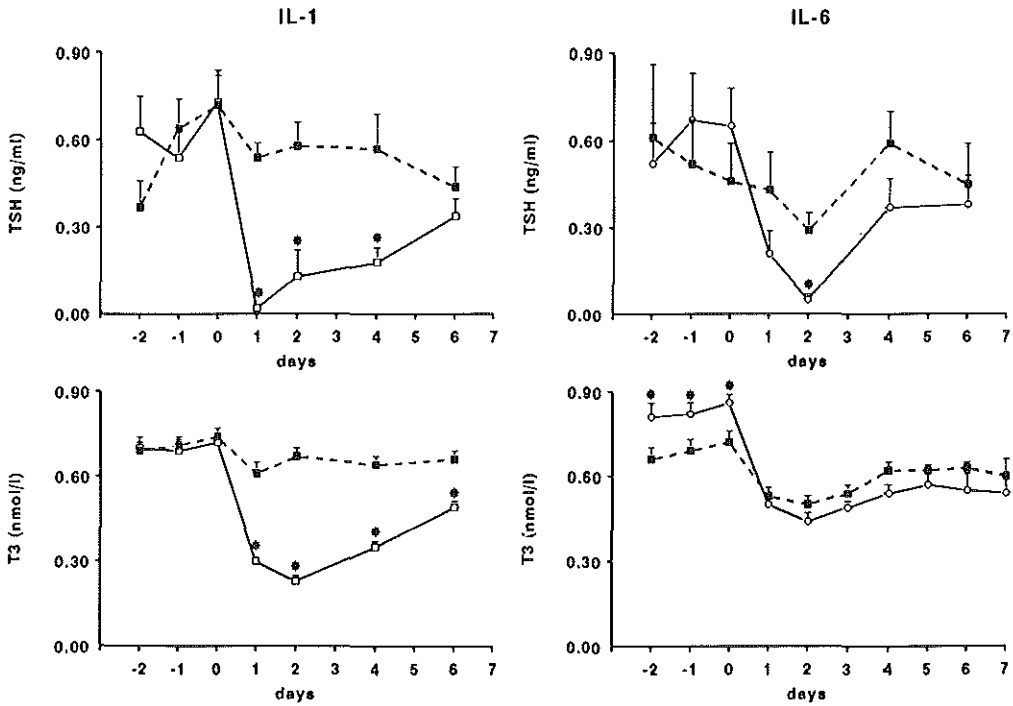

FIG. 5. Effects of continuous infusion of 4 μg IL-1/day (□), 16 μg IL-6/day (○), or saline (■) for 1 week on plasma T_4 and FT_4 levels. Blood samples were taken daily from an indwelling jugular venous cannula between 10–12 h. Data are presented as the mean ± SEM of 7–17 rats. *, $P < 0.05$ compared to saline-infused rats.

TABLE 1. The effects of continuous infusion of IL-1 (4 μg/day) or saline for 1 or 2 days were measured on plasma TSH, T_3, and T_4 levels in male rats

Parameter	Treatment	Day 1	Day 2
TSH (ng/ml)	Saline	0.38 ± 0.08	0.81 ± 0.09
	IL-1	0.11 ± 0.02*	0.06 ± 0.03*
T_3 (nmol/liter)	Saline	0.70 ± 0.06	0.61 ± 0.03
	IL-1	0.25 ± 0.02*	0.20 ± 0.02*
T_4 (nmol/liter)	Saline	40.7 ± 3.5	33.7 ± 1.4
	IL-1	12.3 ± 1.3*	4.5 ± 0.8*

Data are presented as the mean ± SEM of six or seven rats. After 1 or 2 days of infusion, trunk blood was collected after decapitation.
* $P < 0.05$ compared to saline-infused rats.

hypophyseal TSH secretion. This may be due to the direct effects of IL-1 and IL-6 on the thyrotroph or to alterations in hypothalamic or peripheral factors involved with TSH regulation. Concerning the latter, plasma FT_4 may be transiently increased acutely after commencement of cytokine administration, resulting in long-lived feedback inhibition of TSH secretion (4). It is remarkable, however, that hypophyseal TSHβ mRNA was not decreased after 1 day of IL-1 administration at the time serum TSH was at its nadir. Although this lack of an acute effect on hypophyseal TSHβ mRNA does not exclude a decrease in TSH synthesis, these results suggest that the IL-1-induced decrease in serum TSH after 1 day of IL-1 infusion is not secondary to a decreased TSH biosynthesis.

Inflammation in general and administration of cytokines such as IL-1 in particular have profound effects on multiple hypophyseal hormones, *e.g.* ACTH secretion is acutely increased (12–16, 24), whereas the secretions of TSH (4, 18), LH (17), and GH (41) are decreased. The effects of IL-1 on ACTH and LH secretion appear to be mediated largely by an increase in the hypothalamic production and secretion of CRF (42–44) and a decrease in the production and secretion of GnRH (45, 46), respectively. Evidence has also been presented that inhibition of GH secretion by IL-1 is due to an increased supply of hypothalamic somatostatin (47, 48). A suprahypophysial action of IL-1 on TSH secretion is supported by observations that intracerebroventricular administration of minute amounts of IL-1 produces a significant decline in plasma TSH in rats (49). The observation that not only basal serum TSH levels, but also their response to TRH stimulation are decreased during IL-1 infusion (18) suggests that a possible suprahypophysial effect of IL-1 on TSH secretion may be mediated by increased hypothalamic release of somatostatin, rather than decreased release of TRH. This is in agreement with the present findings that serum TSH

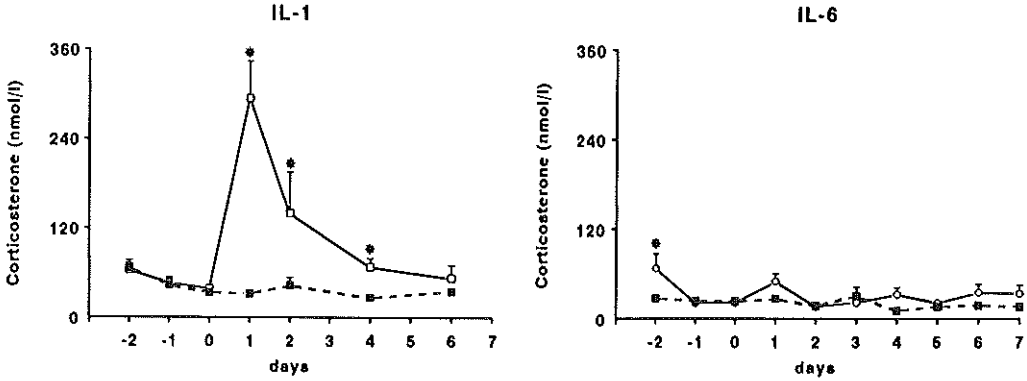

FIG. 6. Effects of continuous infusion of 4 μg IL-1/day (□), 15 μg IL-6/day (○), or saline (■) for 1 week on plasma corticosterone levels. Blood samples were taken daily from an indwelling jugular venous cannula between 10–12 h. Data are presented as the mean ± SEM of 7–17 rats. *, $P < 0.05$ compared to saline-infused rats.

TABLE 2. Effects of IL-1 (4 μg/day) infusion for 1, 2, or 7 days on the levels of hypothalamic pro-TRH mRNA, median eminence (ME) content of TRH, hypophysial TSHβ mRNA, and hepatic type I deiodinase in male rats

Parameter	Treatment	Day 1	Day 2	Day 7
Pro-TRH mRNA	Saline	100 ± 33	100 ± 25	100 ± 27
	IL-1	135 ± 17	125 ± 56	27 ± 7*
TRH in ME (ng)	Saline	1.3 ± 0.25	ND	ND
	IL-1	1.4 ± 0.28	ND	ND
TSHβ mRNA	Saline	100 ± 33	100 ± 14	100 ± 10
	IL-1	76 ± 30	38 ± 8*	38 ± 2*
Deiodinase (pmol/min·mg)	Saline	306 ± 25	208 ± 20	195 ± 13
	IL-1	243 ± 9*	159 ± 8*	123 ± 13*

Results are presented as the mean ± SEM ratios of the optical densities of pro-TRH mRNA over GAPDH mRNA or of TSHβ mRNA over β-actin mRNA, and expressed as a percentage of the mean of the respective control values. Groups contained five to nine rats. ND, Not determined.

* $P < 0.05$ compared to saline-infused rats.

TABLE 3. Effects of IL-6 (15 μg/day) infusion for 7 days on the levels of hypothalamic pro-TRH mRNA and hypophysial TSHβ mRNA in male rats

Parameter	Treatment	Day 7
Pro-TRH mRNA	Saline	100 ± 9
	IL-6	103 ± 22
TSHβ mRNA	Saline	100 ± 31
	IL-6	64 ± 7

Results are presented as the mean ± SEM ratios of the optical densities of pro-TRH mRNA over GAPDH mRNA or of TSHβ mRNA over β-actin mRNA, and expressed as a percentage of the mean of the respective control values. Groups contained six to eight rats.

and hypophyseal TSHβ mRNA are decreased before an effect of IL-1 is observed on hypothalamic pro-TRH mRNA. However, the lack of short term effects of IL-1 infusion on hypothalamic pro-TRH mRNA levels and median eminence TRH content does not exclude the possibility that IL-1 acutely inhibits TRH release into hypophyseal portal blood.

Direct effects of cytokines on anterior pituitary cells in culture have been reported, although this includes, paradoxically, stimulation of the secretion of TSH, LH, and GH (14). In this respect it is worthwhile to mention that both IL-1 and IL-6 are produced in the anterior pituitary and may, thus, act as paracrine factors in the regulation of hypophysial hormones (50, 51). In our study, IL-6 did not appear to act on the hypothalamus, as it failed to induce fever, nor did it stimulate the hypothalamic-hypophyseal-adrenal axis. It seems likely, therefore, that the effect of IL-6 on TSH secretion does not involve an action at the hypothalamic level, but, rather, a direct effect on the thyrotroph. As IL-1 induces the production of IL-6 (33), the effect of IL-1 infusion on TSH secretion may be mediated in part by this action of IL-6 on the pituitary.

As pro-TRH gene expression is only suppressed after 7 days of IL-1 infusion, it is likely that this effect is mediated by factors other than IL-1 itself. As discussed above, hypothalamic CRF gene expression is acutely stimulated by IL-1. As CRF neurons lie adjacent to TRH neurons in the paraventricular nucleus (PVN) (52, 53), the effects of IL-1 on TRH neurons may be mediated by local factors produced by CRF neurons. Kakucska *et al.* (54) showed by *in situ* hybridization a reduction of pro-TRH mRNA in the PVN 24 h after a constant intracerebroventricular infusion of IL-1, at the same time when pro-CRF mRNA in the PVN was increased. This inverse relationship between the levels of pro-TRH mRNA and CRF mRNA in PVN neurons has also been observed during hypothyroidism (55). Furthermore, high concentrations of glucocorticoids due to activation of the pituitary-adrenal axis may influence hypothalamic TRH production and secretion. In our study we demonstrated an increase in plasma corticosterone during at least 4 days of IL-1 infusion, whereas IL-6 infusion had no effect. A suppressive effect of plasma corticosterone on TRH gene expression would explain the different effects of IL-1 and IL-6 on pro-TRH mRNA. This hypothesis is supported by 1) the

reduction in pro-TRH mRNA in the PVN after chronic high dose glucocorticoid treatment (56), 2) the presence of a consensus glucocorticoid response element in the TRH gene promoter (57), and 3) the coexistence of glucocorticoid receptors in TRH neurons in the PVN (58).

In conclusion, our findings suggest that in addition to the direct inhibition of thyroid hormone production by IL-1, the multiple effects of this cytokine on the hypothalamus-pituitary-thyroid axis include 1) a decrease in plasma T_4 binding; 2) an acute decrease in TSH secretion, followed by a decrease in TSH synthesis; and 3) only after prolonged IL-1 administration, a decrease in hypothalamic pro-TRH gene expression. The transient decrease in plasma T_4 binding and the acute decrease in TSH secretion are also observed during IL-6 infusion. The acute decrease in TSH secretion occurs before (IL-1) or even without (IL-6) a decrease in hypothalamic pro-TRH mRNA and, therefore, does not appear to be the result of decreased hypothalamic TRH synthesis, although a decrease in hypothalamic TRH release is not excluded. The decreased TSH secretion may also involve an increased supply of hypothalamic somatostatin as well as an effect via IL-6 directly on the thyrotroph. The decrease in pro-TRH gene expression by prolonged infusion of IL-1 may be mediated by the high plasma corticosterone levels.

Acknowledgments

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STRAIN-SPECIFIC EFFECTS OF STREPTOZOTOCIN-INDUCED DIABETES MELLITUS ON THE HYPOTHALAMUS-PITUITARY-THYROID AXIS IN RATS

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Abstract

Streptozotocin-induced diabetes mellitus causes a decrease in the release of thyrotropin (TSH) and thyroid function. We hypothesized that the reduced thyroid function in diabetic rats could be due to an altered hypothalamic synthesis of TSH-releasing hormone (TRH). Therefore, proTRH mRNA was measured in male rats of two Wistar substrains, e.g. R-Amsterdam (R-A) and RxU rats, 3 successive weeks after *iv* injection with vehicle or streptozotocin (STZ, 65 mg/kg body weight). Furthermore, we determined TRH content in the median eminence, pituitary TSHß subunit mRNA, plasma TSH, corticosterone and thyroid hormones, and liver enzymes involved in thyroid hormone metabolism. Hypothalamic proTRH mRNA showed a time-dependent decrease in diabetic R-A rats, but not in RxU rats, although the values were not significantly different from those in controls. In both rat strains, the TRH content in the median eminence increased significantly after STZ injection. In diabetic R-A rats pituitary TSHß mRNA decreased in time, whereas it was unchanged in RxU rats 3 weeks after diabetes-induction. Plasma TSH showed a strong decline in both rat strains at all times after STZ injection. In contrast to RxU rats, plasma corticosterone increased significantly at 1 and 2 weeks after STZ injection in R-A rats. In diabetic R-A rats plasma total T4 decreased while the free T4 fraction increased, resulting in normal plasma free T4 levels. Plasma total T3 and free T3 fraction showed a decline in both rat substrains, resulting in strongly decreased free T3 levels. Hepatic type I deiodinase activity decreased and T4 UDP-glucuronyltransferase activity increased in R-A rats. The parallel decrease in hypothalamic proTRH mRNA and hypophyseal TSHß mRNA in association with the increase in plasma corticosterone in R-A rats, but not in RxU rats, suggests strain-dependent inhibition of proTRH and TSHß gene expression in STZ-induced diabetes mellitus, mediated by the increased corticosterone. Additional mechanisms must exist for the diabetes-induced suppression of TSH secretion to explain the decrease in plasma TSH in diabetic RxU rats. The opposite effects of STZ-induced diabetes on the free fractions of plasma T4 and T3 are explained by an increase in thyroxine-binding globulin and a decrease in thyroxine-binding prealbumin. Decreased hepatic T4 to T3 conversion and increased T4 glucuronidation contribute to the generation of the low T3 syndrome in STZ-induced diabetic rats.

Introduction

STZ-induced diabetes mellitus, which is frequently used as a model to generate the low T3 syndrome (1, 2), affects various sites of the hypothalamic-pituitary-thyroid axis. In STZ diabetic rats lower (3, 4) or unchanged (5, 6) hypothalamic TRH concentrations and decreased in vivo (4) and in vitro (7) TRH secretion have been reported. Plasma thyroid stimulating-hormone (TSH) in diabetic rats is decreased in diabetic rats and the TSH response to TRH has been reported to be normal (4) or diminished (8), while plasma TSH clearance is unaltered. Furthermore, plasma T4 and T3 levels and plasma T4 and T3 production rates as well as T4 conversion to T3 in peripheral tissues and in the pituitary gland have been found to decrease in STZ-diabetic rats (4, 9-11).

Using two locally bred Wistar substrains, e.g. R-Amsterdam (R-A) and RxU rats, we investigated if the STZ-induced suppression of thyroid function originates from a decrease in hypothalamic proTRH gene expression. Therefore, hypothalamic proTRH mRNA and pituitary TSHß subunit mRNA were measured in control and STZ-induced diabetic rats. Plasma corticosterone was determined to identify its possible role in mediating the down-regulation of the hypothalamus-pituitary-thyroid axis. In order to elucidate peripheral mechanisms involved in the generation of the low T3 syndrome, we also investigated the effects of diabetes on thyroid hormone-binding proteins and on liver enzymes involved in thyroid hormone metabolism.

Materials and methods

Animals

Male R-A and RxU rats were used in this study. They were housed under controlled conditions (20-22 C; lights on between 05.00 and 19.00 h) and allowed free access to water and food. For all experiments, approval was obtained from the Animal Welfare Committee (DEC) of the Erasmus University.

Experimental design

Under ether anesthesia, rats were injected into the external jugular vein with 65 mg/kg body weight STZ (Sigma, St. Louis, MO, USA) in 0.2 ml citrate-buffered saline (pH 4.5) or with 0.2 ml vehicle. To avoid degradation, STZ was dissolved immediatly before injection. Body weights of the rats were determined before, and

at weekly intervals during the experiment. At 1, 2 or 3 weeks after administration of STZ or vehicle, groups of rats were decapitated between 10.00 and 13.00 h. Trunk blood was collected for measurement of blood glucose using the Haemo-Glukotest (Reflolux, Boehringer, Mannheim, Germany) and to determine plasma hormone levels. Livers were removed, cut into small pieces, frozen in liquid nitrogen, and kept at -80 C until estimation of enzyme activities. The skull was opened to isolate the pituitary gland, the median eminence, and the remainder of the hypothalamus as described previously (12). The anterior pituitary gland was frozen in liquid nitrogen and stored at -80 C until estimation of TSHß mRNA. The median eminence was extracted with 2 ml methanol for measurement of TRH. The remaining part of the hypothalamus was snap frozen in liquid nitrogen and kept at -80 C until RNA isolation.

Measurement of proTRH mRNA and TSHß mRNA

RNA was isolated from the hypothalamus and the anterior pituitary, and proTRH mRNA and TSHß mRNA were determined by Northern blotting, as described previously (13, 14). Results were calculated as the ratio of proTRH mRNA or TSHß mRNA over ß-actin mRNA, and expressed as the percentage of the mean in control rats.

Enzyme assays

Liver microsomes were prepared and microsomal type I iodothyronine deiodinase (ID1) and UDP-glucuronyltransferase (UGT) activities were determined essentially as previously described (15). Conditions for the ID1 assay were: 1 µM rT3 (substrate), 5 mM dithiothreitol (cofactor), 25 µg/ml microsomal protein, and 30 min incubation at 37 C. UGT activities were assayed at 1 (T4, rT3) or 100 µM (bilirubin) substrate, 5 mM UDP-glucuronic acid (cofactor), 1 (T4, bilirubin) or 0.25 (rT3) mg/ml microsomal protein, and 60 (T4, rT3) or 15 (bilirubin) min incubation at 37 C.

Hormone determinations and agar electrophoresis

Plasma TSH was measured by radioimmunoassay with materials and protocols supplied by the NIDDK, with rat-TSH-RP-2 as standard. Levels of T4 and T3 were estimated by specific radioimmunoassays in unextracted plasma. The plasma free T4 fraction (FFT4) was measured by equilibrium dialysis (16), and plasma free T4 (FT4) was calculated as the product of total T4 and FFT4. A similar procedure was

followed to measure plasma free T3 (FT3). Corticosterone was estimated by radioimmunoassay (17). The radioimmunoassay for TRH was performed with antiserum #8880 (18, 19). Detection limits were 0.2 µg/l RP-2 TSH, 2 nmol/l T4, 0.1 nmol/l T3, 0.05 nmol/l rT3, 1 nmol/l corticosterone, and 3-5 fmol TRH/tube. Intra- and interassay coefficients of variation for the assays varied between 5 and 15%.

Agar gal electrophoresis was performed using 0.9% Agar Noble (Difco, Detroit, MI, USA) and 0.2 M glycine, 0.13 M sodium acetate buffer (pH 8.6) was performed as described by Docter et al (20) to determine the distribution of serum T4 and T3 over their binding proteins.

Statistical analysis

Results are presented as means±SEM. Analysis of variance was used for statistical evaluation of the data. Provided significant overall effects were obtained, comparisons between groups were made by Duncan's new multiple range tests. Differences were considered to be significant at $p \leq 0.05$.

Results

Effects of diabetes on body weight and blood glucose

Changes in body weight and blood glucose after intravenous administration of vehicle or STZ in both rat substrains are presented in Table 1. Compared to vehicle-injected controls, body weight in R-A rats was reduced at 2 and 3 weeks after STZ injection. In RxU rats body weight in the experimental animals was lower from the first week of STZ injection. In both Wistar substrains blood glucose was increased in STZ diabetic rats.

Effect of diabetes on hypothalamic proTRH and TRH content in the median eminence

Hypothalamic proTRH mRNA in R-A rats decreased significantly between 1 and 3 weeks after STZ injection (Fig. 1A), although values were not significantly different from controls. RxU rats showed no significant change in the levels of hypothalamic proTRH mRNA after STZ injection. In both rat strains, the TRH content in the median eminence increased significantly after STZ injection, compared to control rats (Fig. 1B).

Strain		R-A rats		RxU rats	
	Week	Control (n=10)	STZ (n=9)	Control (n=15)	STZ (n=18)
Body weight (g)	1	296±10	259±15	328±11	295±7[a]
	2	304±8	253±14[a]	347±8	292±6[a]
	3	316±7	244±14[a]	358±8[b]	294±7[a]
Glucose (mmol/l)	1	N.D.	22.3±0.9	N.D.	24.3±2.5
	2	N.D.	27.6±1.9[b]	N.D.	28.2±0.7
	3	6.7±0.2	28.6±2.0[ab]	5.1±0.3	26.9±1.0[a]

Table 1. Body weights and blood glucose in male control and STZ injected R-A and RxU rats (mean ± SEM).
[a] P≤0.05 vs. control rats; [b] P≤0.05 vs. week 1; N.D. not determined

Effects of diabetes on anterior pituitary TSHß mRNA and plasma TSH
Streptozotocin-induced diabetes mellitus caused a highly significant decrease in the levels of anterior pituitary TSHß mRNA in R-A rats, which reached minimum levels 3 weeks after STZ treatment (Fig. 1C). No change was seen in RxU rats, 3 weeks after STZ injection. Plasma TSH showed a significant decrease in both rat strains at all times after STZ injection, although the effects were more pronounced in R-A rats (Table 2).

Effects of diabetes on plasma thyroid hormones, binding proteins and corticosterone
At all times investigated, plasma T4 was significantly lower in diabetic than in control R-A rats, while plasma FFT4 was significantly increased after 1 and 3 weeks. As a consequence, plasma FT4 remained unchanged (Table 2). As for RxU rats, only plasma T4 was measured which was significantly decreased after STZ injection (Table 2). Plasma T3, FFT3 and thus FT3 were all decreased in diabetic R-A rats, which changes were significant after 2 and 3 weeks. In diabetic RxU rats plasma T3, FFT3 and FT3 were significantly decreased at all time points, except for

FFT3 which showed a significant increase 3 weeks after STZ injection.

Plasma of control and diabetic R-A and RxU rats was incubated with [^{125}I]T4 or [^{125}I]T3 and analysed by agar gel electrophoresis. In contrast to human serum (20), rat albumin and thyroxine-binding prealbumin were not separated by this method. Therefore, the first peak represents thyroxine-binding globulin (TBG) and the second peak contains both albumin and TBPA. In control rats radioactive T3 was bound to TBG and albumin-TBPA in a ratio of 1:5 which changed to 1:1 in diabetic rats, 3 weeks after STZ injection. A single peak of protein-bound labelled T4 was found in the albumin-TBPA region in control rats. In diabetic rats a small second radioactive peak was found in the TBG region.

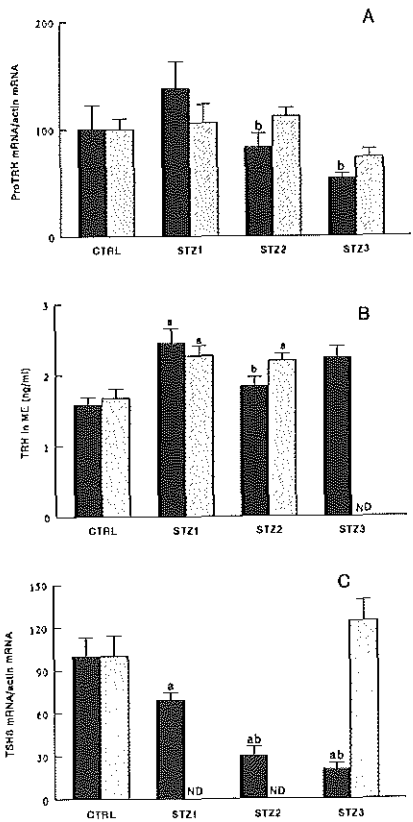

Figure 1 *Effect of STZ-induced diabetes mellitus on hypothalamic proTRH mRNA (A), TRH content in the median eminence (B) and pituitary TSHß mRNA (C) 1, 2 or 3 weeks after injection in male R-A (solid bars) and RxU (hatched bars) rats. mRNA data are presented as the mean ± SEM ratio of the optical densities of proTRH mRNA and TSHß mRNA over ß-actin mRNA, expressed as a percentage of the mean of the control values. Groups contained 5-10 rats. [a] P≤0.05 vs. control rats; [b] P≤0.05 vs. week 1; N.D. not determined*

In contrast to RxU rats, plasma corticosterone increased significantly at 1 and 2 weeks after STZ injection in R-A rats (Table 2). Although the mean in R-A rats was still elevated after 3 weeks, it was not significantly different from controls.

Effects of diabetes on hepatic UGT and ID1 activities

Effects of STZ-induced diabetes on the activity of liver enzymes involved in thyroid hormone metabolism were measured in R-A rats and are presented in Fig. 2. The T4 , reversed T3 (rT3) and bilirubin UGT activities increased gradually after induction of diabetes mellitus, which changes were already significant 1-week after STZ injection (Fig. 2A-C). ID1 activity was significantly lower in diabetic rats than in vehicle-injected controls, reaching a minimum 2 weeks after STZ treatment (Fig. 2D).

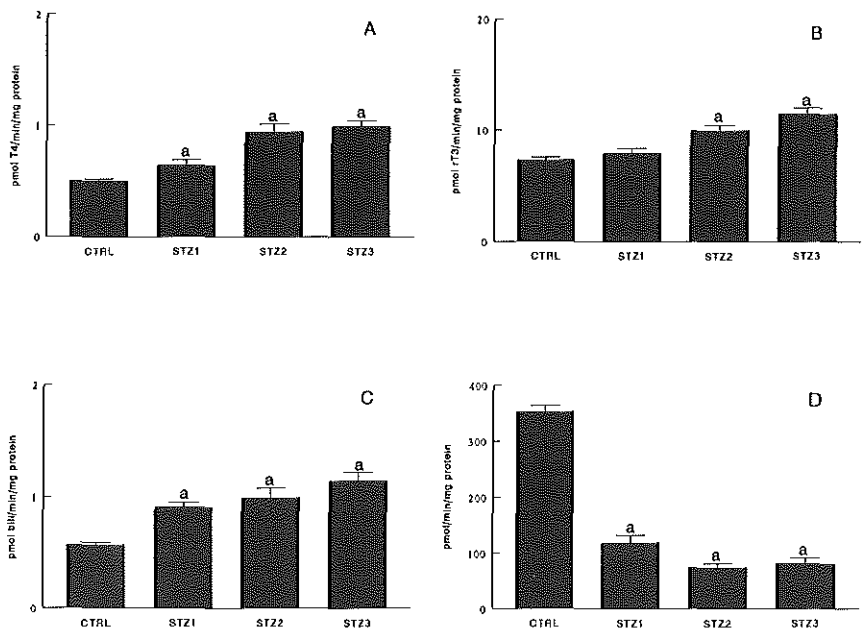

Figure 2 *Effect of STZ-induced diabetes mellitus on UGT activities for T4 (A), rT3 (B), bilirubin (C), and ID1 (D) activity in liver microsomes 1, 2 or 3 weeks after injection in male R-A rats.*
Results are means ± SEM per group (n=3-5).
[a] *P≤0.05 vs. control rats*

	R-A rats				RxU rats			
Parameter	Control	STZ 1 week	STZ 2 weeks	STZ 3 weeks	Control	STZ 1 week	STZ 2 weeks	STZ 3 weeks
T4 (nmol/l)	57.7±6.9 (n=10)	16.5±2.5[a] (n=10)	19.6±4.8[a] (n=10)	20.1±4.9[a] (n=8)	80.7±1.5 (n=25)	49.3±7.6[a] (n=4)	45.4±3.7[a] (n=5)	40.1±3.2[a] (n=18)
FFT4 (%)	0.03±0.001 (n=5)	0.093±0.02[a] (n=5)	0.055±0.012 (n=5)	0.064±0.006[a] (n=5)	N.D.	N.D.	N.D.	N.D.
FT4 (pmol/l)	23.07±1.33 (n=5)	18.05±1.99 (n=5)	20.33±3.5 (n=5)	20.77±5.0 (n=8)	N.D.	N.D.	N.D.	N.D.
T3 (nmol/l)	1.06±0.14 (n=10)	0.77±0.14 (n=10)	0.67±0.08[a] (n=10)	0.72±0.07[a] (n=8)	1.22±0.02 (n=25)	0.71±0.07[a] (n=4)	0.53±0.05[a] (n=5)	0.64±0.04[a] (n=18)
FFT3 (%)	0.60±0.05 (n=10)	0.79±0.10 (n=10)	0.53±0.07[a] (n=10)	0.33±0.09[a] (n=10)	0.71±0.03 (n=25)	0.45±0.09[a] (n=4)	0.58±0.06 (n=5)	0.92±0.04[a] (n=18)
FT3 (pmol/l)	5.87±0.53 (n=10)	4.99±0.68 (n=10)	3.45±0.58[a] (n=10)	2.09±0.61[a] (n=8)	8.62±0.34 (n=25)	3.20±0.67[a] (n=4)	3.69±0.77[a] (n=5)	6.37±0.64[a] (n=18)
TSH (ng/ml)	0.87±0.19 (n=10)	0.20±0.12[a] (n=10)	0.25±0.11[a] (n=10)	0.39±0.14[a] (n=7)	1.33±0.12 (n=25)	0.63±0.26[a] (n=4)	0.70±0.45[a] (n=5)	0.53±0.11[a] (n=18)
corticosterone (nmol/l)	75.9±16.6 (n=10)	155.9±27.5[a] (n=10)	193.2±45.1[a] (n=10)	197.5±91.8 (n=8)	92.0±17.3 (n=20)	90.8±32.1 (n=4)	95.4±50.7 (n=5)	149.9±24.2 (n=13)

Table 2. Plasma T4, FT4, FFT4, T3, FT3, FFT3, TSH and corticosterone in control and in 1, 2 or 3 week STZ-treated male R-A and RxU rats. Data are presented as the mean ± SEM per group.

[a] P≤0.05 vs. control rats; N.D. not determined

Discussion

Uncontrolled diabetes as well as several other conditions causing severe non-thyroidal illness are frequently associated with a suppression of TSH secretion and thyroid function. In this study we investigated the role of central changes in the hypothalamus-pituitary-thyroid axis and of changes in thyroid hormone metabolism in the diabetes-induced generation of the low T3 syndrome in 2 Wistar substrains, e.g. R-A and RxU rats.

Hypothalamic proTRH mRNA showed a decrease between 1 and 3 weeks after STZ injection in R-A rats, but not in RxU rats, suggesting a time- and strain-dependent decrease in TRH synthesis. did not change after STZ injection compared to control rats, but there was a significant reduction of proTRH mRNA in time in these diabetic rats (Fig. 1A). This decrease in proTRH synthesis is in line with the earlier reported reduction of in vivo and in vitro hypothalamic TRH release (4, 7). The lack of change in hypothalamic proTRH mRNA in RxU diabetic rats suggests a strain-dependent effect of STZ-induced diabetes on the hypothalamus. However, in contrast to total hypothalamic TRH content (3-6), median eminence TRH content is increased in diabetic animals of both substrains, which is compatible with a decreased TRH release as previously reported (4,7). Although this reduction in TRH release seems to occur independent of changes in proTRH gene expression, it may contribute to the profound decline in plasma TSH in STZ treated diabetic rats.

Together with the decrease in proTRH mRNA a gradual decrease in pituitary TSHß mRNA was observed in R-A rats, suggesting a causal relationship. However, because of the lack of effect of diabetes on both parameters in RxU rats, additional factors must be involved in the downregulation of plasma TSH that also occur in diabetic RxU rats. Previous studies have also reported low plasma TSH levels in diabetic rats together with a normal (4) or diminished (8) TSH response to TRH and an unaltered TSH clearance. Bestetti et al. (5) explained the low secretory activity of the pituitary in diabetic rats by demonstrating a morphological shift from type I thyrotrophs in control pituitaries, which readily release TSH granules, to type II thyrotrophs in diabetic pituitaries, which mainly accumulate TSH granules. Such morphological changes may contribute to the decrease in plasma TSH levels in diabetic rats both substrains.

In this study, plasma corticosterone was measured to determine its role in the

suppression of thyroid function during STZ-induced diabetes mellitus. Whereas in diabetic R-A rats a significant increase was demonstrated 1 and 2 weeks after STZ injection, levels of plasma corticosterone in RxU diabetic rats were hardly affected. This suggests that the activation of the hypothalamus-pituitary-adrenal axis as a response to stress or systemic illness may be strain-dependent. The association of increased plasma corticosterone with decreased hypothalamic proTRH mRNA and pituitary TSHß mRNA in R-A diabetic rats and the lack of effect on all these parameters in diabetic RxU rats suggest that plasma corticosterone plays a role in the regulation of hypothalamic proTRH and hypophyseal TSHß gene expression. Previous studies have alsosuggested a negative relationship between proTRH mRNA and corticosterone in rats after short-term starvation (13), after continuous administration of interleukin-1 (12), or during lactation[1]. The decrease in proTRH mRNA in starved rats is prevented if serum corticosterone is kept constant by adrenalectomy and corticosterone substitution (13). The present findings of strain-dependent differences in proTRH mRNA and plasma corticosterone further substantiate this negative correlation between TRH gene expression and corticosterone.

Hypothalamic somatostatin is known to have an inhibitory effect on pituitary TSH release (21, 22). As previously reported, induced diabetes mellitus in rats increases hypothalamic and peripheral somatostatin concentrations (23-26) which may contribute to the decline in TSH release.

Many studies have demonstrated reductions in plasma T4 and T3 in STZ-induced diabetes mellitus (3, 4, 6, 27, 28), while changes in free thyroid hormone levels have received less attention. In agreement with a previous study from our laboratory (7), we observed that the decrease in plasma total T4 in diabetic rats is accompanied by an increase in FFT4, resulting in normal plasma FT4 levels. Plasma total T3 and FFT3 showed a parallel decline in diabetic animals, resulting in strongly decreased FT3. These results suggest an selective effect of STZ-induced diabetes mellitus on T4 and T3 binding to plasma proteins. In this study the decrease of plasma FFT3 was correlated with an increased TBG, a minor thyroid hormone-binding protein in normal adult rats (29, 30). The increase in plasma FFT4 may be caused in part by a decrease in plasma TBPA, the major binding protein in normal adult rats, which has also been shown to decrease during fasting (31).

In addition, we studied the principal metabolic pathways for thyroid hormone by measuring hepatic UGT and ID1 activities in R-A rats. Two UGT isoenzymes

catalyzing the glucuronidation of T4 and rT3 have been identified (15). In addition to these iodothyronines, type I UGT glucuronidates bilirubin, while type II UGT glucuronidates 'bulky' phenols. Tunon et al. (32) have previously reported an increased bilirubin UGT activity in STZ-diabetic rats. Our findings that not only bilirubin but also T4 and rT3 UGT activities are increased in diabetic rats point to the induction of the type I UGT isoenzyme. In agreement with others (33), we also found that hepatic ID1 activity is strongly decreased STZ-diabetic rats. Since this enzyme is very important for the peripheral T4 to T3 conversion, the decrease in its activity may contribute to the low plasma T3, characteristic for patients with non-thyroidal illness (34). A reduced activity of this enzyme may contribute to the low plasma T3, characteristic for patients with non-thyroidal illness (34). The above-mentioned increase in hepatic T4 UGT activity suggests that the decreased conversion to T3 in diabetic rats is accompanied by an increased routing of T4 through the glucuronidation pathway.

In conclusion, both hypothalamic proTRH mRNA and hypophyseal TSHß mRNA gradually decrease and plasma corticosterone increases in diabetic R-A rats, but none of these parameters changes in RxU diabetic rats. This suggests strain-dependent inhibition of proTRH and TSHß gene expression in diabetes mellitus, mediated by the increased corticosterone. However, additional mechanisms must exist for the diabetes-induced suppression of plasma TSH that occurs in both substrains. The opposite effects of STZ-induced diabetes on plasma FFT4 and FFT3 are explained by an increase in TBG and a decrease in TBPA. Finally, the decreased hepatic T4 to T3 conversion and increased T4 glucuronidation may be important factors contributing to the generation of the low T3 syndrome in STZ-induced diabetic rats.

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Footnote

[1] van Haasteren GAC, van Toor H, Klootwijk W, Handler B, Linkels E, van der Schoot P, van Ophemert J, de Jong FH, Visser TJ, de Greef WJ 1995 Studies on the role of thyrotrophin-releasing hormone and corticosterone in the regulation of prolactin and thyrotrophin secretion during lactation. J of Endocrinol (submitted for publication)

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CHAPTER 3

THE ROLE OF TRH IN THE REGULATION OF PRL SECRETION DURING LACTATION

STUDIES ON THE ROLE OF THYROTROPHIN-RELEASING HORMONE AND CORTICOSTERONE IN THE REGULATION OF PROLACTIN AND THYROTROPHIN SECRETION DURING LACTATION

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Abstract

This study describes the effects of litter size and acute suckling on hypothalamic synthesis and release of thyrotrophin (TSH)-releasing hormone (TRH) as indirectly estimated by determination of hypothalamic proTRH mRNA and median eminence TRH content. Litter size effects (5 or 10 pups) were studied throughout lactation, while suckling-induced acute changes were analyzed on day 13 of lactation in dams with 10 pups. In view of the enhanced adrenal activity during lactation and the recent evidence that corticosteroids exert negative effects on hypothalamic TRH release, we also examined the effects of the suckling-induced enhanced plasma corticosterone in dams with 10 pups by removal of the adrenals on day 2 followed by treatment with corticosterone in the drinking water (0.2 mg/ml) to maintain basal plasma corticosterone levels.

In addition to a strongly increased plasma prolactin (PRL) level, adrenal weight and plasma corticosterone increased, while plasma levels of TSH, T_3, T_4 and free T_4 (FT_4) decreased during lactation. Litter size correlated positively with plasma PRL, adrenal weight and plasma corticosterone. No effect of litter size was observed on plasma T_3, but rats with 10 pups had lower plasma TSH, T_4 and FT_4 than rats with a 5-pup litter. Compared to dioestrous rats, lactating rats showed an increased hypothalamic content of proTRH mRNA on day 2, but not on days 8 and 15 of lactation. On days 8 and 15, rats with 10 pups had somewhat higher proTRH mRNA levels than mothers with 5 pups. Median eminence TRH levels in lactating rats gradually increased until day 15 and decreased thereafter. Acute suckling, after a 6-h separation of mother and pups, rapidly increased plasma PRL and corticosterone in the mothers, but had no effects on plasma TSH and thyroid hormone levels. Hypothalamic proTRH mRNA increased two-fold after 0.5 h of suckling, and then gradually returned to presuckling values after 4-6 h. Compared to sham-operated rats, corticosterone-substituted adrenalectomized (ADX) rats had increased plasma PRL and TSH, hypothalamic proTRH mRNA and pituitary TSHß mRNA on day 15 of lactation. Moreover, while acute suckling did not enhance TSH release in sham-operated rats, it not only provoked PRL release but also TSH release in corticosterone-substituted ADX dams.

It is concluded that suckling exerts a rapid, positive effect on hypothalamic proTRH mRNA. However, the concurrent enhanced adrenal activity during acute and continued suckling has negative effects on hypothalamic proTRH gene expression resulting in a suppressed hypophysial-thyroid axis during lactation. While

TRH appears to play a role in the release of PRL during the first days of lactation and during acute suckling, TRH seems not important to maintain PRL secretion during continued suckling.

Introduction

The secretion of PRL from the anterior pituitary gland is controlled by factors released from the hypothalamus and neurointermediate lobe of the pituitary gland (see for reviews de Greef & van der Schoot 1985, Neill 1988, Lamberts & MacLeod 1990, Frawley 1994). Although the identity of the neurointermediate lobe factors is still unknown (Frawley 1994), there is evidence that TRH and α-melanocyte-stimulating hormone are involved (Lackoff & Jackson 1981, Murai & Ben-Jonathan 1987, Hill et al. 1993). Hypothalamic factors known to affect PRL secretion include dopamine and, again, TRH. While evidence has been obtained that a decreased supply of dopamine is important for suckling-induced PRL release (de Greef et al. 1981, de Greef & Visser 1981, Selmanoff & Wise 1981, Plotsky et al. 1982, Rondeel et al. 1988, Wang et al. 1993), the physiological role of TRH for PRL release remains enigmatic for several reasons.

Firstly, in many physiological conditions there is a dissociation between the release of PRL and TSH. Indeed in contrast to PRL, plasma TSH is not consistently increased by acute suckling (Blake 1974, Riskind et al. 1984, Sheward et al. 1985, de Greef et al. 1987). Moreover, immunoneutralization of TRH had no or only modest effects on suckling-induced PRL release (Harris et al. 1978, Riskind et al. 1984, Sheward et al. 1985, de Greef et al. 1987), while studies on hypothalamic TRH release and synthesis are equivocal. Whereas acute suckling did not increase TRH in the push-pull perfusate of the mediobasal hypothalamus (Rondeel et al. 1988), mammary nerve stimulation increased TRH in hypophysial portal blood (de Greef & Visser 1981). Although acute suckling transiently enhanced proTRH mRNA levels in the hypothalamic paraventricular nucleus (Uribe et al. 1993), the amount of proTRH mRNA decreased from day 1 to day 5 of lactation in mothers with 8 pups (Uribe et al. 1991).

In view of the enhanced adrenal corticosterone secretion during continued suckling (Voogt et al. 1969, Walker et al. 1992) and the negative effects of corticosteroids on thyroid function (Kakucska & Lechan 1991, van Haasteren et al. 1994, 1995), the reduced hypothalamic levels of proTRH mRNA during lactation may be caused by the suckling-induced enhanced corticosterone secretion. We have addressed this issue in this study by evaluating parameters of hypothalamic TRH synthesis and release during acute and continued suckling in lactating rats in which the suckling-induced increase in plasma corticosterone was prevented by adrenalectomy and subsequent treatment with corticosterone to maintain basal

plasma levels of corticosterone.

Materials and methods

Animals

Locally bred hooded (RxU) F_1 rats have been employed in these studies except for one experiment in which locally bred albino R-Amsterdam rats were used since (RxU) F_1 rats were no longer bred in our animal facility. R-Amsterdam rats have similar plasma PRL levels as (RxU) F_1 rats, but they have lower TSH and thyroid hormone levels. Rats were housed under controlled conditions (lights on between 05.00-19.00 h; temperature between 20-22 °C) and they had free access to food and water. Female rats weighing 220-250 g were caged with male rats of proven fertility. At the end of pregnancy the females were caged individually, and only rats which gave birth to at least 9 pups were included in the experiments. Litter size was adjusted to 5 or 10 pups between 09.00 and 10.00 h on day 2 of lactation (day 1 is day of parturition). During the experiments, the weights of mothers and litters were monitored daily. Normal suckling was established by direct observation, and the time the mothers spent with their litter and the number of milk ejections were recorded (van der Schoot et al. 1978). Long-term mother-young interactions were measured with an automatic device (Croskerry et al. 1976, de Greef et al. 1987, 1989). In two experiments, adrenalectomized (ADX) rats were used to study possible effects of the increased plasma corticosterone levels during lactation. To maintain basal plasma levels of corticosterone, ADX rats were treated with corticosterone in their drinking water. Adrenalectomy or sham-operation was performed on day 2 of lactation using ether as anaesthetic. The ADX rats received corticosterone in their drinking water (0.2 mg/ml (w/v), Sigma, St. Louis, MO) as described previously (van Haasteren et al. 1995). Corticosterone was dissolved in ethanol and then added to saline (0.9% NaCl in water), yielding a final concentration of 4% ethanol (v/v). Sham-operated rats received similar water without corticosterone. Since water consumption follows a circadian rhythm, this procedure ensures diurnal corticosterone rhythms in ADX rats leading to normal plasma adrenocorticotrophin (ACTH) levels (van Haasteren et al. 1995). Based on the water consumption, the ADX rats received 4-8 mg corticosterone daily. Unless otherwise indicated, blood was obtained within 1 min after removal of the rat from the cage by decapitation or from the orbital plexus of lightly ether-anaesthetized animals. For all experiments, approval was obtained from the Animal Welfare

Committee (DEC) of the Erasmus University.

Effect of litter size on hypothalamic proTRH mRNA, median eminence TRH and plasma hormone levels throughout lactation

Effects of litter size (5 or 10 pups) on hypothalamic proTRH mRNA, median eminence TRH and hormonal changes were investigated in lactating rats on days 8, 15 and 22 of lactation. Another group of lactating rats with 11.0±0.5 pups (means±SE) was sacrificed on day 2. Female rats decapitated at the dioestrous stage of the ovarian cycle were used as controls (dioestrous rats). Furthermore, dams separated from their litter at day 2 of lactation and decapitated 6 days later were also included in this experiment (non-lactating dams). Rats were decapitated between 10.00 and 13.00 h and trunk blood was collected to measure plasma hormone levels. The adrenal glands were removed and weighed. The skull was opened, the brain was removed, and median eminence and remainder of the hypothalamus, which includes the paraventricular nuclei, were isolated as described previously (van Haasteren et al. 1994, 1995). The median eminence was placed immediately in 2 ml methanol to prevent a possible tissue degradation of TRH (Bauer et al. 1990), whereas the remainder of the hypothalamus was snap frozen in liquid nitrogen and kept at -80 °C until RNA isolation, or was also transferred to 2 ml methanol. Tissue collected in methanol was homogenized, subsequently dried under a stream of nitrogen, redissolved in phosphate buffer (pH 7.4) and stored at -20 °C until assayed for TRH.

Hypothalamic proTRH mRNA, pituitary TSHß mRNA and plasma hormone levels in corticosterone-substituted ADX dams

Sham-operated or corticosterone-substituted ADX lactating rats with 10 pups were used. In the first part of the experiment, (RxU) F_1 dams were employed. Blood was taken between 10.00 and 13.00 h from the orbital plexus on days 8, 15 and 22 of lactation to estimate plasma levels of PRL, TSH and corticosterone. For the second part of this study, lactating R-Amsterdam rats were used, which were decapitated on day 15 of lactation. As control, non-lactating dams, R-Amsterdam rats separated from their litter on day 2 of lactation and decapitated 6 days later were used. Trunk blood, collected between 10.00 and 13.00 h, was used to determine plasma hormone concentrations. Hypothalamus and pituitary gland were isolated, snap frozen in liquid nitrogen, and stored at -80 °C until RNA isolation. Adrenal glands

were also isolated and weighted.

Effect of acute suckling on hypothalamic proTRH mRNA and plasma hormone levels in lactating dams

Dams were separated from their 10-pup litter between 04.30 and 08.30 h on day 13, and they were reunited 6 h later. Lactating rats were decapitated between 14.00 and 17.00 h after they had been suckled for 0.5, 1, 4 or 6 h. Mothers not reunited with their pups, and decapitated between 14.00 and 16.00 h served as controls (0 h). Trunk blood was collected to measure plasma hormone levels. The median eminence was removed and processed as describe above to measure its TRH content. The rest of the hypothalamus was isolated, snap frozen in liquid nitrogen and kept at -80 °C until RNA isolation.

Effect of acute suckling on PRL and TSH levels in corticosterone-treated ADX lactating dams

Sham-operated and corticosterone-substituted ADX lactating dams with 10 pups received an indwelling cannula (0.96 mm outer diameter, 0.58 mm inner diameter) in the jugular vein (Popovic & Popovic 1960) on day 11 of lactation. On day 13, the pups were removed between 07.30 and 08.30 h and reunited with their mothers 6 h later. Blood samples of about 0.5 ml were taken from the jugular vein cannula just before and after 1, 4 and 6 h of reunion to measure plasma PRL and TSH concentrations.

Measurement of pituitary TSHß mRNA and hypothalamic proTRH mRNA

Pituitary and hypothalamic RNA was isolated by acid guanidinium thiocyanate-phenol-chloroform extraction (Chomczynski & Sacchi 1987), and the amount and purity of the isolated RNA was determined by absorbance at 260/280 nm. Pituitary TSHß mRNA was estimated by Northern blotting as described before (van Haasteren et al. 1994, 1995). In short, 10 µg total RNA was subjected to denaturing agarose gel electrophoresis, blotted onto Hybond N^+ filter (Amersham International PLC, Amersham, UK), and hybridized with a ^{32}P-labelled 420-basepair (bp) fragment of the rat TSHß cDNA (Chin et al. 1985). Variation in loading was accounted for by normalizing to the ß-actin mRNA content which was hybridized with a ^{32}P-labelled hamster ß-actin cDNA probe (Dodemont et al. 1982). Hypothalamic proTRH mRNA was estimated using an RNase protection assay (van

Haasteren et al. 1994) using 10 µg total RNA. Hybridization was carried out with a labelled 351-bp antisense cRNA probe transcribed from a 981-1322 bp rat proTRH cDNA fragment (Lechan et al. 1986, van Haasteren et al. 1994). Variations in procedures were accounted for by normalization to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA using a cRNA probe transcribed from a 410-bp Pst1/SauA1 GAPDH cDNA fragment (van Haasteren et al. 1994). Autoradiographs were scanned densitometrically, and the resulting signals were integrated by computer using custom-made software written by Dr. R. Docter (Department of Internal Medicine and Clinical Endocrinology). The ratios between the integrated optical densities of TSHß and ß-actin mRNA or proTRH and GAPDH mRNA were calculated for each sample. Results are presented as the percentage of the mean of control rats. The validation of the methods used to isolate and measure proTRH and TSHß mRNA have been described previously (van Haasteren et al. 1995).

Hormone determinations

Plasma TSH was measured by RIA with materials and protocols supplied by the NIADDK, with rat-TSH-RP-2 as standard. Levels of T_4, T_3 and reverse T_3 (rT_3) were estimated in unextracted plasma by RIA. The plasma T_4 dialysable fraction was measured by equilibrium dialysis (Sterling & Brenner 1966), and plasma free T_4 (FT_4) was calculated as the product of total T_4 and the dialysable fraction. The same procedure was used to measure plasma free T_3 (FT_3). Plasma PRL was determined by RIA (de Greef & Zeilmaker 1978) using rat-RP-1 as standard. Corticosterone was estimated by RIA (Marzouk et al. 1991). The RIA for TRH was performed with antiserum 4319 as reported before (Visser et al. 1977). Detection limits were 0.2 µg/l RP-2 TSH, 2 nmol/l T_4, 0.1 nmol/l T_3, 0.05 nmol/l rT_3, 5 µg/l RP-1 PRL, 1 nmol/l corticosterone and 3-5 fmol TRH/tube. Intra- and interassay coefficients of variation for the assays varied between 5 and 15%.

Statistical analysis

Results are presented as means±SEM. Analysis of variance (ANOVA) was used for the statistical evaluation of the data. Provided significant overall effects were obtained, comparisons between groups were made by Duncan's new multiple range tests. Differences are considered to be significant at $p \leq 0.05$.

Results

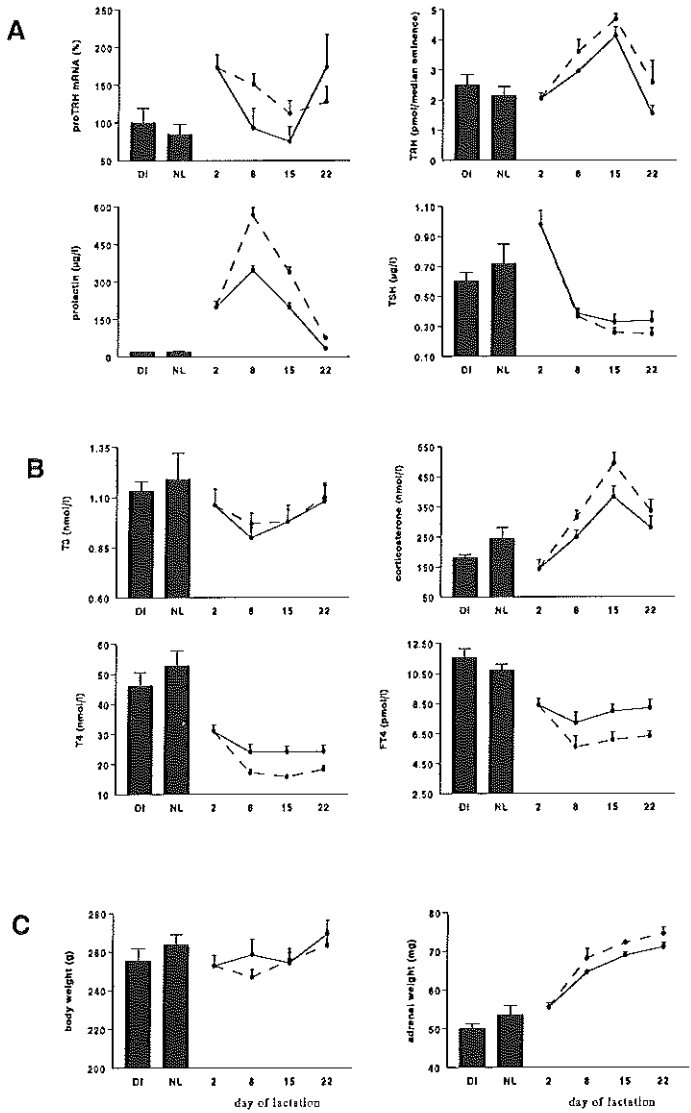


*Figure 1. Hypothalamic proTRH mRNA, median eminence TRH and plasma PRL and TSH (**A**), plasma levels of thyroid hormones and corticosterone (**B**), and body and adrenal weight (**C**) in lactating rats with 5 (o--o) or 10 (o--o) pups. For comparison data from dioestrous rats (DI) and dams separated from their pups at day 2 of lactation and decapitated 6 days later (NL) are included. Values are means±SEM (n=8-24). *P≤0.05 compared to dioestrous rats, †P≤0.05 compared to rats with 10 pups*

Effect of litter size on hypothalamic proTRH mRNA, median eminence TRH and plasma hormone levels throughout lactation

Results are summarized in Figure 1. Plasma PRL was raised throughout lactation, and a larger litter was associated with higer PRL levels. On day 2 of lactation plasma TSH concentrations were higher than the values observed in dioestrous female rats, but after day 2 plasma TSH decreased in lactating rats and became lower than that in dioestrous rats. Overall plasma TSH was somewhat lower in mothers with 10 pups than in dams nursing 5 pups ($p \leq 0.05$, ANOVA), but at the individual time points studied, litter size had no significant effect on plasma TSH. Plasma T_4 and FT_4 levels were significantly lower in lactating than in dioestrous rats, and mothers nursing 10 pups had generally lower plasma T_4 and FT_4 during lactation than dams with 5 pups. Litter size had no effect on plasma T_3 during lactation, and while plasma T_3 in lactating rats tended to be lower on days 8 and 15 than in dioestrous rats, this was significant only in rats with 5 pups on day 8. Plasma rT_3 was low in all groups of rats, and no differences were found between control and lactating rats (data not shown). Plasma corticosterone was usually higher in lactating than in dioestrous rats, and mothers with 10 pups tended to have higher levels than rats nursing 5 pups. Adrenal weight increased gradually during lactation, and mothers nursing 10 pups generally had larger adrenals than rats with 5 pups. Lactating rats had higher hypothalamic proTRH mRNA than dioestrous rats on day 2 of lactation, but proTRH mRNA gradually decreased in the subsequent 2 weeks. In dams nursing 10 pups hypothalamic proTRH mRNA remained similar between days 8 and 22, but it increased in mothers with 5 pups between days 15 and 22. Hypothalamic proTRH mRNA on days 8 and 15 was somewhat higher in dams with 10 pups than in rats nursing 5 pups ($p \leq 0.02$, ANOVA). However, at the individual days, litter size had no significant effect on proTRH mRNA. One week after parturition, hypothalamic proTRH mRNA content was higher in dams with 10 pups (151.1±14.0%) than in rats from which the pups had been removed on day 2 of lactation (84.6±3.2%, $p \leq 0.05$). During lactation, median eminence TRH content gradually increased until day 15 and decreased thereafter, and dams with 10 pups had overall somewhat higher levels than mothers with 5 pups ($p \leq 0.05$).

The body weight of the mothers gradually increased during lactation, and no difference between dams with 5 or 10 pups was observed. At the time of weaning (day 22), 10-pup litters weighed 422±12 g and 5-pup litters 260±6 g. The time the mothers spent with the pups was similar in both groups (data not shown), *i.e.* 16-21

hours/day between days 2 and 15, gradually declining to 10-12 hours/day.

	Non-lactating dams	Lactating dams	
		Sham-operated	ADX
proTRH mRNA[+]	100.0±3.8	115.3±6.5	149.6±9.5[ab]
TRH (pmol/ME)	2.16±0.31	3.14±0.35[a]	2.97±0.65
TSHß mRNA[++]	100.0±13.1	81.4±11.3	135.3±15.8[b]
PRL (µg/l)	25±3	396±39[a]	527±32[ab]
TSH (µg/l)	0.32±0.06	0.11±0.04[a]	0.21±0.06
T_4 (nmol/l)	27.1±3.9	12.3±1.1[a]	17.6±2.2[a]
FT_4 (pmol/l)	9.38±0.93	4.12±0.35[a]	5.57±0.76[a]
T_3 (nmol/l)	0.89±0.05	0.57±0.02[a]	0.71±0.04[ab]
FT_3 (pmol/l)	3.09±0.14	2.33±0.09[a]	2.77±0.15[b]
Cort.(nmol/l)	182±20	379±34[a]	138±35[b]
Adrenal weight (mg)	48.5±1.1	60.9±1.7[a]	---

Table 1. *Hypothalamic proTRH mRNA, median eminence TRH, pituitary TSHß mRNA, plasma hormone levels and adrenal weight on day 15 of lactation in sham-operated or corticosterone-substituted ADX R-Amsterdam rats nursing 10 pups. For comparison, data from dams separated from their litter on day 2 of lactation, and sacrificed 6 days later, are included (non-lactating dams). Values are means±SEM (n=9-10).*

[a] P≤0.05 compared to non-lactating dams, [b] P≤0.05 compared to sham-operated dams

[+] relative to GAPDH mRNA, expressed as percentage of non-lactating dams

[++] relative to actin mRNA, expressed as percentage of non-lactating dams

Hypothalamic proTRH mRNA, pituitary TSHß mRNA and plasma hormone levels in corticosterone-substituted ADX lactating rats

To prevent the suckling-induced increase in plasma corticosterone in lactating rats, adrenal glands were removed on day 2 of lactation and the ADX dams were subsequently treated with corticosterone in their drinking water to maintain basal plasma levels of corticosterone. In the first part of the study, (RxU) F_1 rats were

used and the results are presented in Figure 2. Corticosterone-treated ADX lactating rats had higher plasma PRL levels than sham-operated mothers on day 15, but plasma PRL was similar in both groups on days 8 and 22 of lactation. Compared to sham-operated dams, plasma TSH was higher in the corticosterone-substituted ADX rats on days 15 and 22 of lactation. Whereas sham-operated dams gained weight (from 233±12 to 270±15 g), the weight of corticosterone-treated ADX mothers remained similar during the period of observation (246±8 vs 250±9 g). Mother-young interactions were similar in both groups (data not shown), but pups nursed by corticosterone-treated ADX mothers gained less weight than pups of sham-operated rats (day 22: 288±17 vs 388±21 g, p≤0.01).

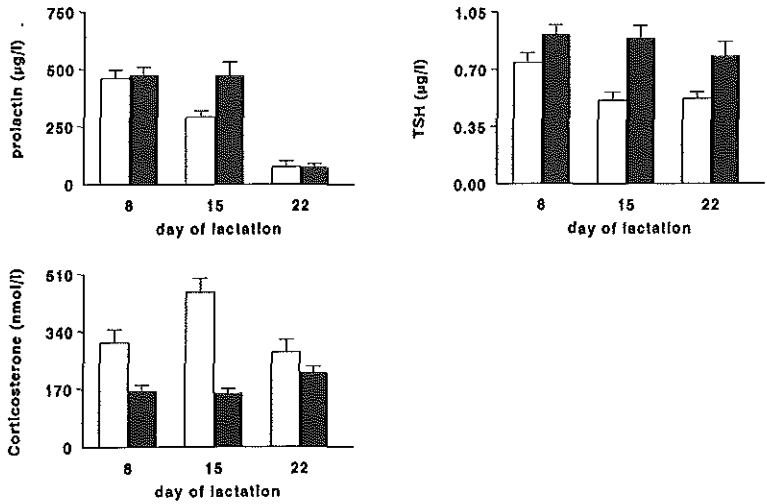


*Figure 2. Plasma levels of PRL, TSH and corticosterone in sham-operated (open bars, n=9) and corticosterone-treated ADX (black bars, n=12) lactating rats with 10 pups. Values are means±SEM. *P≤0.05 compared to sham-operated rats*

For the second part of the experiment, R-Amsterdam rats were studied on day 15 of lactation, and the results are given in Table 1. When compared with values in non-lactating dams, sham-operated lactating rats had similar levels of proTRH mRNA and TSHß mRNA, lower plasma TSH and thyroid hormone concentrations, higher median eminence TRH content, higher PRL and corticosterone levels, and increased adrenal weight. Prevention of the lactation-induced corticosterone release resulted in higher levels of proTRH mRNA and TSHß mRNA, and increased plasma concentrations of PRL, TSH and thyroid hormones, although effects on plasma

TSH, T_4 and FT_4 were not significant. In corticosterone-substituted ADX rats, median eminence TRH content on day 15 of lactation was not different from the levels in non-lactating dams.

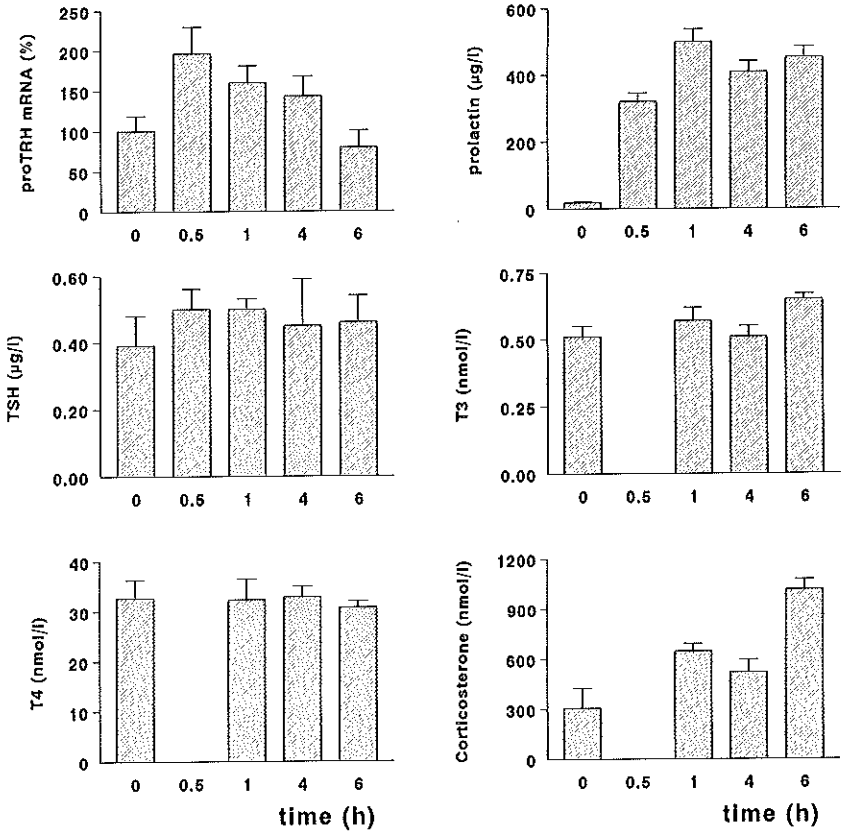


*Figure 3. Suckling-induced changes in hypothalamic proTRH mRNA content and in plasma hormone levels on day 13 of lactation in lactating rats. Mothers and 10-pup litters had been separated for 6 h, and were reunited at 0 h. Values are means±SEM of 9-11 rats. *P≤0.05 compared to presuckling values*

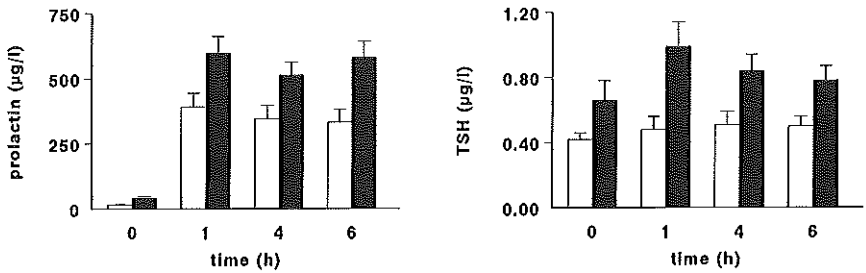
Effect of acute suckling on hypothalamic proTRH mRNA and plasma hormone levels in lactating rats

On day 13 of lactation, dams were separated from their 10-pup litters and reunited 6 h later to estimate acute suckling-induced changes in hypothalamic proTRH mRNA, median eminence TRH content, and plasma hormone levels (Figure 3). Hypothalamic proTRH mRNA had increased significantly after 30 min of suckling,

and gradually returned to presuckling values. While acute suckling strongly increased plasma PRL and corticosterone, no significant effects on plasma TSH, T_3 and T_4 levels were found.

Effect of acute suckling on PRL and TSH levels in corticosterone-treated ADX lactating rats

Possible effects of the increased plasma corticosterone on PRL and TSH secretion induced by acute suckling were studied on day 13 in sham-operated and corticosterone-treated ADX lactating rats nursing 10-pup litters (Figure 4). Acute suckling increased plasma PRL in both groups of rats, but absolute levels became higher in corticosterone-substituted ADX dams. Acute suckling had no effect on plasma levels of TSH in sham-operated mothers, but increased plasma TSH in corticosterone-treated ADX lactating rats.



*Figuur 4. Effect of acute suckling on day 13 of lactation on plasma PRL and TSH in sham-operated (open bars, n=7) or corticosterone-treated ADX (black bars, n=6) lactating dams nursing 10 pups. Mothers and their litters had been separated for 6 h, and were reunited at 0 h. *P≤0.05 compared to presuckling values, +P≤0.05 compared to sham-operated rats*

Discussion

In the present study we investigated the effects of suckling on parameters of hypothalamic TRH synthesis and release. It has been argued that TRH is not involved in the suckling-induced release of PRL, since *a)* suckling evokes only a modest increase in plasma TSH compared with that of PRL (Blake 1974, Riskind et al. 1984, Sheward et al. 1985, de Greef et al. 1987), *b)* immunoneutralization of TRH has only small effects on suckling-induced PRL release (Harris et al. 1978, Riskind et al. 1984, Sheward et al. 1985, de Greef et al. 1987), and *c)* studies on suckling-induced changes in hypothalamic TRH synthesis and release are equivocal

(de Greef & Visser 1981, Rondeel et al. 1988, Uribe et al. 1991, 1993). However, a dissociation between PRL and TSH secretion does not necessarily imply that hypothalamic TRH release is not stimulated by suckling since other factors could be involved in the dissociation between PRL and TSH release. For instance, oxytocin has been found to attenuate the TRH-induced TSH release from pituitary cells (Frawley et al. 1985), indicating that the suckling-induced increase in oxytocin could also be responsible for an inhibition of suckling-induced TSH release. Furthermore, TRH receptors and TRH-degrading ectopeptidase activity may be regulated independently on lactotrophs and thyrotrophs (Bauer et al. 1990). Finally, the value of immunoneutralization studies is not always clear, since passive immunization has been reported to sometimes enhance the biosynthesis of hypothalamic peptides (van Oers et al. 1991, Strbák et al. 1993).

Another factor which may cause a dissociation between PRL and TSH release is corticosterone (van Haasteren et al. 1995). Therefore we studied the effects of the suckling-induced increase in corticosterone secretion on the hypothalamic-hypophysial-thyroid axis. Since endocrine changes during lactation are related to the suckling stimulus (van der Schoot et al. 1978, 1982), it is essential to establish that treatment-induced effects are not due to an altered suckling stimulus. None of the experimental conditions, however, interfered with normal suckling and nursing behaviour. Thus, the present results are unlikely caused by treatment-induced alterations in mother-young interaction.

It has been reported that proTRH mRNA in the paraventricular nucleus decreases from day 1 to day 5 of lactation in rats nursing 8 pups (Uribe et al. 1991), and this study confirms this observation since hypothalamic proTRH mRNA levels were found to decrease after day 2. Although, hypothalamic proTRH mRNA in lactating rats was only slightly affected throughout lactation when compared with proTRH gene expression in dioestrous rats, it was also observed that rats nursing 10 pups had somewhat higher hypothalamic levels of proTRH mRNA on day 8 and 15 than rats with 5 pups. Moreover, one week after parturition hypothalamic proTRH mRNA was nearly twice as high in dams suckled by a 10-pup litter than in dams from which the pups had been removed on day 2 of lactation. Besides an effect of the number of pups on hypothalamic proTRH mRNA during continued suckling (this study), acute suckling also induces a rapid, but transient, increase in proTRH mRNA (Uribe et al. 1993, this study). It is unlikely the observed effects of acute suckling on proTRH mRNA are due to circadian influences (Covarrubias et al.

1988, Zoeller et al. 1990), since the variation in hypothalamic proTRH mRNA in control rats during the same time period (14.00-17.00 h) is small (unpublished data). Thus, we conclude that suckling stimulates proTRH gene expression. In this context, it has to be realized that changes in hypothalamic proTRH mRNA are modest even in rats made severely hypothyroid by thyreostatic drugs or thyroidectomy (Koller et al. 1987, Zoeller et al. 1988, Shi et al. 1994, van Haasteren et al. 1995).

To study possible effects of the enhanced corticosterone secretion during lactation, the adrenal glands were removed from rats after parturition and the dams were subsequently treated with corticosterone in the drinking water to maintain basal plasma corticosterone levels. Using this experimental approach it appeared that the increased corticosterone levels during lactation suppress hypothalamic proTRH gene expression, and PRL and TSH secretion. While acute suckling hardly affected plasma TSH in sham-operated lactating rats, it increased TSH release in corticosterone-substituted ADX dams. Previously, it was found that the synthetic glucocorticoid dexamethasone rapidly reduced hypothalamic TRH and pituitary TSH release (van Haasteren et al. 1995). We therefore suggest that the negative effect of high levels of corticosterone on plasma TSH during lactation could be due to a reduced hypothalamic TRH synthesis and release. The presence of a glucocorticoid responsive element in the promotor region of the proTRH gene (Lee et al. 1988) and the occurrence of glucocorticoid receptors in TRH-synthesizing cells in the hypothalamic paraventricular area (Ceccatelli et al. 1989) support this conclusion. Besides an inhibition of hypothalamic TRH synthesis and release (Kakucska & Lechan 1991, van Haasteren et al. 1995), corticosteroids have also been found to reduce TRH-induced TSH release (Pamenter & Hedge 1980) and to increase the hypothalamic synthesis and release of somatostatin (Nakagawa et al. 1987, 1992), a hormone known to inhibit TSH secretion.

There is evidence that the TRH content both in the median eminence and in the posterior pituitary gland may serve as an index for hypothalamic TRH release (Mori & Yamada 1987, Bruhn et al. 1991, Rondeel et al. 1995). The TRH content of the median eminence was found to increase after day 2 of lactation, and to decrease again after day 15. A similar profile has been reported for TRH in the posterior pituitary gland of lactating rats (Uribe et al. 1991). The TRH content both in median eminence and posterior pituitary gland increases in conditions in which the hypothalamic TRH release has been found to decrease (Rondeel et al. 1995).

However, since the TRH content in the median eminence is a resultant of proTRH synthesis and processing in the paraventricular nucleus (Lechan et al. 1986) with subsequent axonal supply of TRH from the paraventricular nucleus to the median eminence and TRH secretion into the hypophysial portal blood, it is difficult to interpret changes in median eminence TRH content unless approximations of TRH synthesis are available. The decrease in hypothalamic proTRH mRNA and the increase in median eminence TRH after day 2 of lactation suggest that hypothalamic TRH secretion becomes reduced after day 2 of lactation. On day 2 of lactation, hypothalamic proTRH mRNA was higher and median eminence TRH was similar to the values observed in dioestrous rats, suggesting that hypothalamic TRH release on day 2 of lactation is higher than during the dioestrous stage of the cycle. This interpretation of the data is supported by the somewhat increased plasma levels of TSH in lactating dams on day 2, followed by a subsequent reduction in plasma TSH during the remainder of the lactation period.

The higher hypothalamic TRH release on day 2 of lactation suggests that the PRL release during the early phase of lactation is also under control of TRH. However, unless the sensitivity of the lactotroph to TRH becomes increased during lactation, the supposed reduction in hypothalamic TRH release after day 2 implies that TRH is not a major factor in the maintenance of PRL secretion after day 2 of lactation.

After the onset of acute suckling, there is a transient increase in hypothalamic proTRH mRNA (this study), a gradual rise in median eminence TRH (Rondeel et al. 1995), and a concurrent decrease of TRH in the medial basal hypothalamus (Uribe et al. 1991). These observations suggest that acute suckling transiently stimulates TRH release from the hypothalamus. A similar situation is observed after exposure to cold, since this stimulus transiently enhances hypothalamic proTRH mRNA content (Rage et al. 1994) and TRH release (Rondeel et al. 1991) together with a simultaneous reduction of TRH in the medial basal hypothalamus (Rage et al. 1994). Hypothalamic proTRH mRNA levels returned to presuckling values after 6 h of suckling (present study), at a time when TRH in the median eminence had increased (Rondeel et al. 1995), suggesting that after 6 h of suckling hypothalamic TRH release had decreased again. Thus, acute suckling on day 13 seems only to increase hypothalamic TRH secretion transiently. This indicates that TRH is perhaps only required for the normal onset of PRL release induced by acute suckling, explaining the delayed onset of PRL secretion induced by acute suckling

after interference with TRH action by passive immunization (de Greef et al. 1987) or TRH secretion through paraventricular area lesions (de Greef et al. 1989).

Plasma levels of thyroid hormones have been reported to decrease during lactation (Fukuda et al. 1980, Kahl et al. 1987, Valverde-R & Aceves 1989). We also found a consistent decrease in plasma T_4 during lactation which correlated with litter size. The lower plasma T_4 can be explained by diminished synthesis and/or by an increased clearance for instance by loss of T_4 in the milk (Oberkotter & Rasmussen 1992). Plasma T_4 and FT_4 show a similar decrease in lactating rats, suggesting that plasma T_4 binding is not greatly affected during lactation. Irrespective of whether T_4 clearance is increased during lactation, thyroid function is not sufficient to maintain normal plasma levels of T_4 and FT_4. The lower thyroid function during lactation is probably due to the lactation-induced decrease in plasma TSH as found consistently in this study both in (RxU) F_1 and R-Amsterdam rats. Our results are in agreement with other reports on effects of lactation on thyroid parameters in Wistar rats, but disagree with the reported increase in plasma TSH in lactating Sprague-Dawley rats (Fukuda et al. 1980). The reason for this difference between several rat strains remains to be resolved.

Plasma PRL levels gradually decrease during lactation in intact dams, but they remain elevated in ADX rats (van der Schoot & de Greef 1983). In addition, the decrease in plasma PRL during lactation is delayed in corticosterone-substituted ADX dams compared with control dams (present study). These findings indicate that the lactation-induced increase in serum corticosterone negatively affects PRL secretion. Since high levels of PRL stimulate the release of ACTH-releasing factor (CRF) and ACTH (Kooy et al. 1990, Weber & Calogero 1991), the enhanced adrenal activity during lactation may not be a direct effect of suckling but due to the suckling-induced hyperprolactinemia. Thus, PRL, CRF, ACTH and corticosterone seem interdependent, suggesting that they are components of a feedback system regulating TRH synthesis and release.

On the basis of the available data it is suggested that the following interactions exist during lactation. Suckling stimulates, through a neuroendocrine reflex, the secretion of PRL from the pituitary gland. Part of this neuroendocrine response is a decrease in the hypothalamic release of dopamine throughout lactation (Selmanoff & Wise 1981, Wang et al 1993). Furthermore, factors from the neurointermediate lobe seem to be involved in PRL release (Murai & Ben-Jonathan 1987, Hill et al. 1993). Hypothalamic TRH release is probably increased during the early phases of

lactation in comparison with the dioestrous stage of the cycle (present study), suggesting that in this stage of lactation TRH is involved in PRL release. The ensuing increase in plasma corticosterone, which may be due to a hyperprolactinemia-induced release of CRF and ACTH (Kooy et al. 1990, Weber & Calogero 1991, de Greef et al. 1995), has negative effects on hypothalamic proTRH gene expression and TRH release causing a decrease in the pituitary-thyroid axis after day 2 of lactation. This supposed decrease in hypothalamic TRH release also implies that TRH is not impoortantly involved in the maintenance of PRL release after day 2 of lactation.

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CHAPTER 4

DISCUSSION

Discussion

In this thesis, the neuroendocrine role of TRH in regulating TSH and PRL secretion from the anterior pituitary was investigated. TRH supply to the anterior pituitary is influenced by its synthesis, processing, transport, secretion and metabolism, while its biological effects are modulated by other factors such as thyroid hormones, dopamine and somatostatin. These various processes and factors need to be considered when the role of TRH within the regulation of the hypothalamo-pituitary-thyroid axis is to be studied under different (patho-)physiological conditions. In the first part of this chapter the role of TRH in the generation of the low T3 syndrome, under the four conditions as described in chapter 2, will be discussed. The second part of this chapter deals with the role of TRH in the regulation of suckling-induced PRL secretion as described in chapter 3.

4.1 Role of TRH in the regulation of TSH secretion

The body's response to starvation and food reduction is the generation of the low T3 syndrome. In order to preserve energy, thyroid function in suppressed. Interleukin (IL-1 and IL-6) administration and STZ-induced diabetes mellitus are experimental models of non-thyroidal illness (NTI), resulting in the low T3 syndrome as well. Next to low levels of plasma T4 and T3, these four models of adaptive hypothyroidism are associated with inappropriately normal or low levels of TSH. A common mechanism has been postulated, originating from the CNS, which may mediate the decrease in thyroid function. In this thesis, the contribution of TRH to the suppressed pituitary-thyroid function in this syndrome was investigated.

A 3-day starvation period decreases hypothalamic proTRH mRNA, hypothalamic TRH content and TRH concentration in portal blood. In contrast to the effect of starvation, long-term food reduction does not affect levels of hypothalamic proTRH, nor TRH content in the ME, despite a profound decline in plasma TSH. Levels of hypothalamic proTRH mRNA are also unaffected after 7 days of IL-6 infusion, while IL-1 infusion causes a significant decrease in these levels after 7 days, but not after 1 or 2 days when plasma TSH reached its nadir. Two and three weeks after induction of diabetes mellitus by STZ, R-Amsterdam rats show decreased levels of proTRH mRNA, while this parameter is unaffected in RxU rats.

Summarizing the effects of these four studies on hypothalamic TRH production and release, it is clear that these parameters are either unaffected or show a trend towards reduction rather than an increase as might be expected,

based on studies showing that thyroid hormone deficiency leads to increased proTRH mRNA in the PVN (1). On the other hand, decreased TSH secretion occurs before (IL-1 treatment and STZ-induction of diabetes mellitus in R-Amsterdam rats) or even without (food reduction, IL-6 treament and STZ-induction in diabetes mellitus in RxU rats) the decrease in proTRH mRNA. The decline in TSH secretion, therefore, cannot be explained by the changes in hypothalamic proTRH mRNA. The simultaneous decrease in serum TSH, T4 and T3 during food deprivation and in the different experimental models of NTI indicates that inhibition of thyroid function is mediated at the level of the hypothalamus and/or pituitary.

An inverse relationship between the concentration of proTRH mRNA and corticotropin-releasing hormone (CRH) mRNA in PVN neurons in response to hypothyroidism, has recently been reported by Ceccatelli et al (2). Furthermore, a suppressive effect of activated CRH neurons on TRH neurons has been suggested, following systemic lipopolysaccharide (LPS) or intracerebroventricular IL-1 administration, since these neurons are adjacent to each other in the PVN (3, 4). In contrast, levels of proCRH mRNA in PVN neurons in fasted and food restricted rats are decreased (5), which indicates that CRH synthesis does not seem to be involved in proTRH mRNA regulation in these models for NTI.

However, starvation, food reduction, interleukin administration, and STZ-induced diabetes mellitus are all associated with increased plasma levels of glucocorticoids. In fasting rats, this is partly due to decreased clearance of corticosterone (6), while interleukin treatment activates the hypothalamo-pituitary-adrenal axis (7, 8). It is postulated that the sustained elevation of plasma corticosterone may influence proTRH gene expression in the hypothalamus. Continuous IL-1 infusion stimulates corticosterone secretion, and proTRH gene expression is reduced after 7 days, but not after 1 or 2 days of infusion. In the same study, IL-6 infusion had no effect on plasma corticosterone nor on hypothalamic proTRH mRNA. A similar observation was made in the study on STZ-induced diabetes mellitus. In R-Amsterdam diabetic rats the increased plasma corticosterone concentration is accompanied by a reduced proTRH gene expression, whereas in RxU diabetic rats neither parameter changed significantly. A suppressive effect of plasma corticosterone on proTRH gene expression would explain the different effects of IL-1 *vs.* IL-6, and in R-Amsterdam *vs.* RxU rats on proTRH mRNA. This hypothesis is supported by 1) the reduction in proTRH mRNA in the PVN following chronic high dose glucocorticoid treatment (9), 2) the presence

of glucocorticoid receptors in TRH neurons in the PVN (10), and 3) the presence of a consensus glucocorticoid reseponse element in the TRH gene promoter (11). Furthermore, a corticotrophin release-inhibiting factor is encoded within the proTRH gene which suggests a coordinated, but inverse, regulation of pituitary-adrenal and pituitary-thyroid functions (12). Indirect evidence for an inverse relationship between hypothalamic proTRH and glucocorticoids in man was obtained by Brabant et al (13). High-dose glucocorticoid injection abolished TSH pulses, and suppressed basal TSH. Together with a normal serum TSH response to TRH, these data suggest that glucocorticoid exerts its effect at a suprapituitary level.

Direct effects of glucocorticoids on pituitary TSH secretion have been described in man and rats (14-17). Samuels et al (17) studied the effects of cortisol infusions over 24 h on the pulsatile secretion of pituitary glycoprotein hormones in healthy subjects. Basal plasma TSH and TSH pulse amplitude decreased after cortisol infusion, while the TSH pulse frequency was unaltered. Considering the fact that TSH pulsatility is predominantly regulated by hypothalamic TRH (18), these data suggest a direct effect of cortisol at the pituitary level. However, in man, the effect of glucocorticoids at the pituitary level depends on the time-span of hypercortisolism, as only prolonged and not acute exposure interferes with TSH secretion (16). The underlying mechanism for acute or prolonged inhibitory effects of hypercortisolism at the pituitary level is still a matter of debate. Using immunocytochemical double labelling techniques colocalization of glucocorticoid receptors and TSH has been demonstrated, whereas only a minority of the PRL-immunoreactive cells expressed the glucocorticoid receptor (15). Glucocorticoids may therefore differentially regulate the secretion and/or synthesis of TSH and PRL by directly affecting the hormone-producing cells of the anterior pituitary.

Another factor involved in the direct control of TSH secretion, might be somatostatin, since somatostatin inhibits basal and TRH-stimulated TSH release in anterior pituitary cells (19, 20). Furthermore, *in vitro* somatostatin antiserum stimulates TSH secretion from pituitary cells, while *in vivo* it increases basal serum TSH levels and serum TSH responses to both cold stress and TRH (21, 22). Fasting, IL-1 administration and diabetes mellitus have been found to increase hypothalamic somatostatin content and release (23-25). Furthermore, passive immunization with somatostatin antiserum resulted in a marked increase in plasma TSH in rats after long-term restricted feeding and starvation (26-28). It has been postulated by Smith et al (29) that increased somatostatin release may be mediated

by a central effect of glucocorticoids. He demonstrated a decrease in hypothalamic somatostatin content 10 days after adrenalectomy in rats, which was reversed by dexamethasone administration. With respect to the studies described in this thesis, the acute and profound decrease in plasma TSH after food deprivation, IL-1 and IL-6 adminsitration and diabetes mellitus induction may reflect alterations in the secretion of the somatostatin, possibly mediated by glucocorticoids.

Neuropeptide Y (NPY) is another candidate peptide that may mediate the reduction in TSH secretion during NTI. However, some studies report a suppressive effect on pituitary TSH secretion, whereas others see no effect of NPY on serum TSH (30-32). On the other hand, NPY administration was found to increase DA release by the ME (32), which may mediate an dopaminergic inhibitory effect of NPY on TSH secretion.

In conclusion, in the generation of the low T3 syndrome the hypothalamus seems the primary site affected. From the data presented in this thesis, it can be concluded that decreased pituitary TSH secretion cannot be attributed to changes in hypothalamic TRH alone, but is more likely caused by a number of concomitant changes at the hypothalamic, pituitary and peripheral level, which may act in concert to manifest impaired thyroid hormone secretion.

4.2 Role of TRH in the regulation of prolactin secretion

The response to suckling includes a) an increase in PRL release from the anterior pituitary and b) sequential changes in the pituitary-thyroid axis, resulting in the decrease in plasma thyroid hormone concentration. The importance of an increased TRH release underlying the stimulated PRL release in lactating rats has been challenged, because of the lack of a concomitant rise in TSH during suckling. In this thesis, variations in levels of hypothalamic proTRH mRNA throughout lactation and after a separation of mothers and pups for 6 h were investigated, in order to elucidate the possible bifunctional role of TRH during lactation.

Given the dual role of TRH as a TSH and PRL releasing factor one expects an increase in hypothalamic TRH synthesis and release underlying the stimulated PRL secretion in lactating rats, whereas the decreased plasma thyroid hormone levels in lactating rats suggest a decrease in TRH release. At the onset of the lactation period and in response to readmission of pups to their mother after a separation period hypothalamic proTRH mRNA was found to be transiently increased . This suggests that additional factors are involved in the central and

peripheral regulation of PRL and TSH secretion during suckling.

Based on our data and the observations of others, we postulate the following sequence of events before and during lactation. High levels of estrogen and decreased plasma T3 levels at the end of pregnancy (33-35) may be responsible for an increased sensitivity of the pituitary lactotrophs to TRH. Estrogens increase the number of lactotrophs during pregnancy (36), and increase the TRH receptor levels in these cells (37, 38). Moreover, estrogens inhibits activity of the T3-stimulated membrane-bound TRH degrading enzyme, which is preferentially located on lactotrophs and participates in the inactivation of extracellular TRH (39, 40). During pregnancy the inhibitory effect of T3 on TRH receptor levels on lactotrophs (37, 41, 42) and the stimulatory effect of T3 on TRH degrading enzyme activities (39, 40) are diminished because of the decrease in plasma T3. However, low levels of T3 are known to increase DA release (43), which may prevent an increased PRL release in pregnant rats prior to lactation.

Early lactation is characterized by pulsatile bursts of PRL secretion in response to suckling. These PRL surges could be secondary to a transient reduction of DA secretion into hypophysial portal blood, which potentiates the prolactin-releasing action of hypothalamic TRH (44-48). The PRL response to the transient increase in proTRH mRNA early in lactation is enhanced by the increased TRH sensitivity of the lactotrophs, in addition to the decreased DA tone early in lactation. In addition, Nagy et al (49) reported morphological shifts in lactotroph populations in response to suckling. According to this group, pituitary tissue is sensitized to PRFs by a decrease in number of cells susceptible to inhibition by DA and an increase in those responsive to PRFs.

The role of TRH as a PRF has been established by 1) PRL secretion in response to TRH administration from rat anterior pituitary cells (50), 2) the presence of specific TRH membrane receptors on lactotrophs (51, 52) and 3) the demonstration of TRH responsiveness of two regions on the 5'-flanking region of the PRL gene (53, 54). The role of TRH as both a TSH and PRL releasing factor has recently been confirmed by Haisenleder et al (55), who measured the expression of PRL and TSH subunit mRNA in response to different TRH pulses. They demonstrated that the pattern of TRH pulsatile signals can influence the expression of the genes of these pituitary hormones in a differential manner. The role of TRH as a PRF during suckling has been supported by the findings that TRH concentration in hypophysial portal blood increases following suckling-induced PRL

release (47, 48).

After the initial increase of the proTRH mRNA levels on day 2 of lactation, and following readmission of pups to mothers after a 6 h separation later during lactation, these levels return to normal. The described changes in plasma PRL until day 8 of lacation cannot solely be explained by changes in TRH synthesis, and hence PRFs other than TRH have to be postulated. The expected high levels of proTRH mRNA during the remaining lactation period may be prevented by the stimulation of the hypothalamo-pituitary-adrenal axis. This suckling-induced increase in plasma corticosterone may be involved in the suppression of the proTRH gene expression (the inverse relationship between corticosterone on proTRH mRNA has been discussed in chapter 4.1).

The increase of hypothalamic TRH synthesis on day 2 of lactation, is accompanied by a increase in pituitary TSH secretion. On day 13 of lactation, the transient increase of hypothalamic proTRH mRNA following reunion of mothers and pups after a 6 h separation, is not accompanied by an increase in TSH secretion. This different response on day 2 and day 13 of lactation to the hypothalamic TRH signal, may be related to an increased sensitivity of the pituitary thyrotrophs to TRH in pregnant rats near term and at the onset of lactation, or to an increase in plasma corticosterone during lactation, or both. As discussed in chapter 4.1, glucocorticoids can exert a direct suppressive effect on pituitary TSH secretion, and levels of plasma corticosterone on day 13 are higher than those on day 2 of lactation. This suppression of pituitary TSH secretion by plasma corticosterone, may prevent the normal pituitary response to increased TRH synthesis on day 13 of lactation.

As lactation progresses, the magnitude of PRL response to suckling decreases in rats (56-58). Mechanisms that could contribute to this decline are reduction in intensity and frequency of suckling, lactotroph refractoriness to PRL-releasing stimuli, and a faster PRL metabolic clearance rate (58-61).

In conclusion, during lactation TRH seems primarily involved in the onset of PRL release and other factors are important for the continuation of the suckling-induced PRL release. Furthermore, our data support the dual role of TRH as a PRL and TSH releasing factor. The differential response of anterior pituitary PRL and TSH secretion to the hypothalamic TRH signal during lactation is regulated by a variety of factors. The role of TRH as a PRF is affected by functional differences between lactotrophs and thyrotrophs influencing the sensitivity and/or response of these cells to hypothalamic and peripheral factors.

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SUMMARY

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Thyrotropin-releasing-hormone (TRH) is produced by hypothalamic neurons, transported to the median eminence, where it is released into the hypophyseal portal blood. At the pituitary gland it stimulates the function of the thyrotrophs and lactotrophs, which synthesize and release thyroid-stimulating-hormone (TSH) and prolactin (PRL), respectively. TSH, in turn, stimulates the secretion of thyroxine (T4) and triiodothyronine (T3) from the thyroid gland. In addition to stimulating milk production, PRL is involved in a broad spectrum of biological activities. In this thesis the role of TRH was studied in the regulation of TSH and PRL secretion under different (patho-) physiological conditions.

The role of TRH in the regulation of TSH secretion under four thyroid function-suppressing conditions - starvation, long-term food reduction, interleukin administration and STZ-induced diabetes mellitus - was investigated (chapter 2). Starvation and food reduction cause a suppression of the metabolic rate, in order to save energy. This adaption of the body is associated with low plasma levels of T3, and is therefore known as the low T3 syndrome. Interleukin-induced systemic illness and diabetes mellitus are experimental models of non-thyroidal illness, also resulting in a low T3 syndrome. In view of the low plasma T4 and/or T3, these four (patho-) physiological conditions are characterized by inappropriatly normal or low levels of TSH. This points to a central mechanism for the inhibition of TSH secretion and thyroid function. Therefore, the contribution of TRH to the suppressed thyroid function in this syndrome was investigated.

A 3-day starvation period decreases hypothalamic proTRH mRNA, hypothalamic TRH content and TRH concentration in portal blood. In contrast to the effect of starvation, long-term food reduction does not affect levels of hypothalamic proTRH mRNA, nor TRH content in the ME, despite a profound decline in plasma TSH. Levels of hypothalamic proTRH mRNA are also unaffected after 7 days of IL-6 infusion, while IL-1 infusion causes a significant decrease in these levels after 7 days, but not after 1 or 2 days when plasma TSH reached its nadir. Two and three weeks after induction of diabetes mellitus with STZ, R-A rats show decreased levels of proTRH mRNA, while this parameter is unaffected in RxU rats.

Summarizing the effects of these four studies on hypothalamic TRH production and release, it is clear that these parameters are either unaffected or show a trend towards reduction rather than an increase as might be expected, if

changes in hypothalamic TRH were secondary to the reduction in plasma thyroid hormone levels. Decreased TSH secretion occurs before (IL-1 treatment and STZ-induction of diabetes mellitus in R-A rats) or even without (food reduction, IL-6 treament and STZ-induction in diabetes mellitus in RxU rats) the decrease in proTRH mRNA. The decline in TSH secretion, therefore, cannot only be explained by the changes in hypothalamic proTRH mRNA. Concomitant changes at the hypothalamic and/or pituitary level seem to be involved in the generation of a low T3 syndrome.

Starvation, food reduction, interleukin administration, and STZ-induced diabetes mellitus are all associated with increased plasma levels of glucocorticoids. An inverse relationship has been demonstrated between levels of plasma corticosterone and levels of proTRH mRNA. Continuous IL-1 infusion stimulates corticosterone secretion, and proTRH gene expression is reduced after 7 days, but not after 1 or 2 days of infusion. In the same study, IL-6 infusion had no effect on plasma corticosterone nor on hypothalamic proTRH mRNA. A similar observation was made in the study on STZ-induced diabetes mellitus. In R-A diabetic rats the increased plasma corticosterone concentration is accompanied by a reduced proTRH gene expression, whereas in RxU diabetic rats neither parameter changed significantly. A suppressive effect of plasma corticosterone on proTRH gene expression would explain the different effects of IL-1 *vs.* IL-6, and strain-dependent responses to STZ-induced diabetes on proTRH mRNA. Negative control of hypothalamic proTRH by plasma glucocorticoids may be one of the central mechanisms underlying the generation of the low T3 syndrome.

However, our data demonstrate that the decreased pituitary TSH secretion cannot be attributed to changes in hypothalamic proTRH alone. Direct effects at the level of the pituitary by e.g. glucocorticoids, somatostatin and NPY may contribute to the decreased TSH secretion. Concomitant changes at the hypothalamic, pituitary and peripheral level may act in concert to inhibit thyroid hormone secretion.

In chapter 3 the dual role of TRH in the secretion of PRL and TSH was investigated during lactation. Lactation is associated with an increase in PRL release from the anterior pituitary and changes in the pituitary-thyroid axis, resulting in the decrease in plasma thyroid hormone concentration. The latter is unexpected if high PRL levels during lactation are due to increased TRH stimulation. This thesis describes the effects of litter size throughout lactation and the effects of acute suckling after a period of separation of mothers and pups, on TRH synthesis and

release.

Suckling-induced increase of plasma PRL was accompanied by a transient increase of hypothalamic proTRH on day 2 of lactation and, at later time points, following readmission of mothers and pups after a 6 h separation. This suggests that during lactation TRH is primarily involved in the onset of PRL release and that other factors are important for the continuation of the suckling-induced PRL release. The return of hypothalamic proTRH to normal levels during continued lactation may be mediated by an inhibitory effect of the suckling-induced increase in plasma corticosterone.

The increase of hypothalamic TRH synthesis on day 2 of lactation, is accompanied by a increase in pituitary TSH secretion. On day 13 of lactation, the transient increase of hypothalamic proTRH mRNA following reunion of mothers and pups after a 6 h separation is not accompanied by an increase in TSH secretion. This different response on day 2 and day 13 of lactation to the hypothalamic TRH signal may be related to an increased sensitivity of the pituitary thyrotrophs to TRH in rats at the onset of lactation, and/or to the increased levels of plasma corticosterone at day 13 of lactation, which may exert a direct inhibitory effect at the pituitary. Furthermore, the differential control of anterior pituitary PRL and TSH secretion by the hypothalamic TRH signal during lactation is related to functional differences between lactotrophs and thyrotrophs, which influence the sensitivity and/or response of these cells to hypothalamic and peripheral factors.

In conclusion, in the generation of the low T3 syndrome, the decreased pituitary TSH secretion cannot be attributed to changes in hypothalamic proTRH alone. Dependent on the (patho-) physiological condition, concomitant changes at the hypothalamic, pituitary and peripheral level act in concert to inhibit thyroid hormone secretion.

SAMENVATTING

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Het tripeptide "thyrotropin-releasing-hormone" (TRH), geproduceerd door hypothalame neuronen, wordt getransporteerd naar de eminentia mediana waar het wordt afgegeven aan het hypofysesteelbloed. Aangekomen bij de hypofyse, stimuleert het vervolgens de functie van de thyrotrofe en lactotrofe cellen, welke respectievelijk schildklier-stimulerend-hormoon (TSH) en prolactine (PRL) synthetiseren en afgeven. TSH, op zijn beurt, stimuleert de secretie van thyroxine (T4) en triiodothyronine (T3) uit de schildklier. Naast stimulatie van de melkproduktie is PRL betrokken bij een breed scala aan biologische activiteiten. In dit proefschrift wordt de rol van TRH bestudeerd in de regulatie van de TSH- en PRL-secretie tijdens verschillende (patho-)fysiologische condities.

De rol van TRH in de regulatie van de TSH-secretie werd onderzocht tijdens vier schildklierfunctie-onderdrukkende condities, nl. vasten, langdurige voedselreductie, interleukinentoediening en STZ-geïnduceerde diabetes mellitus (hoofdstuk 2). Vasten en voedselreductie veroorzaken een onderdrukking van het basaal metabolisme waardoor energie wordt bespaard. Deze aanpassing van het lichaam wordt geassocieerd met lage plasma-T3-spiegels en staat daardoor bekend als het lage-T3-syndroom. Interleukinen-geïnduceerde systemische ziekte en diabetes mellitus zijn experimentele modellen voor niet-schildklier aandoeningen (non thyroidal illness) welke ook leiden tot het lage-T3-syndroom. De lage plasma-T3-spiegels tijdens deze vier (patho-)fysiologische condities gaan gepaard met onwaarschijnlijk normale of lage plasma-TSH-spiegels. Dit duidt op een centraal mechanisme dat verantwoordelijk is voor de remming van de TSH-secretie en schildklierfunctie. Daarom werd de bijdrage van TRH in de ontwikkeling van onderdrukte schildklierfunctie onderzocht.

Drie dagen vasten veroorzaakte een daling in hypothalaam proTRH mRNA, hypothalame TRH-content en TRH-concentratie in het portale bloed. In tegenstelling tot vasten, veroorzaakte langdurige voedselreductie geen veranderingen in hypothalaam proTRH mRNA en TRH-content in de eminentia mediana, ondanks de sterke daling in plasma-TSH. Hypothalaam proTRH mRNA-spiegels bleven eveneens onveranderd na 7 dagen interleukine-6-infusie, maar interleukine-1-infusie veroorzaakte een daling van deze spiegels na 7 dagen, zij het niet na 1 of 2 dagen, wanneer plasma-TSH het laagst was. Twee en drie weken na inductie van diabetes mellitus met STZ, daalde het proTRH mRNA in de hypothalamus significant in de

R-A ratten, maar bleef onveranderd in RxU ratten.

De effecten van deze vier condities op de hypothalame TRH-produktie en -afgifte samengevat, tonen aan dat deze parameters onveranderd of verlaagd zijn. Deze resulaten zijn in strijd met de te verwachten stijging, ervan uitgaande dat veranderingen in hypothalaam TRH secundair zijn aan de afname in plasma-schildklierhormoonspiegels. De verlaging in TSH-secretie werd waargenomen voor (IL-1-infusie en STZ-geïnduceerde diabetes mellitus in R-A ratten) of zelfs zonder (voedselreductie, IL-6-infusie en STZ-geïnduceerde diabetes mellitus in RxU ratten) een daling in proTRH mRNA. De daling in TSH-secretie kan daardoor niet volledig verklaard worden op basis van de veranderingen in hypothalaam proTRH mRNA. Waarschijnlijk zullen andere veranderingen op hypothalaam en hypofysair niveau bijdragen aan de ontwikkeling van het lage-T3-syndroom.

Vasten, voedselreductie, interleukinentoediening, en STZ-geïnduceerde diabetes mellitus gingen alle gepaard met een stijging in de plasma-glucocorticoïdenspiegels. Een inverse relatie tussen deze spiegels en proTRH mRNA werd aangetoond. Continue IL-1-infusie stimuleerde corticosteronsecretie en onderdrukte de proTRH-genexpressie na 7 dagen, maar niet na 1 en 2 dagen. IL-6-infusie had daarentegen geen effect op plasma-corticosteron, noch op proTRH mRNA. In de studie aangaande STZ-geïnduceerde diabetes mellitus namen we een stijging in plasma-corticosteron en een daling in proTRH mRNA waar in de R-A ratten, terwijl in de RxU ratten de beide parameters niet significant veranderden. Een onderdrukkend effect van plasma-corticosteron op de proTRH-genexpressie zou de verschillende effecten van de IL-1- *vs.* de IL-6-behandeling en de stam-afhankelijke effecten van STZ-geïnduceerde diabetes mellitus op proTRH verklaren. Een negatieve controle van hypothalaam proTRH door plasma-glucocorticoiden zou een van de centrale mechanismen kunnen zijn die ten grondslag liggen aan de ontwikkeling van het lage-T3-syndroom.

Onze data tonen echter aan dat de verlaagde hypofysaire TSH-secretie niet alleen verklaard kan worden door veranderingen in hypothalaam proTRH. Directe effecten op het niveau van de hypofyse door bv. glucocorticoiden, somatostatine en NPY kunnen eveneens bijdragen aan de verlaging van de TSH-secretie. Waarschijnlijk leiden veranderingen op hypothalaam, hypofysair en perifeer niveau tesamen tot een verlaagde schildklierhormoonsecretie.

In hoofdstuk 3 wordt de dubbele rol van TRH in de secretie van PRL en TSH bestudeerd tijdens zogen. Lactatie wordt enerzijds geassocieerd met een toename

in de PRL-afgifte van de hypofysevoorkwab, en anderzijds met veranderingen binnen de hypofyse-schildklier-as, resulterend in verlaagde schildklierhormoonspiegels. Dit laatste is onverwacht als veranderingen in hypothalaam TRH secundair zouden zijn aan de verlaging in schildklierhormoonspiegels in plasma. Dit proefschrift beschrijft de effecten van nestgrootte tijdens de gehele lactatieperiode en de effecten van een acute zoogstimulus na een periode waarin moeders en pups gescheiden zijn geweest, op TRH-synthese en -afgifte.

De door zogen geïnduceerde toename in PRL-afgifte gaat samen met een korte stijging van hypothalaam proTRH mRNA op dag 2 van lactatie en op dag 13 van lactatie, na terugplaatsing van moeders en pups nadat zij 6 uur gescheiden waren geweest. Dit suggereert dat tijdens lactatie TRH voornamelijk betrokken is bij de aanzet tot de verhoogde PRL-afgifte, en dat andere factoren verantwoordelijk zijn voor het verdere verloop van de PRL-spiegels. Het snelle herstel van hypothalaam proTRH mRNA zou gemediëerd kunnen worden door een remmend effect van de door zogen geïnduceerde hoge plasma-corticosteron-spiegels.

De toename van de hypothalame TRH-synthese op dag 2 van lactatie gaat samen met een toename in plasma-TSH. De korte toename van proTRH mRNA na terugplaatsing van moeders en pups op dag 13 van lactatie, gaat niet samen met een toename in plasma-TSH. Dit verschil in respons op dag 2 en dag 13 van lactatie op de TRH-stimulus, zou gerelateerd kunnen zijn aan een toegenomen gevoeligheid van de hypofysaire thyrotrofe cellen voor TRH in ratten aan het begin van de lactatie-periode, en/of aan de verhoogde plasma-corticosteron-spiegels op dag 13 van lactatie, welke een direct remmend effect op de hypofyse zouden kunnen uitoefenen. Bovendien is het verschil in regulatie van de hypofysaire TSH- en PRL-secretie door hypothalaam TRH tijdens lactatie gerelateerd aan functionele verschillen tussen lactotrofe en thyrotrofe cellen, welke de gevoeligheid en/of respons van deze cellen beïnvloeden op hypothalame en perifere factoren.

Tijdens de ontwikkeling van het lage-T3-syndroom, kan de verlaagde TSH-secretie niet volledig worden toegekend aan veranderingen in hypothalaam proTRH. Afhankelijk van de (patho-)fysiologische conditie, zullen gelijktijdige veranderingen op hypothalaam, hypofysair en perifeer niveau tesamen, leiden tot de uiteindelijke remming van schildklierhormoonsecretie.

ACTH	Adrenocorticotropic hormone
Arg	Arginine
BSA	Bovine serum albumin
BW	Body weight
CNS	Central nervous system
CRH	Corticotropin-releasing hormone
DA	Dopamine
DKP	Diketopiperazine
DNA	Deoxyribonucleic acid
F	Free (not protein bound)
FF	Free fraction
FFA	Free fatty acids
Gln	Glutamine
Glu	Glutamic acid
Gly	Glycine
His	Histidine
HPLC	High performance liquid chromatography
ID1	Type 1 iodothyronine deiodinase
IL-1	Interleukin-1
IL-6	Interleukin-6
iv	Intravenous
LPS	Lipopolysaccharide
Lys	Lysine
ME	Median eminence
MMI	Methimazol
NTI	Non-thyroidal illness
NPY	Neuropeptide Y
Pro	Proline
Ps4	Spacer peptide 4
proTRH	Prohormone of TRH
PRL	Prolactin
PVN	Paraventricular nucleus
R-A	R-Amsterdam
RIA	Radioimmunoassay
mRNA	Messenger ribonucleic acid
SEM	Standard error of the mean
STZ	Streptozotocin
T3	Triiodothyronine
rT3	Reverse triiodothyronine
T4	Thyroxine
TBG	Thyroxine-binding globuline
TBPA	Thyroxine-binding prealbumin
TRH	Thyrotropin-releasing hormone
TSH	Thyroid-stimulating hormone
UDP	Uridine diphosphate
UGT	UDP-glucuronyltransferase

Het is een goede gewoonte te onderstrepen dat het schrijven van een proefschrift nooit een individuele zaak is, maar veeleer het resultaat van het werk van een collectief. Tijdens dit onderzoek dat uiteindelijk heeft geleid tot deze dissertatie, wist ik mij gesteund door velen. De verantwoordelijkheid voor de tekst ligt bij mij, maar zonder de hulp, inzet en steun van anderen was deze misschien nooit verschenen.

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Goedele van Haasteren werd geboren op 3 mei 1967 te Rotterdam. In 1975 verhuisde zij met haar ouders naar Hilvarenbeek, waar zij tot 1985 bleef wonen. In dat jaar behaalde zij haar vwo-diploma aan het Mill-Hillcollege te Goirle. Aansluitend verhuisde zij naar Maastricht om Gezondheidswetenschappen te gaan studeren aan de Rijks Universiteit Limburg. Al snel koos zij voor de richting *Biologische Gezondheidskunde*, werd lid van de *curriculumgroep* van deze studierichting en begeleide menige studiegroep. Ter oriëntatie op de verschillende vakgebieden binnen de specialisatie *Biologische Gezondheidskunde*, liep zij allereerst stage bij Prof. Dr. A.C. Nieuwehuijzen-Kruseman op de afdeling Endocrinologie van het Academische Ziekenhuis van Maastricht. Ze bestudeerde daar de rol van alfa-glucosidaseremmers als additionele behandelingswijze voor patiënten met type II diabetes mellitus. Vervolgens vertrok zij voor een jaar naar Leuven (België) om stage te gaan lopen op de afdeling Experimentele Geneeskunde en Endocrinologie van de Katholieke Universiteit van Leuven. Onder begeleiding van Prof. Dr. R. Bouillon vergeleek zij drie eiwitten in de urine van type I diabetes-mellituspatiënten als markers voor diabetische nefropathie. Voor haar laatste stage verhuisde zij naar Rotterdam om daar onder begeleiding van Dr. A.M. Verkerk onderzoek te doen naar de moleculaire basis van het fragiele-X-syndroom.

Nog voor de uitreiking van haar doctoraaldiploma in juni 1991, begon zij te werken als assistent in opleiding bij de afdeling Endocrinologie en Voortplanting van de Erasmus Universiteit in Rotterdam. In dit proefschrift staat het onderzoek beschreven dat zij daar gedurende vier jaar verrichtte. Tijdens de zomer van 1994 bracht zij een werkbezoek van 3 maanden aan de afdeling Endocrinologie en Metabolisme van de C.H.U.V. in Lausanne (Zwitserland), onder de begeleiding van Dr. M. J. Reymond. Haar eerste ervaring als *post-doc* zal zij per oktober 1995 opdoen aan de Fondation de Recherche Médicale te Genève (Zwitserland) bij Prof. Dr. W. Schlegel.