REGULATION OF THE HYPOTHALAMIC RELEASE OF THYROTROPIN-RELEASING HORMONE (TRH)

Regulatie van de hypothalame afgifte van thyrotropin-releasing hormone (TRH)

PROEFSCHRIFT

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Voor mijn ouders
Voor Jolande

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CHAPTER I.

GENERAL INTRODUCTION AND SCOPE OF THESIS.

A. INTRODUCTION.

Thyrotropin-releasing hormone (TRH) is a tripeptide (pyroglutamyl-histidyl-proline amide; pGlu-His-ProNH_2) that was chemically characterized in 1970 by the groups of Guillemin (1) and Schally (2). It is synthetized from a high-molecular weight prohormone (3-5), the genetic code of which was recently unravelled by Lechan et al. (4) and Lee et al. (5). In the rat one molecule of this prohormone generates five molecules of TRH. The processing of one TRH-generating sequence in the prohormone is shown in **Figure 1**. The sequence glutaminyl-histidyl-prolyl-glycine (Gln-His-Pro-Gly) is flanked by two basic amino acids, lysine (Lys) and arginine (Arg). After removal of these residues by a trypsin-like protease and a carboxypeptidase (6), Gln is cyclized to pGlu (7) and Pro is amidated by modification of Gly (8). This last step is rate-limiting and catalyzed by a peptidyl α-amidating monooxygenase (PAMase) requiring oxygen, copper and ascorbate as cofactors (9).

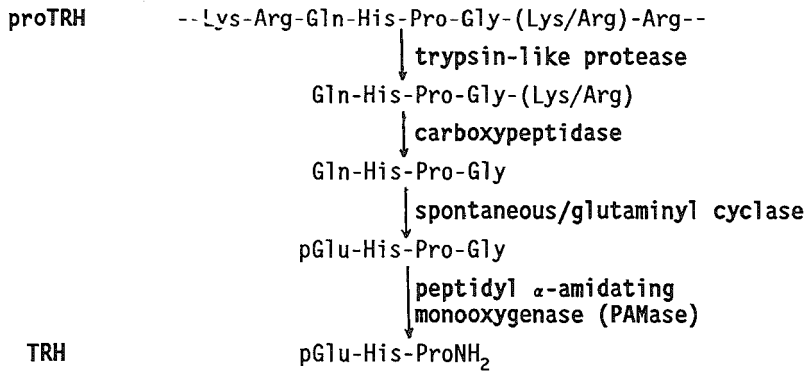


Figure 1: Biosynthesis of thyrotropin-releasing hormone (TRH).

It should be stressed that processing of proTRH may give rise to a family of peptides other than TRH, some of which may be of biological significance (10-13). Moreover, there is a striking difference in proTRH processing patterns among various tissues, suggesting tissue-specific differential regulating mechanisms for TRH (10-13). TRH is ubiquitous in the body and has diverse endocrine and non-endocrine effects on different organ systems (for review see Refs. 14 and 15). Also biosynthesis of proTRH

has been found to occur throughout the central nervous system (12,13), in organs like pancreas (16) and prostate (17), in a medullary thyroid carcinoma cell line (18) and in fetal hypothalamic cells in culture (see next section). It is believed that this diversity in location and effects of TRH stems from two distinct functions of TRH. The extrahypothalamic location and non-endocrine effects of TRH point to a neuromodulating role, whereas its hypothalamic site and endocrine functions relate to a classical hypophysiotropic role, which is dealt with in this thesis.

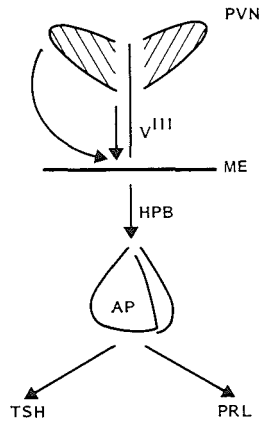
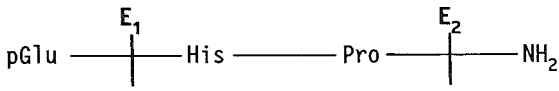


Figure 2: Schematic view of hypothalamus and transport of TRH to anterior pituitary leading to TSH and PRL secretion (PVN=paraventricular nucleus, V^{III}=third ventricle, ME=median eminence, HPB=hypophysial portal blood, AP=anterior pituitary).

The hypophysiotropic TRH is synthetized in the medial parvocellular part of the paraventricular nucleus in the hypothalamus (19) (**Figure 2**) and transported via anterolateral and medial pathways along the third ventricle to the external zone of the median eminence (20), from where it is released into hypophysial stalk blood. In this way TRH reaches its receptor on the thyrotroph and lactotroph of the anterior pituitary. Peptides derived from proTRH are also transported along the axons with TRH to the external zone of the median eminence (12,21) and may be released into hypophysial portal blood as well. Binding of TRH to its receptor leads to phospholipase C activation (22), resulting in hydrolysis of phosphatidylinositol-4,5-diphosphate. The resulting products diacylglycerol and inositol-1,4,5-triphosphate increase protein kinase C activity and cytoplasmic calcium

concentrations, respectively, which ultimately lead to secretion of TSH.



E_{1c} non-specific pyroglutamate aminopeptidase (cytosol)
E_{1s} TRH-specific pyroglutamate aminopeptidase (serum)
E_{1p} particulate TRH-degrading enzyme (plasma membrane)
E_2 post-proline cleaving enzyme (cytosol)

Figure 3: Metabolism of TRH (from Ref. 23).

The bioavailability of TRH is also regulated by its metabolism in plasma and tissue (23) **(Figure 3)**. Cleavage at the C-terminal Pro-NH_2 bond occurs only in tissue. It is catalyzed by a post-proline cleaving enzyme which is not TRH-specific, as is the cytoplasmic pyroglutamate peptidase. Because of their intracellular localization these two enzymes cannot be important for the inactivation of TRH after its release. Hydrolysis of the pGlu-His bond results in the formation of His-ProNH_2 which is further degraded to its constituent amino acids or cyclized spontaneously to the diketopiperazine (cyclo(His-Pro) or DKP) which is claimed to be biologically active (24). The TRH-degrading serum enzyme is TRH-specific and regulated by estrogens and thyroid hormone, as is the particulate, plasma membrane-bound pyroglutamyl peptidase in the anterior pituitary (23). This enzyme may thus exert a biologically important function in the inactivation of plasma TRH and the regulation of the TRH effect at the target sites in the pituitary. The TRH-degrading brain enzyme is located on the outer surface of the nerve terminal membranes and may be important for the inactivation of synaptically released TRH.

This thesis deals with the two foremost endocrine effects of TRH: its role as a classical hypothalamic hypophysiotropic releasing hormone of TSH (25) and PRL (26), thus controlling thyroid function and lactation, respectively. The body may regulate the bioavailability and therefore the effect of TRH by altering the aforementioned processes, i.e.: synthesis and processing of the TRH-precursor, transport of TRH in the hypothalamus, its release into hypophysial portal blood and its metabolism at the neuronal synapse, in the blood or at the target sites in the anterior pituitary. Regulation of hormonal release, however, seems to be the most important

factor by which the body can control the effect of a hormone. Therefore, the scope of this thesis is to discuss the way hypothalamic TRH release is regulated with regard to thyroid function and lactation. In **Section B** of this chapter neuro-endocrine techniques are discussed that enable the measurement of the release of hypothalamic substances. Part of our own results are integrated in this section and described in greater detail in **Chapter II. Sections C and D** of this chapter deal with the role of TRH in the regulation of TSH and PRL secretion, respectively. Again, our previously published results are integrated in these sections and described in detail in **Chapters III and IV**, respectively.

B. THE MEASUREMENT OF HYPOTHALAMIC TRH RELEASE.

Table 1 shows the most common techniques for the measurement of the release of hypothalamic substances. Some techniques are considered to specifically measure hypothalamic release (push-pull perfusion, collection of hypophysial portal blood, intracerebral microdialysis), while others only represent an indirect estimate of release of hypothalamic substances (tissue content, peripheral blood levels).

Table 1: Measurement of the release of hypothalamic substances.

In vivo techniques:
-levels of TRH in peripheral blood
-collection of hypophysial portal blood
-push-pull perfusion of the median eminence or the anterior pituitary
-intracerebral microdialysis
-content of TRH in hypothalamus or pituitary

In vitro techniques:
-static incubation or superfusion of hypothalamic fragments or synaptosomes
-hypothalamic neurons in culture
-cell lines

In vivo techniques.

Measurement of TRH immunoreactivity (TRH-IR) in **peripheral blood** has frequently been used as an estimate of hypothalamic TRH release, both in man (27-30) and in the rat (31-33). Indeed, TRH-IR in peripheral blood

shows the same diurnal rhythm as TRH levels in the hypothalamus (34) and some studies show that plasma TRH-IR changes with thyroid status (28,30), cold exposure (31,32), experimentally induced diabetes mellitus (35,36) and starvation (37). These effects, however, may also stem from alterations in the metabolic clearance of TRH in these conditions (23). Moreover, only part of the TRH in peripheral blood is derived from the hypothalamus (27,38,39) and only part of the TRH-IR recovered from peripheral blood is authentic TRH (40,41). In comparison, De Greef et al. showed that all TRH-IR in hypophysial stalk blood comigrates with synthetic TRH on HPLC (42). Moreover, most if not all TRH in hypophysial portal blood is of hypothalamic origin (43). Therefore, measurement of immunoreactive TRH in peripheral blood provides a poor estimate of hypothalamic release of TRH, and collection of hypophysial portal blood is more conclusive.

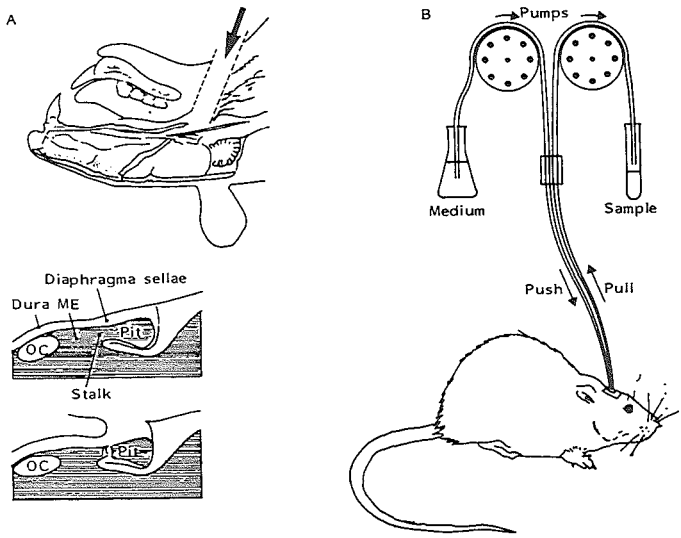


Figure 4: Collection of hypophysial portal blood (**A:** ME=median eminence, Pit=pituitary, OC=optic chiasm), and push-pull perfusion technique (**B**).

Collection of **hypophysial portal blood (Figure 4A)** into methanol or ethanol to minimize enzymatic TRH degradation is a reliable means to determine hypothalamic TRH release (33,39,44-48). However, the major disadvantages of this technique are the stressful surgery and the cutting of the hypophysial stalk, which excludes the measurement of hypophysial

hormones in peripheral blood during the experiment. Moreover, the use of anesthesia may alter secretion of TSH (49) and PRL (50,51), presumably by affecting release of different hypothalamic substances such as somatostatin (52) and TRH (47).

Push-pull perfusion (Figure 4B) of the median eminence seems to overcome these disadvantages, since no anesthesia is needed, the rat can freely move in its cage and the hypothalamic-pituitary axis remains intact during the perfusion (53). Indeed, this technique has been used to measure hypothalamic release of various substances, such as catecholamines (54,55), LHRH (54,56), somatostatin (57), CRF (58) and TRH (54,55,59-63). The technique is based on the principle that neurohormones diffuse from the nerve terminals from which they are released in the median eminence to the push-pull perfusate. It is believed that these substances are released into the push-pull perfusate in much the same way as into portal blood, since for example LHRH release during perfusion is pulsatile (56) and can be stimulated by potassium (56) and amphetamines **(Figure 5).** However, the experimental success rate of this technique is low and clotting of the cannula is the foremost cause of perfusion failure (64). Moreover, because only approximately 20% of the median eminence is perfused, sensitive assays are obligatory to measure the low concentrations of hypothalamic substances in the perfusate.

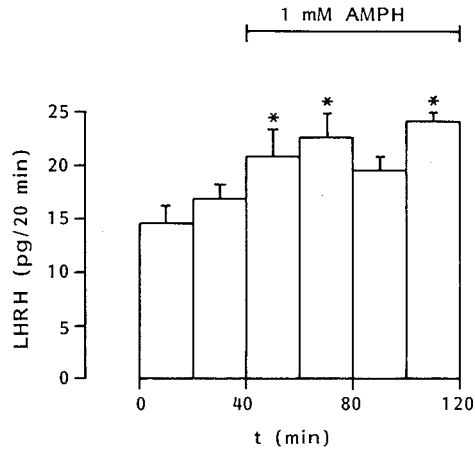


Figure 5: Release of LHRH from the median eminence determined by push-pull perfusion. Basal (0-40 min) and 1 mM amphetamine-induced (AMPH; 40-120 min) release was measured during 20 min intervals (means±SEM, n=5; *$p \leq 0.05$ vs 0-40 min period).

In our experiments it was shown that hypothalamic TRH release into hypophysial portal blood was approximately 200 pg/15 min, while during push-pull perfusion of the median eminence release amounted to around 4 pg/15 min (42,43,48,54,55,63). Moreover, suckling (55) or 56 mM potassium (Figure 6) did not increase TRH release during push-pull perfusion, whereas these conditions are well-known stimuli of TRH release in vivo (48) and in vitro (see below), respectively. A much better correlation is seen between the levels in hypophysial portal blood and in push-pull perfusate with regard to other hypothalamic substances such as LHRH and dopamine (46,56).

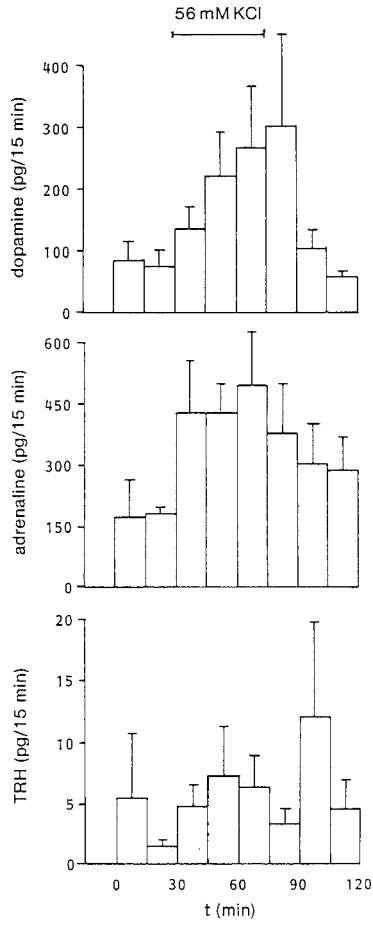


Figure 6: Release of TRH, dopamine and adrenaline (means±SEM, n=6) from the median eminence determined by push-pull perfusion. Rats were anesthetized with pentobarbital (35 mg/kg BW i.p.) and release was measured before (0-30 min), during (30-75 min) and after (75-90 min) a depolarization stimulus (56 mM KCl).

Moreover, during push-pull perfusion release of LHRH and dopamine can clearly be stimulated by amphetamines (**Figure 5**; Ref. 54) and potassium (**Figure 6**; Refs. 54 and 56). Therefore, it seems that measurement of TRH during push-pull perfusion of the median eminence underestimates in vivo TRH release into hypophysial portal blood. Some factors that may contribute to this large difference are inherent to the push-pull perfusion technique such as the small tissue compartment being perfused, and disruption of nerve terminals by an inflammatory reaction round the tip of the push-pull cannula. Other factors are specific for TRH such as disruption of nerve fibers transporting TRH to the median eminence by the push-pull cannula, enzymatic degradation of TRH during push-pull perfusion, and the physicochemical properties of TRH influencing its diffusion from nerve terminals to the push-pull perfusate. Electrical stimulation of the paraventricular area increases TRH release during perfusion of the median eminence by the same factor as TRH release into hypophysial portal blood (**Table 2**; Ref. 63). Other stimuli that increase TRH release during perfusion of the median eminence are acute cold (59,61,62) and noradrenaline or phenylephrine (60). Therefore, hypothalamic pathways transporting TRH to the median eminence seem to remain at least partly intact during perfusion.

Table 2: Hypothalamic TRH release into hypophysial portal blood (HPB) or during push-pull perfusion (PPP) of the median eminence before (basal) and during electrical stimulation of the paraventricular nucleus (PVN).

TRH (pg/15 min)	basal	PVN stimulation
HPB (n=6)	217±25	530±90*
PPP (n=12)	4±1	15±4*

From Ref. 63; means±SEM, *$p \leq 0.05$ vs basal

Recovery of synthetic (54) or radioiodinated (59) TRH added to the push-pull perfusate during perfusion is near 100%. Therefore, enzymatic degradation of TRH in the perfusate is excluded. Most interestingly, however, this finding also shows that little TRH diffuses from the medium into the perfused tissue compartment. This in vivo finding is nicely corroborated in vitro, since tritium-labeled TRH is neither taken up by synaptosome preparations of the rat brain (65). Therefore, it seems that the physicochemical properties of TRH severely limit its diffusion to and

from the perfusate and hence the amounts of TRH detected.

Intracerebral microdialysis seems to overcome many of the disadvantages of the push-pull perfusion technique (66). The experimental success rate is much greater, since tedious pump-balancing between infusion and withdrawal is not necessary, the flowing medium is not in contact with tissue and the outer diameter of the cannula is much smaller. Therefore, tissue damage is minimal and occlusions and hydrostatic pressure changes are not possible. Very low flow rates should be used in this system to obtain maximal exchange and to avoid damage to the dialysis membrane. However, the greater exchange surface allows for greater recovery and detectability of peptide. This method has not yet been applied to the determination of TRH release patterns, but gains more interest in neuroendocrinologic research.

Turnover of hypothalamic TRH is high and amounts to between 34% (45) and 80% (47) per hour. One would assume then that **hypothalamic TRH content** rapidly changes if release is altered. Conflicting results exist with regard to TRH content in hypothyroidism (60,89-93) and diabetes mellitus (35,36,72). This could be related to the effect of age and diurnal variations of hypothalamic TRH content (73). Roti et al. (69) and Bassiri and Utiger (67) show that hypothalamic TRH content is reduced in hypophysectomized rats. Maintenance of normal serum T4 levels did not prevent the reduction in hypothalamic TRH content (67,69), but when TSH was administered simultaneously with T4, hypothalamic TRH content was restored to normal (69). Therefore, Roti et al. conclude that there is a short loop feedback regulation of hypothalamic TRH content (69). It has been shown that total hypothalamic TRH content remains constant under conditions that are believed to alter hypothalamic TRH release such as cold (67,71) and hyperthyroidism (43,67-69). Presumably, synthesis of TRH and processing of proTRH are highly effective in maintaining hypothalamic TRH content in a narrow range in these conditions (74). Therefore, total hypothalamic TRH content does not reflect hypothalamic TRH release. TRH content in discrete hypothalamic areas, such as the median eminence, however, may change under various conditions (75-77) and may therefore be used as an indirect estimate of TRH release. Indeed, it has been reported that hypothyroidism decreases TRH content in the median eminence but not in the whole

hypothalamus, and that thyroid hormone substitution restores TRH content (76,77).

Hypothalamic TRH released into hypophysial stalk blood reaches the anterior pituitary and therefore, **hypophysial TRH content** (78), and push-pull perfusion (63,79,80) or microdialysis (66) of the anterior pituitary might also be used as indicators of hypothalamic neuropeptide release. However, only part of the hypophysial TRH is receptor-bound hormone on the thyrotroph or lactotroph derived from the hypothalamus (81). There is also in situ processing of preproTRH in plurihormonal cells of the anterior pituitary (82,83). Moreover, most hypophysial TRH resides in the posterior pituitary and this TRH probably stems from parvocellular cell bodies in the magnocellualr divisions of the paraventricular nucleus (84). Because of vascular connections between the anterior and posterior pituitary (85), the exact source of hypophysial TRH becomes even more speculative and the physiological meaning of changes in hypophysial TRH content under pathological conditions such as hypothyroidism (77,78) remains inconclusive.

In vitro techniques.

Static incubation (86-92) and **superfusion** (60,93,94) of hypothalamic fragments or synaptosomes have been used to measure hypothalamic TRH release. It has been shown that TRH release is stimulated in a calcium-dependent way by depolarizing agents such as potassium **(Figure 7)**, ouabain or veratridine (86-88,92). Secretion of TRH by hypothalami in static incubation amounts to approximately 0.3-0.5% of total hypothalamic TRH content per hour in male rats (92). Release of TRH into hypophysial portal blood amounts to 34-80% of hypothalamic content per hour (45,46). It should be stressed, however, that the hypothalami in static incubation or superfusion are isolated from their afferent and efferent axonal and vascular connections. Moreover, they are bathed in simple medium, the composition of which is totally different from the in vivo plasma composition, that can change profoundly in pathological conditions. Clearly, these changes are not maintained in vitro. Nevertheless, there seems a good correlation between TRH release in vivo and in vitro under

conditions such as hyperthyroidism (43,92), the oestradiol-stimulated prolactin surge (42,89), opioid inhibition (62,90), and α_1-adrenergic stimulation (60,61).

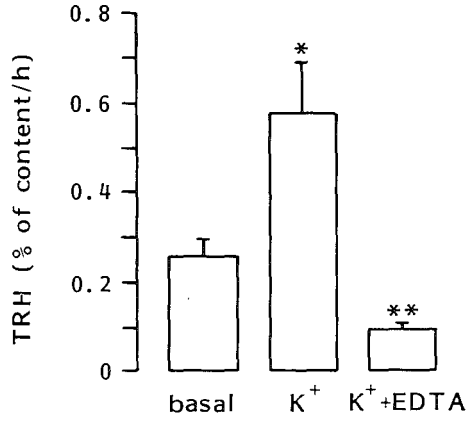


Figure 7: Secretion of TRH during 1 h incubations of hypothalamic fragments under basal conditions (n=20), after addition of 56 mM KCl (K^+; n=14) and after addition of 56 mM KCl and 0.05 M EDTA (K^++EDTA; n=14). Values are expressed as % of hypothalamic TRH content. (From Ref. 92; means±SEM, *$p \leq 0.05$ vs basal, **$p \leq 0.05$ vs basal and K^+).

TRH is also detected in the medium of fetal **hypothalamic neurons in culture** (95-103). Synthesis of TRH by these cells is dependent on ascorbate (97,99,101) and release of TRH is further enhanced by supplementation of the serum-free culture medium with triiodothyronine, corticosterone and polyunsaturated fatty acids (98). Until now only one cell line has been reported to synthesize and secrete TRH and proTRH-derived peptides (18,103a). TRH production in this **medullary thyroid carcinoma cell line (CA77)** increases dose-dependently after addition of dexamethasone (103a). Hypothalamic cells in culture and the CA77 cell line provide a good model to study synthesis and processing of preproTRH and cellular mechanisms leading to TRH release. Only few studies, however, report on regulation of TRH release by these cultures. These studies show that TRH release can be stimulated by depolarizing agents in a calcium-dependent way (97-99,102).

In conclusion, various in vivo and in vitro techniques are used to measure hypothalamic TRH secretion. While some techniques provide poor estimates of hypothalamic release of TRH, others have minor or major

drawbacks that should be taken into consideration in the interpretation of results.

C. THE ROLE OF TRH IN THE REGULATION OF TSH SECRETION.

Numerous conditions have been shown to alter levels of TSH in peripheral blood (reviewed in Refs. 25,104,105), thyroid disease of course being most notorious clinically (105,106). Secretion of TSH may also change in several non-thyroidal diseases that also affect levels of circulating thyroid hormone. These conditions are characterized by low levels of T3 and it is believed that the changes in thyroid hormone concentrations represent an adaptive hypothyroidism that might benefit the sick patient (for review see Ref. 107). These conditions are known as 'the low T3 syndrome' or 'the sick euthyroid syndrome'. Also physiological conditions can influence thyroid function via an effect on TSH secretion. Perhaps most frequently studied is the stimulation of thyroid activity by acute cold exposure (for review see Ref. 108), which may represent an adaptive hyperthyroidism by which the body responds to cold. Less known is that substantial fluctuations in serum levels of TSH may stem from the circadian variation in TSH secretion. Levels of TSH in the rat are lowest at midnight and highest in the afternoon and the same diurnal variation in the hypothalamic TRH content has been observed in some studies (34,73,109). Moreover, it should be stressed that serum levels of TSH are higher in male than in female rats (109), and age also affects secretion of TSH (91,108,111).

In all the above situations it is not known to what extent the changes in the secretion of TSH are mediated by TRH. In this section the role of TRH in the regulation of TSH secretion will be discussed with regard to alterations in thyroid status, and the body's adaptive response to non-thyroidal illness and cold.

Thyroid function.

The importance of TRH in the regulation of thyroid function has been studied in two ways. Firstly, the influence of antagonizing the effect of TRH on thyroid function has been investigated. Since no pharmacologically

specific TRH antagonists are available, the effect of TRH has been neutralized by anti-TRH antiserum or by reduction of TRH secretion by hypothalamic lesions and transections. Secondly, hypothalamic TRH release has been directly measured in altered thyroid states.

Binding of antibodies to TRH is a dynamic process resulting in an equilibrium between bound and unbound TRH. It is, therefore, not known to what extent the hypophysiotropic effects of TRH will be inhibited. Studies show that administration of antiserum to TRH effectively lowers serum TSH levels in euthyroid rats (112-114). In hypothyroid rats, however, serum TSH is only partially suppressed (114-116).

Several studies have investigated the effect of hypothalamic lesions on thyroid function. Electrolytic lesions in the paraventricular area (PVA) or hypothalamic deafferentation have been shown to reduce levels of TRH in the median eminence (117-122) and TRH release into hypophysial portal blood by 85% (43). The effects on plasma TSH and thyroid function produced by lesions in the PVA or in the median eminence, and by deafferentation of the mediobasal hypothalamus are less pronounced. Serum levels of TSH decrease within 2-7 days by 30-60% (43,106,109,123-126), and this decrease is sustained for at least 62 days (123). Also in hypothyroid rats hypothalamic lesions reduce TSH levels by 20-95% (106,120,121,123-125,127), dependent on the type and extent of the lesion. However, the hypothyroidism-induced rise in TSH levels and the concomitant goitrogenesis are not abolished but merely delayed. It should be stressed, however, that no quantitative relationship between hypothalamic TRH release and levels of TSH in peripheral blood can be established from these studies. Hypothalamic lesions and deafferentation have dramatic effects on the secretion of other hypothalamic substances, such as corticotropin-releasing factor (128), somatostatin (129) and dopamine (43). Therefore, it is unknown whether the decrease of TSH levels in lesioned rats results solely from the reduced hypothalamic TRH release. Moreover, hypothalamic lesions in the PVA induce a lower setting of the pituitary thyrostat that might also account for depressed plasma TSH levels. Sensitivity of the thyrotroph to TRH (117,126) and circulating levels of thyroid hormones (123) is increased. It is not known what mechanisms are responsible for these phenomena since PVA lesions do not change the pituitary TRH receptor state (121), nor the activity of the type II thyroxine 5'-deiodinase in the pituitary (118).

The introduction of several neuroendocrine techniques enabled the

direct measurement of hypothalamic TRH release in vivo into hypophysial portal blood and in vitro by hypothalamic fragments. Levels of TRH have also been measured in peripheral blood, hypothalamus and pituitary. There are, however, major drawbacks to the use of these parameters as an estimate of hypothalamic TRH release. These have been discussed in the previous section. Some studies report that TSH secretion may be suppressed by intraventricular or intrahypothalamic administration of thyroid hormone in quantities that are ineffective if given systemically or intrahypophysially (130,131). It is not known, however, if this effect stems from a direct inhibition of hypothalamic TRH release by thyroid hormone. For instance, previous studies have shown that T3 may stimulate somatostatin release in vitro (132), which in turn could inhibit TSH secretion from the pituitary directly (25,104) or via a reduced TRH release from the median eminence (93).

In only a few studies the secretion of hypothalamic TRH in altered thyroid states has been measured directly. In vitro experiments of Iriuchijima et al. reported that the ouabain-stimulated TRH release from hypothalamic fragments in static incubations is affected by thyroid status (130). They noted that the release of TRH from hypothalami of hyperthyroid rats is reduced by approximately 15%, whereas hypothyroidism was found to increase TRH secretion by approximately 35%. The same workers showed that in vitro addition of T3 to incubations of hypothalami from normal rats reduces TRH release in a dose-dependent way by 30-45% (133). However, these results could not be confirmed by other workers (93).

Several studies have determined in vivo release of TRH into hypophysial portal blood of hypo- and hyperthyroid rats. Ching and Utiger (39) could not find significant changes in portal blood TRH concentrations in long-term (21-35 d) hypothyroidism. Treatment with thyroid hormones seemed to reduce TRH levels in stalk blood, but these changes were not significant. Eskay et al. (33) reported treatment of rats with T4 appeared to lower the secretion rate of TRH, since the range of the TRH values in portal plasma from hyperthyroid rats was less than that seen in euthyroid or hypothyroid animals. Neither Guillaume et al. (134) nor Eckland et al. (135) could find significant effects of thyroid status on hypothalamic TRH release into hypophysial portal blood. It should be mentioned that these studies differ greatly in the reported TRH concentrations. Levels of TRH in stalk blood range from as low as 20 pg/ml (134) to as high as 3000 pg/ml

(39). In the study of Eckland et al. (135) TRH release even amounted to around 2500 pg/15 min but HPLC of extracts of hypophysial portal blood revealed a complex pattern of at least four TRH-immunoreactive peaks, independent of thyroid status. The same group reported that portal plasma flow was increased with 140% in rats treated with propylthiouracil (PTU) and they suggest that regulation of pituitary stalk blood flow might constitute a novel mechanism in the regulation of anterior pituitary function (138).

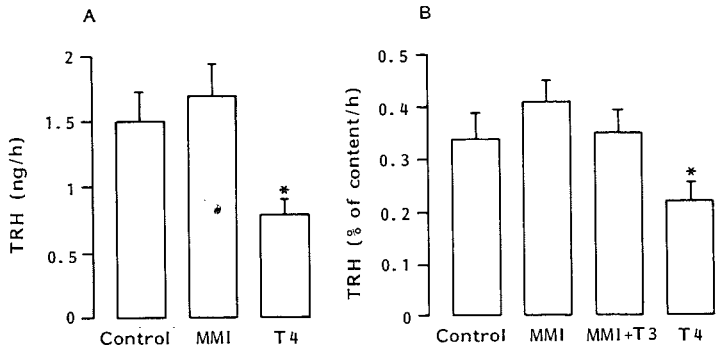


Figure 8: A. Hypothalamic release of TRH into hypophysial portal blood (means±SEM, n=8) of rats treated for 8 days with methimazole (MMI; 0.05% in drinking water) or T4 (10 μg/100 g BW per day i.p.). Untreated rats served as controls. **B.** Secretion of TRH (means±SEM; n=6) during 1 h incubations of hypothalamic fragments of rats treated for 3 weeks with MMI or T4 as above. Hypothalami of untreated rats served as controls. To some incubations of hypothalami from hypothyroid rats 10 nM T3 was added. (From Refs. 43 and 92; *$p \leq 0.05$ vs control).

We recently found that in hyperthyroid rats in vivo TRH release is decreased by 44% (43). In short-term (i.e. 8 d) hypothyroidism, however, TRH release is not significantly altered, although it tends to increase. We could confirm these findings in in vitro experiments: hypothalami of hyperthyroid rats secrete 40% less TRH than normal hypothalami, whereas hypothyroidism does not affect TRH secretion (**Figure 8**; Ref. 92). Several mechanisms may be responsible for the lack of a significant effect of hypothyroidism on in vivo TRH release. Firstly, the duration and severity of the induced hypothyroidism may be important factors in the regulation of hypothalamic TRH release. Indeed, it has been shown that the effect of hypothyroidism on TRH gene expression is significant after 3 weeks. Secondly, it is known that in hypothyroidism intracerebral T3 production is

increased by an enhanced conversion of T4 to T3 by the type II deiodinase enzyme (136,137). Therefore, intracerebral T3 levels may be maintained in the normal euthyroid range in mild hypothyroidism. Until now, however, it is not known to what extent the process of local T4 to T3 conversion is involved in the regulation of hypothalamic TRH release.

Since the characterization of the genetic code of proTRH by Lechan et al. (4), several studies have investigated the effect of thyroid hormones on synthesis and processing of proTRH in the hypothalamus (See Introduction: **Figure 1**). These studies reveal a negative feedback of thyroid hormone on the transcription of the proTRH gene. In hypothyroidism proTRH mRNA and protein levels in the paraventricular nucleus (PVN) increase (139-142). Hence, both transcription and translation of proTRH are increased in hypothyroidism. This increase can be prevented by local (142) or systemic administration of thyroid hormones (139-141). Also administration of thyroid hormone to euthyroid rats reduces mRNA levels of proTRH (140,141) and TRH concentrations in the PVN (143). Synthesis of TRH outside the PVN is not influenced by thyroid status (139,140,142).

These studies raise two important questions: firstly, why is the increased biosynthesis of proTRH during hypothyroidism not reflected by an increased hypothalamic secretion of TRH? Hypothyroidism significantly increases synthesis of proTRH (139-142), whereas its effect on the secretion of hypothalamic TRH is only minimal or absent (33,39,43,92,130,133-135). It seems, therefore, that posttranslational processes counteract the increased synthesis of proTRH. Indeed, variations in proTRH mRNA levels are more prominent than those in hypothalamic TRH content (74). Increased tissue degradation of TRH cannot be responsible for this finding, since thyroid status does not affect the activity of the cellular TRH-degrading enzymes in the hypothalamus (23). Perhaps α-amidation of TRH-Gly to TRH by PAMase is the rate-limiting step in TRH biosynthesis and, therefore, regulation of the PAMase activity might be an important process influencing the releasable pool of TRH (74,97). The increased mRNA levels of proTRH in hypothyroidism could also be reflected in an increased synthesis of proTRH fragments other than TRH, as discussed in the introductory section. The physiological relevance of these peptides in the regulation of TSH secretion, however, remains obscure (144,145).

Secondly, local and systemic administration of T3 reduces synthesis

of proTRH in the PVN (139-143). Formerly, it was thought that intracellular T3 levels in the central nervous system are mainly derived from circulating T4 after intracellular conversion to T3 by the type II thyroxine 5'-deiodinase (137). Synthesis of proTRH, however, can be inhibited directly by high levels of T3 and, therefore, may be largely independent of intracellular conversion of T4 to T3. Indeed, Riskind et al. found that the PVN in contrast to the the median eminence lacks type II deiodinase activity (146). Thus, synthesis of proTRH may be one of the few cerebral processes which are independent of intracellular conversion of T4. The biological relevance of synthesis and processing of proTRH in the regulation of TSH secretion remains an intriguing subject for future studies.

In conclusion, studies employing antiserum to TRH or hypothalamic lesions show that TRH plays an important role in the regulation of TSH levels in the euthyroid rat. In hypothyroidism, however, the hypersecretion of TSH does not seem to be secondary to an increased hypothalamic release of TRH. Thyroid hormones may affect numerous processes involved in the regulation of TRH bioavailability at its pituitary receptor such as hypothalamic synthesis (139-143) and transport (19) of TRH, blood flow through the pituitary stalk (138) and enzymatic degradation in the plasma and the pituitary (23). Hypothalamic secretion of TRH, however, appears barely affected by thyroid status and the mechanisms that are responsible for this finding remain obscure.

The low-T3 syndrome.

Non-thyroidal illness (NTI) is frequently associated with changes in plasma thyroid hormone levels. It is not the aim of this section to extensively review the wide spectrum of these changes and the intricate central and peripheral mechanisms that contribute to the origin of this clinical entity (for excellent reviews see Refs. 107,147,148). Consistent findings in sick euthyroid patients are low concentrations of serum T3 and high levels of rT3. This syndrome is, therefore, called 'the low T3 syndrome', or 'the sick euthyroid syndrome'. It has been shown that the changes in circulating levels of thyroid hormones correlate well with the

severity of the underlying disease. It is believed that these changes represent a spectrum of adaptive hypothyroidism which may benefit the patient because of the preservation of energy. In man this syndrome is also seen during starvation, and after administration of drugs such as propranolol, dexamethasone, amiodarone and cholecystographic agents.

It has been shown that tissue uptake and/or intracellular deiodination of T4 and rT3 are decreased in NTI (149-151). As a consequence, production of T3 and metabolic clearance of T4 and rT3 are reduced, causing serum levels of T3 to decrease and those of rT3 to increase. Furthermore, binding of T4 to serum proteins is inhibited, presumably by free fatty acids (FFA) (152), whereas -at least in the rat- serum binding of T3 seems to be increased because of the production of a thyronine-binding globulin (153). In mild disease these changes may result in increased free T4 (FT4) and decreased free T3 (FT3) levels. It is suggested that the increased levels of FT4 decrease both basal and TRH-induced TSH secretion and, thus, thyroidal T4 production (151,154). Total T4 levels are the net result of thyroidal T4 production and metabolic clearance of T4 in liver and kidney and may be low, normal or high.

In seriously sick patients, serum concentrations of FT4 and total T4 are reduced, while serum TSH levels remain normal or decrease as well (155). The finding of low serum levels of T3 and T4 in patients whith normal or low serum TSH concentrations suggests central inhibition of TSH secretion. During recovery of the disease there is a concomitant rise in the serum levels of TSH, T3 and T4 (156,157). Therefore, it seems that central mechanisms regulating TSH secretion may play a substantial role in the generation of the low T3 syndrome, i.e. in the body's adaptation to disease.

Several models are used to induce the low T3 syndrome in the rat (158,159), e.g starvation (159,160), tumor transplantation (158), experimentally-induced diabetes mellitus (73), lactation (161) and intraperitoneal administration of turpentine (159,162) or cachectin (163). Changes in serum levels of thyroid hormones in the fasting rat reflect those in seriously sick patients: T3 and T4 concentrations are reduced, while serum TSH is normal or low. Although the dialyzable FT4 concentration (DFT4) increases, FT4 levels decrease. DFT3 and FT3 levels are both reduced (160). Interestingly, refeeding completely reverses the changes in circulating thyroid hormones (164,165). It was found that refeeding

increased serum TSH even to above prefasting levels (164); subsequently, serum T3 and T4 are restored to normal. This finding, and the fact that starvation increases TRH-stimulated TSH levels in rats (160,166,167), unlike starvation in man, strongly suggests that the starvation-associated changes in thyroid function are centrally mediated. It should be mentioned that the effects of starvation on thyroid function depend on the dietary composition, and primarily on dietary carbohydrate reduction (168). Indeed, refeeding with glucose alone restores thyroid function in fasted rats (168).

Although starvation has been shown to reduce serum TRH levels (37), no data are available on hypothalamic TRH release. Several studies suggest that other hypothalamic factors such as somatostatin or dopamine may play a role in the diminished TSH release during fasting (169-171). These studies, however, remain inconclusive.

Also untreated diabetes mellitus is associated with the low T3 syndrome (107,159). In rats with streptozotocin-induced diabetes mellitus (STZ-DM) serum levels of T3, T4 and TSH fall (35,36,72,172,173). It is not known whether these changes are mediated by disease-specific alterations in the release of hypothalamic factors, or nonspecifically by the diabetes-associated weight loss. Bestetti et al., however, showed that STZ-DM induces morphological changes in the mediobasal hypothalamus (174), and indeed also the release of LHRH (174) and somatostatin (175) is reduced in experimental diabetes. Morphological and functional changes in the mediobasal hypothalamus are specific to STZ-DM and may, therefore, play a role in the origin of neuroendocrine impairments that frequently associate diabetes mellitus (176).

In conclusion, the low T3 syndrome represents an adaptive hypothyroidism that may benefit the patient with non-thyroidal illnes. Starvation and STZ-DM in the rat profoundly affect thyroid function and provide excellent models to study central mechanisms in the generation of the low T3 syndrome.

Acute cold exposure.

Acute cold exposure is a well-known stimulus of thyroid function in rats (for an excellent review see Ref. 108). Serum levels of TSH rise

within 30 min after transfer to 4 C (75,177-179). The body's response to cold seems mediated by catecholamines which may influence TSH secretion directly at the level of the pituitary or indirectly by acting on TRH secretion (108,180). The rise in serum levels of TSH is not stress-induced, since this has been shown to reduce TSH levels (110,179,181). Serum levels of T3 and T4 rise within 1-2 h of cold exposure (75,177-179).

Several studies provide evidence that the cold-induced TSH rise is TRH-dependent. Antiserum to TRH (114,116,182), hypothalamic lesions (183) or deafferentation of the mediobasal hypothalamus (126,127) all abolish the increase in TSH concentrations during cold. Levels of TRH in peripheral blood have been found to rise (31,33,184) or remain normal (32) during cold, while hypothalamic TRH content has been reported to increase (75,185), decrease (186) or remain unchanged (67,71) during cold exposure. But again the drawbacks to the measurement of TRH in peripheral blood or hypothalamus as estimates of hypothalamic TRH release should be stressed. Finally, Arancibia et al. reported an increased cold-induced TRH secretion in push-pull perfusates of the median eminence (59,61,62). The rise in TRH release could be abolished by α-adrenergic antagonists (61), thus confirming the role of catecholamines in the body's response to cold. The cold-induced TRH rise was, however, not confirmed by Guillaume et al. who measured TRH levels in hypophysial portal blood (134).

In conclusion, acute cold exposure causes serum levels of TSH, T3 and T4 to increase in rats. These changes may represent an adaptive hyperthyroidism in the defense against cold. Although it is proved that the cold-induced rise in TSH is TRH-dependent, studies on the hypothalamic TRH release during acute cold are sparse and remain inconclusive.

D. THE ROLE OF TRH IN THE REGULATION OF PRL SECRETION.

Numerous conditions stimulate PRL release (for review see Ref. 26). The most well-known is suckling, but also cervical stimulation (187), estrogens (188) and stress (50) induce PRL secretion. In the unanesthetized rat PRL levels increase within 15-30 min after the beginning of suckling (48,189-191). Anesthesia seems to delay and attenuate this response (48,189). In the anesthetized rat electrical stimulation of a mammary nerve is considered to simulate the suckling stimulus. Release of PRL, however,

is considerably lower than during normal suckling (192,193). Since cutting of the hypophysial stalk or transplantation of the pituitary under the renal capsule result in hyperprolactinemia, basal PRL release is thought to be under tonic inhibition. It is now established that dopamine (DA) is a PRL-inhibiting factor (PIF) (48,187,190,192,194-196). The suckling-induced PRL release is thought to consist of two phases (197,198): a fast depletion-transformation phase in which PRL is released from intracellular compartments and thus made available for secretion. This phase seems to be controlled by DA (199). The second, so-called steady release phase seems controlled by PRL-releasing factors (PRFs), which induce ultimate secretion of available hypophysial PRL. Indeed, several studies show that DA release is acutely lowered after the beginning of suckling (48,192,195). Despite continued suckling and although plasma PRL levels remain high, DA levels in hypophysial portal blood return quickly to baseline values. Therefore, other factors play an important role in the sustained suckling-induced PRL release (200-202).

Since TRH stimulates in vivo PRL release in man (202,203), and in vitro PRL secretion from adenohypophysial cells (204), this tripeptide has received much attention as a physiological PRF. The effect of administration of TRH on plasma PRL levels in the rat is, however, variable. In some studies PRL levels rise after intravenous injection of TRH (205,206), but others could not confirm this finding (51,207,208). Some workers found that TRH augments PRL levels only after short-term suckling (206,209) or after a transient decrease of DA levels (48,210).

Also administration of antiserum to TRH has been shown to variably affect PRL levels in peripheral plasma. Whereas effects on plasma TSH levels are unequivocal, reported PRL levels are either unaffected (113,114) or decreased (112,199) after administration of anti-TRH antiserum. The paraventricular nucleus (PVN), however, seems to play a pivotal role in the secretion of PRL during suckling or ether stress (191,211,212). Electrical lesions in the PVN reduce hypothalamic TRH release (43). While basal PRL levels are not affected by PVN lesions (191,211,212), the suckling-induced release of PRL is reduced, although not abolished (191,212). This effect seems to be mediated by the hypothyroidism that results from these lesions, since substitution with T4 completely restores PRL levels during suckling (191). Several workers showed that hypothalamic release of DA is increased in hypothyroid and in PVN-lesioned rats (43,46,213). It is, therefore,

suggested that the reduced suckling-induced PRL release in PVN-lesioned rats results from an increased hypothalamic secretion of DA, rather than from a decreased hypothalamic TRH release.

Many workers have hypothesized that if TRH is a physiological PRF, and if suckling induces hypothalamic TRH release, plasma levels of TSH should also rise during suckling in parallel with PRL levels. Indeed, some studies reported a suckling-induced rise in plasma TSH (51,205,209). In other studies, however, TSH secretion was not found to change (203,208). These findings and the unequivocal effect of TRH or TRH-antiserum on plasma TSH in comparison with their variable effect on basal or suckling-induced plasma PRL, prompted many workers to conclude that TRH is not a physiological PRF and that suckling does not increase hypothalamic TRH release. This conclusion, however, is overhasty since the response of the thyrotroph and lactotroph to hypothalamic factors, such as TRH may be modulated differently by central and peripheral factors. Indeed, it has been shown that oxytocin, that is also released during suckling, blunts the TSH response to TRH in vitro (214). Therefore, the fact that plasma levels of TSH and PRL are not intricately correlated during suckling, or after administration of TRH or anti-TRH, does not exclude a physiological role of TRH in the regulation of the suckling-induced PRL release. In this sense, the anterior pituitary should be regarded as the final common pathway of central and peripheral mechanisms that may differently modulate the sensitivity of the thyrotroph and lactotroph to hypothalamic releasing and inhibiting factors.

Also under conditions such as stress (50,179,205,216), cold (114,178,179,217,218) and primary hypothyroidism (219-222) PRL and TSH levels are variably correlated. The stress-induced PRL release seems to be mediated by vasoactive intestinal polypeptide (VIP) (223), rather than by TRH. The hyperprolactinemia that infrequently accompanies primary hypothyroidism is the result of a decreased metabolic clearance of PRL (224), rather than an increased hypothalamic release of TRH. Moreover, in the rat primary hypothyroidism is frequently associated with hypoprolactinemia (213), presumably resulting from an increased hypothalamic DA turnover. Therefore, studies in which the role of TRH in the regulation of PRL release is determined by correlating plasma levels of PRL to TSH under various conditions should be regarded speculative and inconclusive.

In only few studies has hypothalamic TRH release been measured directly. De Greef and Visser have shown that during electrical stimulation of a mammary nerve in urethane-anesthetized rats DA release into hypophysial portal blood is decreased transiently, whereas TRH release is increased (48). In their study the effectiveness of TRH in releasing PRL in the lactating rat was enhanced when a temporary decrease of DA levels occurred before treatment with TRH. This finding was confirmed by Plotsky and Neill (209). Therefore, it seems that a transient suckling-induced decline of DA release enhances the sensitivity of the pituitary gland to PRFs such as TRH (197-199). Also the estrogen-stimulated surge of plasma PRL is associated with a rise in hypothalamic TRH secretion in vivo into stalk blood (42) as well as in vitro using hypothalamic fragments (89). Other studies however could not confirm a suckling-induced rise of TRH levels into stalk blood (47,225). In only one study was hypothalamic release of TRH and DA measured in unanesthetized freely moving rats during normal suckling (55). Confirming previous studies in anesthetized rats during a simulated suckling stimulus (48,192,195), it was shown that DA secretion was transiently depressed within 15-30 min after the beginning of suckling with a subsequent return to baseline levels. Release of TRH, however, did not rise during a 60 min suckling period (55).

In conclusion, studies in which the role of TRH in the regulation of PRL release has been investigated provide variable results. Whereas the role of DA as a PIF is clearly established, the role of TRH as a physiological PRF remains obscure.

E. SUMMARY AND SCOPE OF THESIS.

The tripeptide TRH has diverse endocrine and non-endocrine functions and is ubiquitously located in the body. Its endocrine functions relate to its role as a hypothalamic factor which stimulates the release of TSH and PRL from the adenohypophysial thyrotroph and lactotroph, respectively.

The role of TRH in the regulation of thyroid function and lactation is exerted by the binding of the hormone to its pituitary receptors. Regulation of the hypothalamic release of TRH may be an important factor in the control of its bioavailability. Indeed, indirect studies suggest that TRH plays an important role in TSH secretion during altered thyroid status,

the low T3 syndrome, and acute cold exposure. Moreover, PRL secretion seems partly dependent on hypothalamic TRH and, therefore, TRH is also considered a PRL-releasing factor. In the litterature, however, only sparse data are available on in vivo and in vitro release of hypothalamic TRH in conditions associated with altered plasma levels of TSH and/or PRL. Moreover, these few studies are not reconciable and the exact role of TRH in the regulation of thyroid function and lactation is far from clear.

Therefore, the scope of this thesis was to investigate how hypothalamic secretion of TRH is regulated with regard to thyroid function and lactation. Firstly, different neuroendocrine techniques were validated to measure hypothalamic release of TRH (Chapter II). Secondly, the regulation of the secretion of TRH was investigated in conditions associated with altered plasma levels of TSH (Chapter III) and PRL (Chapter IV). The following questions have been addressed:

A) With regard to the role of TRH in the regulation of TSH secretion:

1. Does hypothalamic TRH release change with thyroid status? (i.e. is there a feedback of thyroid hormone at the hypothalamic level?).
2. Does TRH play a role in the body's response to disease? Streptozotocin-induced diabetes mellitus and starvation were used as models to study central mechanisms in the generation of the low T3 syndrome as an adaptive hypothyroidism.
3. Does TRH play a role in the body's response to acute cold as an adaptive hyperthyroidism?

B) With regard to the role of TRH in the regulation of PRL secretion:

1. Does hypothalamic TRH release change during suckling?
2. Does reduction of the secretion of TRH by hypothalamic lesions influence the suckling-induced PRL release?

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CHAPTER II.

THE MEASUREMENT OF HYPOTHALAMIC TRH RELEASE.

A.

In Vivo Hypothalamic Release of Thyrotropin-Releasing Hormone after Electrical Stimulation of the Paraventricular Area: Comparison between Push-Pull Perfusion Technique and Collection of Hypophysial Portal Blood

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ABSTRACT. Unilateral electrical stimulation for 15 min of the paraventricular area of anesthetized rats induced a 2- to 3-fold increase in plasma TSH levels and caused an increased release of TRH into hypophysial stalk blood from 217 ± 25 to 530 ± 90 pg/15 min (n = 6). This experimental model was then used to determine the *in vivo* hypothalamic release of TRH by push-pull perfusion of either the mediobasal hypothalamus (MBH) or anterior pituitary (AP).

Before stimulation, TRH release per 15 min was 4.2 ± 0.7 pg from the MBH (n = 18) and 3.5 ± 0.3 pg from the AP (n = 13). Unilateral electrical stimulation of the paraventricular area led to higher plasma TSH levels in 27 of 31 rats, and levels during stimulation increased from 0.89 ± 0.04 to 1.86 ± 0.10 ng/ml (n = 31). No significant increase in TRH in the perfusates was observed when push-pull perfusion was done in the MBH contralateral to the site of stimulation (n = 6). However, TRH release increased 2- to 3-fold during the perfusion of the MBH ipsilateral to the site of stimulation (15.4 ± 4.3 pg/15 min; n = 12) or when the AP was perfused (9.5 ± 2.0 pg/15 min; n = 13). In conclusion, push-pull perfusion of the MBH or AP can be used to estimate hypothalamic TRH release. However, the output of TRH by push-pull perfusion is low and varies considerably between individual rats. Thus, the practical value of push-pull perfusion for measurement of *in vivo* TRH release seems limited. (*Endocrinology* **125:** 971–975, 1989)

SEVERAL techniques have been used to measure the *in vivo* release of hypothalamic substances: collection of hypophysial portal blood, push-pull perfusion of the mediobasal hypothalamus (MBH) or anterior pituitary (AP), and intracerebral microdialysis. In a recent paper hypothalamic TRH release measured by push-pull perfusion of the MBH was found not to increase during suckling (1), whereas TRH release into hypophysial portal blood had been found to increase during a simulated suckling stimulus (mammary nerve stimulation) by 70% (2). Thus, there seems no such good correlation between output of TRH in push-pull perfusates and hypophysial portal blood, a correlation that has been described for catecholamines and LHRH (3, 4). Moreover, the amount of TRH in push-pull perfusates is low and varies considerably between individual rats (1, 5, 6). Thus, it remains to be established whether push-pull perfusion of the MBH or AP can be used to estimate *in vivo* hypothalamic TRH release. Since electrical stimulation of the paraventricular area (PVA) has been reported to increase TSH levels in peripheral blood (7, 8), we have used this experimental model to investigate the value of push-pull perfusion for the measurement of *in vivo* hypothalamic TRH release. This study was made possible by our finding, described in the current paper, that electrical stimulation of the PVA indeed increased TRH release into hypophysial stalk blood.

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Materials and Methods

Male Wistar rats (200–300 g BW) were used. They were housed under controlled conditions (lights on, 0500–1900 h; 20–24 C) and had free access to food and tap water.

Experimental procedures

Electrode placement and peripheral blood sampling. Rats were anesthetized with tribromoethanol (Avertin, Merck, Darmstadt, Germany; 250 mg/kg BW, ip) and a cannula was inserted in the right jugular vein (9). They were then placed in a

stereotaxic instrument (David Kopf Instruments, Tujunga, CA), and a unipolar insulated stainless steel electrode was inserted in the right PVA [coordinates according to de Groot (10): 6.4 mm anterior and 3.5 mm dorsal from the interaural line, and 0.5 mm from the midline]. Then, five blood samples were taken from the jugular venous cannula at 15-min intervals, and the PVA was stimulated between 15 and 30 min after the start of blood collection using a biphasic rectangular stimulus with the following characteristics (7): trains of 30 sec on, 30 sec off, a frequency of 50 Hz, a pulse amplitude of 500 μamp, and a pulse duration of 1 msec.

Collection of hypophysial portal blood. Two to 3 days after placement of a stimulation electrode in the PVA (see above), the animals were anesthetized with urethane (1.2 g/kg BW), and hypophysial portal blood was collected for 1 h at 15-min intervals into methanol and processed as described previously (2, 11). The PVA was stimulated, as described above, between 15 and 30 min after the start of the portal blood collection.

Push-pull perfusion. A stainless steel push-pull cannula with a removable stylette protruding 0.3 mm was implanted in the MBH (coordinates: 5.2 mm anteroposterior, 0.2 mm mediolateral, 0.2 mm ventrodorsal above base of skull, and upper incisor bar −3 mm above interaural line) and provisionally fixed on the skull with screws and dental cement. Dimensions of this cannula are 7 × 7 mm with a 10-mm needle of 0.5 mm od (for details, see Ref. 3). Then, the upper incisor bar was set at +5 mm, and a stimulation electrode was placed in the PVA as described above. Both electrode and cannula were then fixed on the skull with dental cement. The electrode was placed in the PVA either ipsilateral or contralateral to the site in the MBH where the push-pull cannula had been implanted. In some rats the push-pull cannula was placed in the AP (coordinates: 3.0 mm anteroposterior, 0.5 mm mediolateral, 0.7 mm ventrodorsal above base of skull, and upper incisor bar −3 mm above interaural line). After surgery rats were caged individually and handled daily. One rat showing signs of infection and continued weight loss was excluded from the experiment. One week after surgery rats were anesthetized with avertin. The stylette was removed and replaced with an inner cannula, which extended 0.3 mm beyond the end of the outer cannula. Push and pull sites of the cannula were connected by polyethylene tubing (id, 0.28 mm) via a homemade double swivel to a peristaltic pump (Ismatec IPN 12, Zürich, Switzerland). Artificial cerebrospinal fluid (CSF) (3) was delivered at a rate of 20 μl/min to the push cannula, and perfusate was collected continuously at the same rate from the pull site into tubes on melting ice. The transit time from the tip of the inner cannula to the sample tubes was 5 min. Every 15 min a new sample was begun, and the first two fractions were disregarded. The PVA was stimulated between 35 and 50 min after the beginning of the first sampled fraction. Blood was taken from the orbital plexus just before and 15 and 25 min after the beginning of electrical stimulation of the PVA. Data were not used when, during perfusion, the tip of the cannula was occluded (n = 2) or the stimulation failed because of a short circuit (n = 4). Plasma and push-pull perfusion samples were frozen immediately after collection and stored at −20 C until assayed.

The animals were killed by decapitation after perfusion, the push-pull cannulas and iron electrodes were gently removed, and the brains were placed in Bouin-Hollande fixative. Serial 10-μm sections were cut and stained with hematoxylin-eosin to confirm correct placement of the cannula and the stimulation electrode. This proved to be the case in all animals. The site of the cannula in the AP was determined macroscopically.

Hormone determinations

TRH was determined by RIA (12), and all samples were assayed in a single run. Sensitivity (defined as the amount of hormone that reduces binding of tracer to 90% of that in the absence of unlabeled hormone) is 1–2 pg. The intraassay coefficient of variation is 4%. TSH was determined by RIA using materials and protocols supplied by the NIDDK and TSH RP-2 was used as standard.

Statistical analysis

Results are presented as the mean ± SEM. Analysis of variance followed by Duncan's multiple range tests were used to establish significant differences. Differences were considered significant at $P \leq 0.05$. For the statistical analysis, undetectable levels of TRH in push-pull perfusate medium (24 of 186 samples) were assigned the value of the detection limit.

Results

Electrical stimulation of the right PVA caused a 2- to 3-fold increase in plasma TSH levels (Fig. 1) and a 2-fold increase in TRH release into hypophysial portal blood from 217 ± 25 to 530 ± 90 pg/15 min (Fig. 2). TSH secretion was stimulated in 8 of 10 rats, and TRH release in 5 of 6 rats.

Before a stimulus was applied, hypothalamic TRH

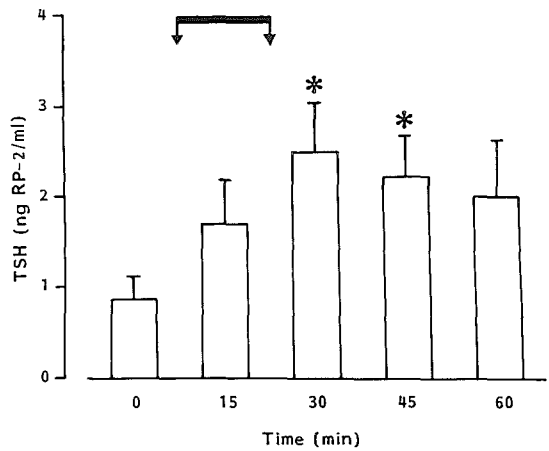

FIG. 1. Effect of electrical stimulation of the right PVA on plasma levels of TSH (mean ± SEM; n = 10) in male rats. *Arrows* indicate the time of stimulation. *, $P \leq 0.05$ compared with the prestimulation level.

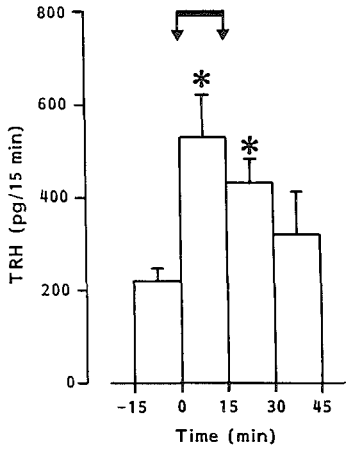

FIG. 2. Release of TRH into hypophysial portal blood (mean ± SEM; n = 6) in response to unilateral electrical stimulation of the PVA. *Arrows* indicate time of stimulation. *, $P \leq 0.05$ compared with the prestimulation level.

release, as estimated by push-pull perfusion, was 4.2 ± 0.7 pg/15 min during perfusion of the MBH (n = 18) and 3.5 ± 0.3 pg/15 min when the AP was perfused (n = 13). Unilateral electrical stimulation of the PVA contralateral to the site where the MBH was perfused increased plasma TSH in all 6 rats studied, but did not result in a significant increase in TRH release during and after stimulation (Fig. 3), although 2 rats showed an increase in TRH in the perfusate. From the 12 rats in which the MBH ipsilateral to the stimulated PVA was perfused, 9 showed elevated TSH levels after stimulation, and TRH release increased 3- to 4-fold in these 12 animals (Fig. 3). Higher plasma TSH levels were observed in 12 of 13 rats in which the AP was perfused after stimulation of the PVA, and this stimulus increased TRH levels in the perfusate to about 250% (Fig. 3).

Discussion

This study was designed to determine if push-pull perfusion is a suitable method for the measurement of *in vivo* hypothalamic TRH release. TRH is synthesized in parvocellular cell bodies of the paraventricular nucleus (13) and transported via anterolateral and medial pathways to the median eminence (13, 14). It has been reported that bilateral electrical stimulation of the PVA of anesthetized rats resulted in increases in plasma TSH concentrations (7, 8). In the present study unilateral electrical stimulation of the PVA was used to increase TSH release. This experimental design made it possible to perform push-pull perfusion in the MBH either contralateral or ipsilateral to the site of electrical stimulation. Also, this approach resulted in increased TSH levels after stimulation. Moreover, the stimulus raised TRH levels in hypophysial portal blood, thus extending previous results of Sheward *et al.* (7).

According to our expectations, no significant effect on TRH levels in perfusate of the MBH was observed during stimulation of the PVA contralateral to the area of perfusion. However, perfusion of the MBH ipsilateral to the site of stimulation or perfusion of the AP led to increased TRH release by approximately 300% after stimulation; this corresponds well with the stimulation-induced 2- to 3-fold increase in hypophysial portal blood TRH levels. Thus, push-pull perfusion of the MBH or AP seems a suitable technique to monitor hypothalamic TRH release. However, the amounts of TRH detected in the perfusates are low.

Basal TRH release into hypophysial portal blood is approximately 200 pg/15 min, whereas only around 4 pg are measured per 15 min during push-pull perfusion (Refs. 2 and 11 and present study). This large difference cannot solely be explained by the fact that only part of the MBH is perfused during push-pull perfusion (3), whereas hypophysial stalk blood represents the effluent of the whole hypothalamus. Moreover, for other hypothalamic substances, such as LHRH or catecholamines, the difference between release measured in push-pull perfusate and that in hypophysial portal blood is much less (3, 4). Degradation of TRH during push-pull perfusion does not appear to be responsible for this difference (5), and addition of bacitracin to artificial CSF to inhibit potential enzymatic degradation of TRH does not lead to higher amounts of TRH in the perfusate (Rondeel, J. M. M., unpublished results). Furthermore, the recovery of TRH, added to the artificial CSF in the push-pull perfusion system, is near 100% (3), indicating that there is little diffusion of TRH from the perfusate to nerve cells in the MBH. One might assume, then, that neither is there diffusion of TRH from nerve cells to the push-pull perfusate. Thus, it may be that the physicochemical properties of TRH prevent diffusion of TRH into the tissue compartiment that is perfused. Another explanation for the low basal TRH release during push-pull perfusion is that insertion of the cannula disrupts nerve fibers projecting from the TRH-producing cell bodies in the paraventricular nucleus to the median eminence. However, this possibility seems unlikely, since electrical stimulation of the PVA ipsilateral to the site where the push-pull cannula was inserted resulted in an increase in plasma TSH levels.

Dluzen and Ramirez (15, 16) found in gonadectomized rats that LHRH release measured by push-pull perfusion was greater if the cannula was located in the AP rather than in the MBH, and they argued that this could be due to a synchronization of LHRH pulses from the MBH. In

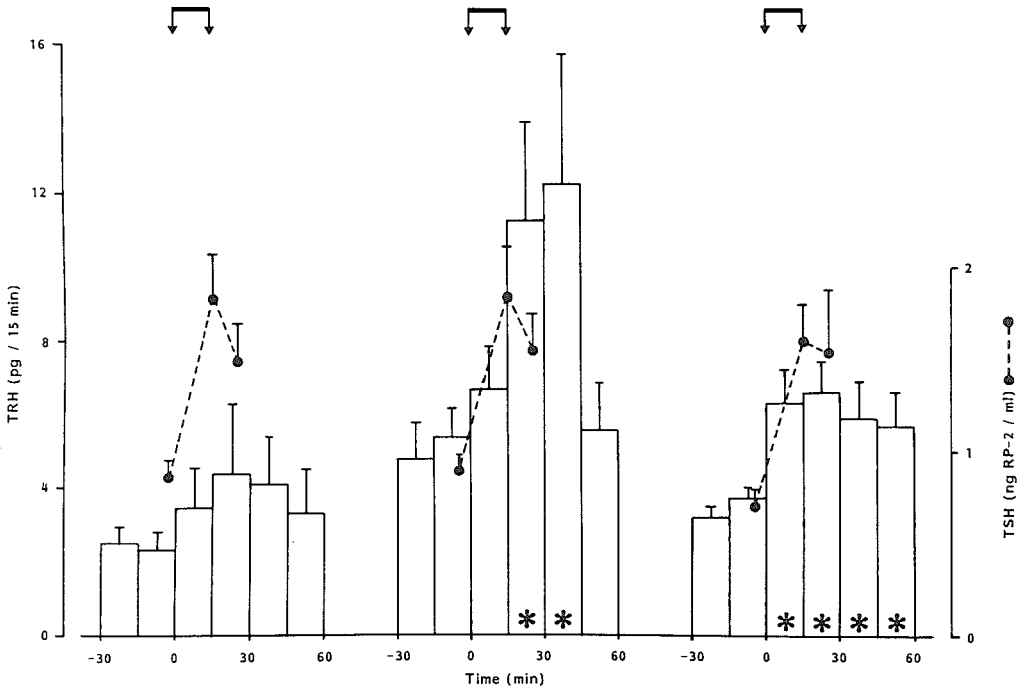

FIG. 3. Plasma concentrations of TSH (- - -) and *in vivo* TRH release into push-pull perfusates (*bars*) in response to unilateral electrical stimulation of the PVA. *Arrows* indicate the time of stimulation. The *right panel* represents the results from perfusion of the anterior pituitary gland (n = 13). The MBH was perfused either contralateral (*left panel*; n = 6) or ipsilateral (*middle panel*; n = 12) to the site of electrical stimulation. The values are the mean ± SEM. *, $P \leq 0.05$ compared with mean of prestimulation levels (−30 to 0 min).

our study basal TRH release was similar during perfusion of the MBH or the AP. With both locations of perfusion, TRH release was approximately 3–4 pg/15 min and increased 2- to 3-fold during stimulation of the PVA. Therefore, perfusion of the AP does not seem to be superior to perfusion of the MBH for measurement of *in vivo* hypothalamic TRH release.

In conclusion, push-pull perfusion of the MBH or AP can be used to monitor hypothalamic TRH release. However, the value of this technique is limited because of the low and variable amounts of TRH that are being measured. Despite its inherent limitations, like the use of anesthetics and the major surgery, the collection of hypophysial portal blood seems the preferred method for the determination of *in vivo* hypothalamic TRH release.

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B.

IMMUNOREACTIVE TRH IN RAT PERIPHERAL BLOOD: AN ESTIMATE OF HYPOTHALAMIC RELEASE OF TRH?

ABSTRACT

The role of hypothalamic thyrotrophin-releasing hormone (TRH) for the release of thyrotrophin and prolactin is not yet resolved, mainly due to difficulties to estimate in vivo hypothalamic TRH secretion. This study aimed to investigate whether immunoreactive TRH (IR-TRH) in peripheral blood is an estimate of hypothalamic TRH release. Values of IR-TRH in peripheral blood, collected into methanol to prevent enzymatic degradation of TRH, were between 14 and 55 pmol/l. Starvation decreased IR-TRH in blood, whereas subsequent refeeding normalized these levels. Hyperthyroid rats had lower and hypothyroid rats tended to have higher IR-TRH levels than euthyroid rats. Rats with bilateral lesions in the paraventricular area had lower IR-TRH levels than sham-lesioned rats.

Clearance of TRH during infusion with TRH was 1.9±0.2, 3.5±0.3, 5.1±0.8 ml/min in hypothyroid, euthyroid and hyperthyroid rats, respectively. These and previous data on TRH release into hypophysial portal blood indicate that in euthyroid rats 8-22 pmol TRH/l peripheral blood is of hypothalamic origin. High performance liquid chromatography revealed that IR-TRH in hypothalamus and hypophysial portal blood coeluted with synthetic TRH, but IR-TRH in peripheral blood consisted of two peaks, one of which corresponded to authentic TRH.

In conclusion, IR-TRH levels in peripheral blood alter during experimental conditions and part of this IR-TRH seems of hypothalamic origin. However, the presence in peripheral blood of TRH-like material not identical to TRH and the observation that experimental conditions may alter the clearance of TRH in blood indicate that IR-TRH in peripheral blood is not a reliable estimate of hypothalamic release of TRH.

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submitted for publication

INTRODUCTION.

Thyrotrophin-releasing hormone (TRH) is a tripeptide synthetized in the hypothalamic paraventricular nuclei (Brownstein, Eskay & Palkovits, 1982), transported to the median eminence (Palkovits, Eskay & Brownstein, 1982) and released into hypophysial portal blood (de Greef & Visser, 1981). Although administration of TRH causes the release of thyrotrophin and prolactin, the role of TRH in the physiologic regulation of the release of these pituitary hormones is still unresolved, mainly due to experimental difficulties to determine in vivo hypothalamic release of TRH. Previously, we have estimated its release by measuring TRH in hypophysial portal blood (de Greef & Visser, 1981; de Greef, Klootwijk, Karels & Visser, 1985; Rondeel, de Greef, van der Schoot, Karels & Visser, 1988; Rondeel, de Greef, van der Vaart, van der Schoot & Visser, 1989) or in push-pull perfusate from median eminence or pituitary gland (Voogt, de Greef, Visser, de Koning, Vreeburg & Weber, 1987; Rondeel, de Greef, Visser & Voogt, 1988; Rondeel et al., 1989). However, both methods require experimental intervention, which may adversely affect in vivo release of TRH. Theoretically, it should be possible to determine the hypothalamic TRH release by measuring TRH in peripheral blood (Visser, 1985), and in this way experimental intervention is kept minimal. In the present study the use of TRH measurements in peripheral blood as an estimate of hypothalamic TRH release was investigated.

MATERIALS AND METHODS.

Animals. Wistar rats (males: 250-300 g; females: 100-120 g) were used. They were housed under controlled conditions (lights on 0.500-19.00 h; temperature 20-24 °C), and had, unless stated otherwise, free access to pelleted food and tap water.

TRH in peripheral blood and thyroid status. Untreated male rats (euthyroid; n=32) and male rats treated for 3 weeks with methimazole [0.05% (wt/vol) in drinking water; hypothyroid; n=68] or thyroxine (daily injections of 10 µg/100 g BW; hyperthyroid; n=25) were used. Approximately 3 ml blood was taken from the orbital plexus while the rats were lightly anesthetized with ether. To minimize enzymatic degradation of TRH (Bauer, 1976; Eskay,

Oliver, Warberg & Porter, 1976), blood was collected into methanol. After collection, the blood was centrifuged for 10 min at 4 °C, the supernatant removed, dried under a stream of nitrogen at 40 °C and dissolved in 1 ml 0.1 M phosphate-buffered saline (pH 7.0) with 0.1% (wt/vol) bovine serum albumin. The samples were stored at -20 °C until assayed for TRH. The residue of blood after extraction with methanol was dried and weighed to estimate the volume of blood collected (de Greef & Visser, 1981). After determination of their immunoreactive TRH (IR-TRH) content, samples of rats treated similarly were pooled for analysis with high performance liquid chromatography (HPLC).

TRH in peripheral blood during starvation and refeeding. Blood was collected from female rats (n=14) deprived of food for 3 days. Other female rats (n=8) were fed again after 3 days of starvation, and blood was taken 2 days later. Normally fed female rats (n=14) were used as controls. The rats were killed by decapitation to collect trunk blood into methanol, and blood was extracted as described above for the estimation of IR-TRH.

TRH in peripheral blood in rats with paraventricular area lesions. Bilateral electrolytic lesions were placed in the paraventricular area (PVA) of 7 male rats (Rondeel et al., 1988a). Control male rats (n=10) underwent a sham-operation. Three weeks later about 3 ml blood was taken from the orbital plexus while the rats were lightly anesthetized with ether. Blood was collected into methanol and processed as described above. After estimation of the IR-TRH content, samples of each group were pooled for analysis by HPLC.

Thyroid status and clearance of TRH. Male rats were made hypothyroid (n=14) or hyperthyroid (n=10) as described above. Untreated males were controls (n=14). Three weeks after the start of the treatment the rats were anesthetized with urethane (ethylcarbamate; 1.2 g/kg BW) and polyethylene cannulas (0.58 mm id, 0.96 mm od) were inserted into a jugular vein to infuse TRH (dissolved in 0.9% NaCl) and in a femoral artery to collect and administer blood. The rats were infused for 4 consecutive periods of 45-min periods with 1.00, 0.50, 0.25 and 0 pmol TRH/min, respectively, at a rate of 20 μl/min. After each 45-min period, 3 ml blood was collected into methanol, and replaced by 3 ml donor blood. The rats were kept at 37 °C

during the procedure. For each rat the amount of TRH in blood measured when no TRH was infused was extracted from the values of TRH during infusion. The corrected values were used to calculate the metabolic clearance (Tait & Burstein 1964):
MCR (ml/min) = (amount infused in pg/min)/(level in pg/ml at equilibrium).

Chromatographic procedures. Pooled extracts of peripheral blood from rats bearing PVA lesions and from hypothyroid, hyperthyroid and euthyroid rats were analysed. For comparison, extracts of hypothalamic tissue and hypophysial portal blood of hypothyroid, hyperthyroid and euthyroid male rats were included. Hypothalamic tissue and hypophysial portal blood were collected as described before (de Greef & Visser, 1981). Before analysis, the IR-TRH content of each pooled sample was estimated. Then, the samples were transferred onto Seppak C18 columns, and after washing these columns with destilled water, TRH was eluted with methanol. The methanol was evaporated and the residues taken up in radioimmunoassay buffer. Part of the purified samples were used for determination of IR-TRH, and at least 90% of the IR-TRH was recovered. The samples were analyzed by isocratic reversed-phase HPLC with a 100x3 mm CP-Spher C18 cartridge (Chrompack, Middelburg, The Netherlands), a 6000A solvent delivery system and a U6k injector (Waters, Meldford, MA, USA). Elution was performed with 4% (v/v) acetonitril in buffer containing 0.1% (v/v) 1-hexane sulfonic acid, 0.0625% (v/v) phosphoric acid and 0.025% (v/v) triethylamine (pH 2.5). The flow rate was 0.6 ml/min, and fractions of 300 μl (0.5 min) were collected for 15 min to measure their IR-TRH content. To prevent cross-over, samples without TRH were injected between each sample with TRH.

TRH determination. TRH was determined by radioimmunoassay (Visser, Klootwijk, Docter & Hennemann, 1977) and all samples from the same experiment were assayed in a single run. Sensitivity (defined as the amount of hormone which reduces binding of tracer to 90% of that in the absence of unlabeled hormone) of this assay is approximately 3 fmol TRH/tube. Intra-assay coefficient of variation is 4%.

Statistical analysis. Results are presented as means±S.E.M. The significance of differences between groups was established with the non-parametric Mann-Whitney U test. Differences were considered to be

significant when $P<0.05$.

RESULTS.

IR-TRH in peripheral blood. In 32 euthyroid male rats the concentration of TRH in peripheral blood was 26.5±2.5 pmol/l. Compared to these values, TRH levels were similar in 68 male hypothyroid rats (32.6±2.2 pmol/l), but were lower in 25 male hyperthyroid rats (14.9±1.9 pmol/l; $P<0.01$). Levels of TRH in young female rats starved for 3 days were lower than those of controls (21.8±2.2 vs 36.2±4.1 pmol/l; $P<0.05$), but had returned to control values after 2 days of refeeding (45.3±12.2 pmol/l).

Three weeks after operation, male rats with bilateral PVA-lesions had lower levels of TRH (37.0±2.5 pmol/l) than sham-lesioned controls (52.5±6.1 pmol/l; $P<0.05$).

Thyroid status and metabolic clearance of TRH. Blood levels of TRH during infusion of TRH are given in **Fig. 1.** The MCRs of TRH calculated from these results were 1.9±0.2 ml/min for hypothyroid, 3.5±0.3 ml/min for euthyroid, and 5.1±0.8 ml/min for hyperthyroid male rats.

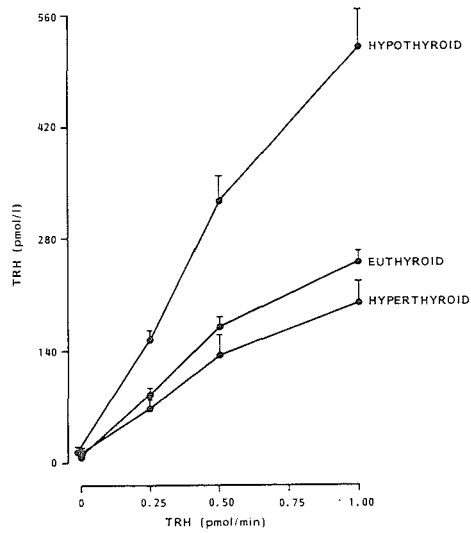

Figure 1: Levels of IR-TRH in peripheral blood during infusion of TRH in hypothyroid (n=14), euthyroid (n=14) and hyperthyroid (n=10) male rats. Rats were anaesthetized with urethane and infused for 4 consecutive 45-min periods. Peripheral blood was collected into methanol after each period.

Chromatography of IR-TRH in hypothalamus and blood. In hypothalamus and hypophysial portal blood one peak of IR-TRH which coeluted with synthetic TRH was found. Two peaks of IR-TRH were observed in peripheral blood (Fig. 2) and the second peak coeluted with authentic TRH. The first peak in the chromatogram of blood from euthyroid rats tended to be higher than that of hypothyroid and hyperthyroid rats. Similar results were obtained when pooled peripheral blood from sham-lesioned or PVA-lesioned rats was analyzed (Fig. 3).

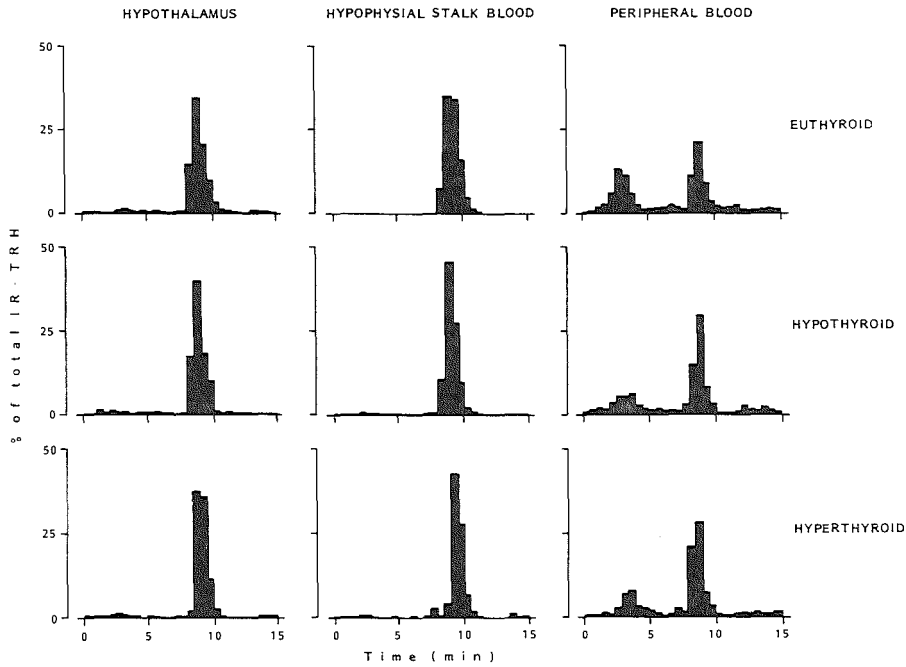

Figure 2: Elution profiles on isocratic reversed phase HPLC of IR-TRH in pooled hypothalamus, hypophysial portal blood and peripheral blood of euthyroid, hypothyroid and hyperthyroid male rats. Each profile represents the mean of at least two observations.

DISCUSSION.

This study aimed to determine whether IR-TRH in peripheral blood is an estimate of hypothalamic release of TRH. Firstly, IR-TRH was measured in peripheral blood during conditions thought to alter hypothalamic TRH release. Then, the MCR of TRH was estimated to establish whether the amount

of TRH released into hypophysial blood was sufficient to yield detectable levels of TRH in peripheral blood. Finally, the identity of the IR-TRH in peripheral blood was analyzed by HPLC.

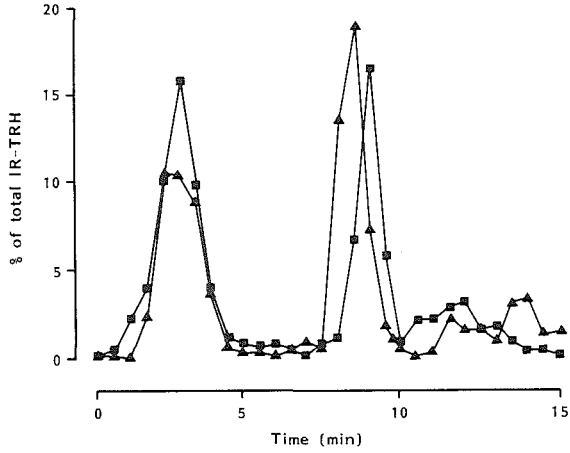

Figure 3: Elution profiles of IR-TRH in pooled peripheral blood from control male rats (■) and PVA-lesioned rats (▲) on isocratic reversed phase HPLC.

Peripheral blood levels of IR-TRH varied between 14 and 55 pmol/l, which is in agreement with the values reported by Eskay et al. (1976). However, other workers found much higher levels (Mallik, Wilber & Pegues, 1982; Mori, Michimata, Yamada, Yamaguchi, Iriuchijima & Kobayashi, 1988; Simard, Pekary, Smith & Hershman, 1989a), which may be due to different antisera used for the measurement of TRH. As in earlier study (Mori et al., 1988) starvation was found to decrease peripheral IR-TRH, whereas refeeding, known to increase thyrotrophin (Hugues, Burger, Grouselle, Voirol, Chabert, Modigliani & Sebaoun, 1983), normalized IR-TRH concentrations. Compared to euthyroid rats, hyperthyroid rats had lower and hypothyroid rats tended to have higher IR-TRH concentrations. These findings corroborate previous results on hypothalamic release of TRH in rats with altered thyroid status (Rondeel et al. 1988a; Rondeel et al., 1989). Rats bearing bilateral PVA-lesions had lower peripheral IR-TRH levels than controls, which agrees with findings on TRH in hypophysial stalk blood of rats with PVA-lesions (Rondeel et al., 1988a). Thus, peripheral IR-TRH levels seem to reflect changes in the hypothalamic

release of TRH.

These changes of TRH in peripheral blood, however, may also be due to altered extrahypothalamic TRH synthesis (Simard, Pekary, Smith & Hershman, 1989b) or may reflect changes in TRH degradation in blood due to differences in the thyroid status during the experimental conditions (Bauer, 1976; Dupont, Labrie, Levasseur, Dussault & Schally, 1976; White, Jeffcoate, Griffiths & Hooper, 1976; Jackson, Papapetrou & Reichlin, 1979; present study).

The MCR of TRH in rats reported by Jackson et al. (1979) is higher than found in this study, likely due to different experimental designs. Jackson et al. (1979) used pentobarbital-anaesthetized rats and calculated the MCR from the disappearance curve of TRH after i.v. administration of 5.5 nmol TRH, whereas in the present study upto 1 pmol TRH/min was infused to determine the MCR of TRH. When 5-10 pmol TRH/min was infused, the MCR of TRH in euthyroid male rats increased to 7-8 ml/min (W.J. de Greef, unpublished). Moreover, urethane used as anaesthetic in this study has been found to lower the clearance of a number of hypophysial hormones by 30-50% (de Greef, de Jong, de Koning, Steenbergen & van der Vaart, 1983).

In euthyroid male rats, the mean TRH concentration in hypophysial portal blood is between 2.5 and 8.5 nmol/l (Mallik et al., 1982; Ching & Utiger, 1983; Rondeel et al., 1988a; Rondeel et al., 1989), corresponding to a release of 2-6 pmol/h (Rondeel et al., 1988a; Rondeel et al., 1989). In this study, male rats had 14 pmol TRH/l peripheral blood when no TRH was infused, whereas infusion with 15 pmol TRH/h increased TRH levels to 80 pmol/l. Assuming that endogenous TRH release does not alter during infusion with TRH, a hypothalamic release of 2-6 pmol/h will result in a level of 8-22 pmol TRH/l peripheral blood in euthyroid males. In hypothyroid and hyperthyroid rats these values are 14-39 pmol/l and 6-14 pmol/l, respectively. Since the MCR of TRH was determined in rats anaesthetized with urethane, which was done because TRH release into hypophysial portal blood was also estimated in urethane-anaesthetized rats (Rondeel et al., 1988a; Rondeel et al., 1989), the calculated level of TRH in peripheral blood may not be valid for non-anaesthetized rats, and may represent an overestimation. Nonetheless, the present data on TRH clearance and previous data on in vivo TRH release (Rondeel et al., 1988a; Rondeel et al., 1989) indicate that at least part of peripheral TRH is of hypothalamic origin.

As in previous reports (Fink, Koch & Ben Aroya, 1982; Ching &

Utiger, 1983; de Greef et al., 1985), most of the IR-TRH in hypothalamus and hypophysial portal blood coeluted on HPLC with synthetic TRH. Other workers, however, reported that hypophysial portal blood eluted in a number of IR-TRH peaks (Sheward, Harmar, Fraser & Fink, 1983; Eckland, Todd, Scott & Lightman, 1988). In this study, analysis of peripheral blood by HPLC revealed two peaks of IR-TRH, a finding also reported for IR-TRH secreted by hypothalamic cells in a perifusion system (Scanlon, Robbins, Bolaffi, Jackson & Reichlin, 1983). Thus, only part of the IR-TRH in peripheral blood seems to correspond to authentic TRH. In peripheral blood of PVA-lesioned rats the amount of IR-TRH was reduced, which corroborates reports that destruction of the PVA reduces the amount of TRH in median eminence (Yamada & Mori, 1989) and in hypophysial portal blood (Rondeel et al., 1988a). However, analysis with HPLC revealed that the peak that coeluted with authentic TRH was not significantly reduced in PVA-lesioned rats compared with results obtained in sham-lesioned animals.

Also in humans, levels of IR-TRH in peripheral blood have been reported, and were found to vary between 80 and 700 pmol/l (Guignier, Pelletier, Touzery & Gaillard, 1981). Most of this IR-TRH seems derived from extra-hypothalamic sources (Mallik et al., 1982), and is probably not identical with authentic TRH (Iversen, 1986).

In conclusion, low amounts of IR-TRH are present in peripheral blood of the rat. Peripheral IR-TRH levels alter during experimental conditions, and may thus reflect changes in TRH release from the hypothalamus. On the other hand, these alterations could also be due to changes in the clearance of TRH induced by the experimental conditions. Moreover, not all IR-TRH in peropheral blood is identical with authentic TRH. Therefore, peripheral levels of IR-TRH seem not a reliable estimate of the hypothalamic release of TRH.

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CHAPTER III.

THE ROLE OF TRH IN THE REGULATION OF TSH SECRETION.

A.

Effect of Thyroid Status and Paraventricular Area Lesions on the Release of Thyrotropin-Releasing Hormone and Catecholamines into Hypophysial Portal Blood

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ABSTRACT. TRH is a potent stimulator of pituitary TSH release, but its function in the physiological regulation of thyroid activity is still controversial. The purpose of the present study was to investigate TRH and catecholamine secretion into hypophysial portal blood of hypothyroid and hyperthyroid rats, and in rats bearing paraventricular area lesions.

Male rats were made hypothyroid with methimazole (0.05% in drinking water) or hyperthyroid by daily injections with T_4 (10 μg/100 g BW). Untreated male rats served as euthyroid controls. On day 8 of treatment they were anesthetized to collect peripheral and hypophysial stalk blood. In euthyroid, hypothyroid and hyperthyroid rats plasma T_3 was 1.21 ± 0.04, 0.60 ± 0.04, and 7.54 ± 0.33 nmol/liter, plasma T_4 50 ± 3, 16 ± 2, and 609 ± 74 nmol/liter, and plasma TSH 1.58 ± 0.29, 8.79 ± 1.30, and 0.44 ± 0.03 ng RP-2/ml, respectively. Compared with controls, hyperthyroidism reduced hypothalamic TRH release (0.8 ± 0.1 *vs.* 1.5 ± 0.2 ng/h) but was without effect on catecholamine release. Hypothyroidism did not alter TRH release, but the release of dopamine increased 2-fold and that of noradrenaline decreased by 20%. Hypothalamic TRH content was not affected by the thyroid status, but dopamine content in the hypothalamus decreased by 25% in hypothyroid rats. Twelve days after placement of bilateral electrolytic lesions in the paraventricular area plasma thyroid hormones and TSH levels were lower than in control rats (T_3: 0.82 ± 0.05 *vs.* 1.49 ± 0.07 nmol/liter; T_4: 32 ± 4 *vs.* 66 ± 3 nmol/liter; TSH: 1.08 ± 0.17 *vs.* 3.31 ± 0.82 ng/ml). TRH release in stalk blood in rats with lesions was 15% of that of controls, whereas dopamine and adrenaline release had increased by 50% and 40%, respectively.

These results suggest that part of the feedback action of thyroid hormones is exerted at the level of the hypothalamus. Furthermore, TRH seems an important drive for normal TSH secretion by the anterior pituitary gland, and thyroid hormones seem to affect the hypothalamic release of catecholamines. (*Endocrinology* **123:** 523–527, 1988)

HIGH LEVELS of TRH exist in various parts of the hypothalamus including the paraventricular nucleus (1, 2). Electrical stimulation of this nucleus causes the release of TSH (3, 4), whereas its destruction decreases plasma TSH and thyroid hormones (5, 6) and median eminence TRH content (7). These results suggest that the paraventricular nucleus is involved in the process of TRH-TSH release, but data on TRH levels in hypophysial portal blood after destruction of this hypothalamic area are lacking. Furthermore, data concerning the effect of thyroid hormones on hypothalamic secretion of TRH are conflicting (7–13), possibly because in most studies only hypothalamic TRH content was determined, which may not be a good estimate for hypothalamic TRH secretion.

Thus, although TRH is a potent stimulator of pituitary TSH secretion, its actual function in the physiological regulation of thyroid activity is still a matter of controversy. It was the aim of the present study to determine 1) the possible feedback action of thyroid hormones on hypothalamic TRH release into hypophysial portal blood, and 2) the effects of destruction of the hypothalamic paraventricular area (PVA) on TRH release. Furthermore, since thyroid hormones seem to influence the activity of tuberoinfundibular dopaminergic neurons (14), we have also determined the effect of the above mentioned experimental conditions on the hypothalamic release of catecholamines.

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Materials and Methods

Animals

Male (R×U) F_1 rats, weighing 250–400 g, were used. They were kept in a temperature-controlled room (20–22 C) with a

light cycle of 14-h light and 10-h darkness. Food and water were always available.

Experimental procedures

Exp 1. Three groups of 8 rats were used. They were made either hypothyroid with methimazole [0.05% (wt/vol) in drinking water] or hyperthyroid with daily ip injections of T_4 (10 μg/100 g BW between 0700 and 0800 h). Untreated rats were used as euthyroid controls. Hypophysial stalk blood was collected for 1 h between 0830 and 1330 h on day 8 of treatment as described before (15). Urethane (1.2 g/kg BW) was used as anesthetic. Before surgery to expose the hypophysial stalk began, a cannula was inserted in the right femoral artery. After the stalk had been exposed, 500 IU heparin were injected through the cannula and then a peripheral blood sample was taken for measurement of thyroid hormones and TSH. Before the hypophysial stalk was cut, extraneous bleeding, if present, in the exposed area was controlled by high-frequency cauterization (Erbotom Bipolar, Erbe Elektromedizin, Tübingen, West Germany). Hypophysial stalk blood for TRH and catecholamine analysis was collected into methanol as reported previously (16, 17), and the transit time for stalk blood to reach the vial with methanol, kept in melting ice, was less than 20 sec. This method of hypophysial blood collection minimizes enzymatic degradation of TRH (9, 16–18). After collection, portal blood samples were centrifuged for 5 min at 2–4 C. The supernatant was divided into two parts. To one part an equal volume of 0.2 M perchloric acid was added and was used for determination of catecholamines. The remainder of the supernatant was used for TRH measurement and was dried at 40 C under a stream of nitrogen; the residue was dissolved in 1 ml 0.1 M PBS (pH 7.0) containing 0.1% (wt/vol) BSA. The precipitate of the portal blood samples was dried and weighed to estimate the collected volume of blood (16).

After collection of portal blood, the rats were killed by decapitation and the hypothalamus (excluding the preoptic region) excised from the brain. The tissue was placed in 2 ml methanol, homogenized with a glass tissue grinder kept in melting ice, and centrifuged for 5 min at 2–4 C. Part of the supernatant was mixed with an equal volume of 0.2 M perchloric acid and used to determine catecholamines. The remainder of the supernatant was dried at 40 C under a stream of nitrogen. The residue was taken up in 1 ml RIA buffer and assayed for TRH. All samples were stored at −20 C until assayed.

Exp 2. Rats (n = 14) were anesthetized with ether and placed in a stereotaxic apparatus to make bilateral electrolytic lesions in the PVA. Using the atlas of de Groot (19), a lesion electrode (diameter 0.1 mm, insulated except for 0.5 mm at the tip) was lowered into the PVA (coordinates: 6.4 mm anterior and 3.5 mm dorsal from the interaural line, and 0.7 mm from the midline) and anodal current (1 mA) was then passed through this electrode for 20 sec. Control rats (n = 7) were subjected to similar surgery, but no current was applied. Collection of peripheral and hypophysial stalk blood took place 12 days after these surgical procedures. After blood collection the animals were killed by decapitation and their brains immersed in Bouin's fixative. The region containing the paraventricular nuclei was embedded in paraffin wax, and 10-μm sections were stained with hematoxylin and eosin. The site of the lesions was determined microscopically.

Hormone determinations

Levels of TRH were determined by RIA as described before (20). The detection limit (defined as the amount of hormone that reduces binding to 90% of that occurring in the absence of unlabeled hormone) of this assay is 2–3 pg, and the intra- and interassay variations are between 4 and 10%. Using HPLC, it was found that at least 70% of the immunoreactive TRH in portal blood was accounted for by synthetic TRH (data not shown). All samples for TRH were run in one assay. Levels of TSH were determined by RIA using materials and protocols supplied by the NIADDK, and TSH-RP-2 was used as standard. Concentrations of T_3 and T_4 were determined by RIA. The catecholamines were measured with a HPLC-electrochemical method. They were separated by an isocratic reversed phase HPLC system with a mobile phase consisting of a water-methanol mixture (70:30, vol/vol) containing 0.1 M citrate acid buffer (pH 5.2), 0.1% (wt/vol) sodium dodecyl sulfate and 0.01% (wt/vol) NaCl and measured with an electrochemical detector (16). The minimal detectable amount of noradrenaline, adrenaline, and dopamine was 10–20 pg (signal to noise ratio of 2).

Statistical procedures

Results are given as means ± SEM. Analysis of variance followed by Duncan's multiple range tests were used to analyze the data. Differences between groups were considered to be significant at $P < 0.05$.

Results

Effect of thyroid status on TRH and catecholamine release

The treatment with methimazole and T_4 was highly effective to induce hypothyroidism and hyperthyroidism, respectively (Table 1). Hypophysial stalk blood was collected for 1 h at 15-min intervals to measure the hypothalamic release of TRH, noradrenaline, adrenaline, and dopamine. Since their release did not change during the period of observation, only mean data for the 1-h period are presented. The release of TRH and dopamine into hypophysial portal blood of hyperthyroid rats was approximately 50% of that in hypothyroid rats, whereas that of noradrenaline was 20% higher (Table 2). The

TABLE 1. Peripheral plasma levels (means ± SEM; n = 8) of thyroid hormones and TSH in rats treated with methimazole (MMI) or T_4 for 8 days

Group	T_3 (nmol/liter)	T_4 (nmol/liter)	TSH (ng RP-2/ml)
1. None	1.21 ± 0.04	50.0 ± 3.0	1.58 ± 0.29
2. MMI	0.60 ± 0.04[a]	15.6 ± 1.6[a]	8.79 ± 1.30[a]
3. T_4	7.54 ± 0.33[a,b]	609.1 ± 74.4[a,b]	0.44 ± 0.03[a,b]

[a] Significantly different from group 1.
[b] Significantly different from group 2.

TABLE 2. Release and concentrations (means ± SEM; n = 8) of TRH, noradrenaline (NA), adrenaline (A), and dopamine (DA) into hypophysial portal blood of rats treated with methimazole (MMI) or T_4 for 8 days

Group	Release (ng/h)				Concentrations (ng/ml)			
	TRH	NA	A	DA	TRH	NA	A	DA
1. None	1.5 ± 0.2	3.4 ± 0.1	1.6 ± 0.1	2.2 ± 0.2	2.2 ± 0.3	5.5 ± 0.9	2.5 ± 0.3	3.6 ± 0.6
2. MMI	1.7 ± 0.2	2.8 ± 0.1[a]	2.1 ± 0.3	4.5 ± 0.6[a]	3.0 ± 0.4	5.1 ± 0.9	3.8 ± 0.6	7.5 ± 1.0[a]
3. T_4	0.8 ± 0.1[a,b]	3.3 ± 0.1[b]	1.6 ± 0.1	2.1 ± 0.3[b]	1.8 ± 0.3[b]	6.5 ± 0.7	3.1 ± 0.4	3.9 ± 0.6[b]

[a] Significantly different from group 1.
[b] Significantly different from group 2.

estimated volume of blood obtained in 1 h was not different between control, hypothyroid, and hyperthyroid rats (740 ± 112, 696 ± 128, and 568 ± 72 μl, respectively).

Hypothalamic TRH content was not affected by the treatments. Compared to the values in hypothyroid rats, the hypothalamic levels in hyperthyroid rats of dopamine and noradrenaline were 50% higher and 25% lower, respectively (Table 3).

Effect of lesions in the PVA on TRH and catecholamine release

Three out of 14 rats died within 2 days after placement of the lesions. Histological examination revealed that in 6 animals the paraventricular nuclei had been lesioned completely whereas in the remaining 5 rats the lesions were just caudal from these nuclei. Since both placements of the lesions gave rise to similar hormone levels, the data of all rats with lesions were combined.

Twelve days after the placement of the lesions the levels of thyroid hormones and TSH in plasma were significantly lower than those in controls (Table 4). The release of TRH into hypophysial stalk blood of PVA-lesioned rats was 15% of that of controls whereas the secretion of dopamine had increased by 50% (Table 5).

TABLE 3. Hypothalamic content (ng ± SEM; n = 8) of TRH, noradrenaline (NA), adrenaline (A), and dopamine (DA) of rats treated with methimazole (MMI) or T_4 for 8 days

Group	TRH	NA	A	DA
1. None	10.42 ± 1.75	4.33 ± 0.21	4.08 ± 0.35	3.58 ± 0.22
2. MMI	11.34 ± 2.81	5.02 ± 0.27	3.22 ± 0.27	2.63 ± 0.10[a]
3. T_4	8.20 ± 1.11	3.76 ± 0.18[b]	3.99 ± 0.25	4.12 ± 0.14[b]

[a] Significantly different from group 1.
[b] Significantly different from group 2.

TABLE 4. Peripheral plasma levels (means ± SEM) of thyroid hormones and TSH in sham-operated rats (n = 7) and in rats bearing bilateral paraventricular area lesions (PVA, n = 11)

Group	T_3 (nmol/liter)	T_4 (nmol/liter)	TSH (ng RP-2/ml)
1. Sham	1.49 ± 0.07	65.9 ± 3.4	3.31 ± 0.82
2. PVA	0.97 ± 0.07[a]	32.7 ± 2.8[a]	1.08 ± 0.11[a]

[a] Significantly different from group 1.

The estimated volume of blood collected in 1 h was 732 ± 128 μl for control rats and 796 ± 90 μl for rats with PVA lesions.

Discussion

The present study was concerned with the effects of thyroid hormones and PVA lesions on the hypothalamic release of TRH and catecholamines. Induction of hyperthyroidism by daily treatment with 10 μg T_4/100 g BW was found to cause a 50% reduction in the release of TRH in hypophysial portal blood, whereas hypothyroidism induced by methimazole led to a slight but insignificant increase in TRH release. These data indicate that thyroid hormones exert a negative feedback control on the hypothalamic release of TRH.

There have been earlier studies (9, 18) on the effects of thyroid hormones on hypophysial stalk blood levels of TRH. From their experiments Ching and Utiger (9) and Guillaume *et al.* (18) concluded that portal blood TRH concentrations did not differ consistently between normal, hypothyroid, or hyperthyroid rats. The difference between these results (9, 18) and the present one may be due to a different degree of hyperthyroidism since in our study severely hyperthyroid rats were used. On the other hand, the conclusion of Ching and Utiger (9) may be true for the individual experiments, but when combining their data it is obvious that portal blood TRH levels of T_4-treated rats are 35–40% lower than those of normal rats. Similarly, combining the data of Guillaume *et al.* (18) shows that portal blood TRH levels of T_4-treated rats are 60% of those of normal rats.

Hypothyroidism has been found to induce an increase of both transcription and translation of the TRH-prohormone in the paraventricular nucleus (21), to increase the number of TRH-positive cells in the paraventricular nucleus (2), and to reduce the amount of TRH in the median eminence (2, 7). These studies implicate that hypothyroidism may affect TRH secretion from the hypothalamus, but direct measurement of TRH in hypophysial portal blood does not reveal an increased hypothalamic TRH release during hypothyroidism (Refs. 9 and 18 and present study). Recently, we have determined

TABLE 5. Release and concentrations (means ± SEM) of TRH, noradrenaline (NA), adrenaline (A), and dopamine (DA) into hypophysial portal blood of sham-operated rats (n = 7) and in rats bearing bilateral paraventricular area lesions (PVA, n = 11)

Group	Release (ng/h)				Concentrations (ng/ml)			
	TRH	NA	A	DA	TRH	NA	A	DA
1. Sham	2.1 ± 0.4	2.9 ± 0.1	2.1 ± 0.2	1.7 ± 0.2	2.9 ± 0.2	4.7 ± 0.7	3.2 ± 0.4	2.5 ± 0.4
2. PVA	0.3 ± 0.1[a]	3.0 ± 0.1	2.9 ± 0.1[a]	2.6 ± 0.1[a]	0.4 ± 0.1[a]	4.3 ± 0.5	4.2 ± 0.7	3.6 ± 0.4[a]

[a] Significantly different from group 1.

TRH levels in hypophysial portal blood in rats treated for 3 weeks with methimazole and again found no different TRH release in hypophysial stalk blood when compared with euthyroid controls (data not shown).

Other groups also have examined whether a possible feedback action of thyroid hormones on hypothalamic TRH release exists. As parameters hypothalamic TRH content or peripheral TRH levels (7–13) were used. The validity of these parameters, however, is questionable. No consistent differences in hypothalamic TRH content have been reported during hypothyroidism or hyperthyroidism. In the present study no effect on hypothalamic TRH content was observed after treatment with methimazole or T_4, although the rats used for collection of portal blood also were used for measurement of hypothalamic TRH levels. The significance of alterations in peripheral TRH induced by thyroid hormones is doubtful since most immunoreactive TRH in peripheral circulation is not of hypothalamic origin (22, 23), and it is questionable whether this immunoreactive material is indeed TRH (24). Furthermore, the clearance of TRH is affected by the thyroid status (25, 26).

Since the clearance of TRH is augmented during hyperthyroidism (25, 26), it is possible that the observed reduction in TRH release into portal blood during hyperthyroidism is due to a change in TRH clearance. However, since the transit time for stalk blood to reach the vial with methanol was less than 20 sec in our experiments it is not likely that the difference in metabolic clearance would account for the observed 50% reduction in TRH release.

Although treatment with methimazole did not affect significantly the release of TRH in portal blood, it was found to decrease hypothalamic dopamine content, and to induce a 2-fold increase of dopamine and a 20% decrease of noradrenaline secretion into hypophysial blood. Earlier it has been reported (14) that the hypothalamic turnover of dopamine was enhanced during hypothyroidism. In hyperthyroid rats the hypothalamic release or content of these two catecholamines was not different from that in euthyroid rats.

The hypothalamo-hypophysial blood flow has been found to increase in hypothyroid rats with the most distinct increase at 3 days of treatment with propylthiouracil (27). In our study no effect of 8 days of hypothyroidism was found on the volume of hypophysial stalk blood collected in 1 h. In hyperthyroid rats this volume was somewhat, but not significantly, lower than that in controls.

In the present study we could confirm that bilateral destruction of the paraventricular nuclei induces a reduction in plasma TSH and thyroid hormones (5, 6). However, lesions just caudal to the paraventricular nuclei, leaving the paraventricular neurons seemingly unaffected, decreased TSH and thyroid hormones levels as well. Thus, both inactivation of the paraventricular nuclei and disruption of the path between paraventricular nucleus and median eminence cause a reduction in adenohypophysial TSH release (6). The fall in plasma TSH and thyroid hormones was accompanied by an 85% reduction in the release of TRH into hypophysial portal blood. Thus, most of the TRH present in stalk blood is derived from the PVA. Earlier, it has been shown that lesions in this area reduced the TRH content of the median eminence (7, 28, 29).

The hypothalamic release of dopamine into hypophysial stalk blood was increased by 50% in PVA-lesioned animals. Since dopamine release into portal blood is also increased in hypothyroid rats (Ref. 14 and present study) and PVA-lesioned rats are hypothyroid, it is tempting to state that the increased dopamine release in lesioned rats is due to their hypothyroid status.

In conclusion, we have provided direct evidence that hyperthyroidism reduces the hypothalamic release of TRH into the hypophysial portal system suggesting that part of the feedback action of thyroid hormones is exerted at the level of the hypothalamus. The effects of ablation of the PVA suggest that TRH is an important drive for normal TSH secretion by the anterior pituitary gland. Furthermore, thyroid hormones seem to affect the hypothalamic release of catecholamines.

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B.

EFFECT OF DRUG-INDUCED HYPOTHYROIDISM ON IN VIVO AND IN VITRO HYPOTHALAMIC RELEASE OF TRH IN MALE RATS.

ABSTRACT.

The aim of this study was to investigate whether severity and duration of primary hypothyroidism influenced hypothalamic TRH release in vivo and in vitro. Hypothyroidism was induced by treating male rats with different thyrostatic drugs for up to 3 weeks. Serum TSH in rats treated for 3 weeks with methimazole (MMI; 0.05% in drinking water) increased 20-fold, but TRH release into hypophysial portal blood remained similar. Treatment with propylthiouracil (PTU; 0.1% in drinking water), which inhibits thyroidal T4 production and the peripheral conversion of T4 to T3, resulted in a more acute and drastic reduction in serum T3 levels and increase in serum TSH after 1 and 2 weeks treatment than in rats treated with 0.1% MMI. Although these differences were not significant anymore after 3 weeks, release of TRH into portal blood of rats treated with PTU was higher than that of MMI-treated rats (1166±81 vs 828±67 pg/h; $p<0.05$). Combined treatment with 0.1% MMI and iopanoic acid (IOP; 4 mg/100 g BW/d i.p.), an inhibitor of both peripheral and central T4 to T3 conversion, resulted in less reduction of serum T4 throughout the 3 week treatment period, whereas it lowered T3 levels more acutely after 1 week compared to MMI treatment. In vivo release of TRH was slightly but not significantly increased by IOP (742±98 vs 1221±245 pg/h, MMI vs MMI plus IOP). Static incubation of hypothalami from rats treated as described above with MMI, PTU or with MMI plus IOP showed that basal and 56 mM K^+-induced TRH release was not influenced by the different drugs.

Interestingly, treatment with MMI or with MMI plus IOP, but not with PTU, decreased pituitary TRH content with 46% and 36% respectively, compared to untreated controls.

In rats treated with 0.05% MMI for 3 weeks after the placement of bilateral electrolytic lesions in the paraventricular area, TSH levels were reduced with 30% compared to intact MMI-treated rats.

These findings indicate that the effects of drug-induced primary hypothyroidism differing in onset, severity and duration on hypothalamic TRH release are minimal and that TRH plays a minor role in the hypersecretion of TSH during primary hypothyroidism. The feedback of thyroid hormone on TSH secretion is mainly exerted at the pituitary level.

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INTRODUCTION.

Thyroid status negatively influences pituitary secretion of TSH mainly by a direct action of thyroid hormone on the thyrotroph, modulating its sensitivity to hypothalamic factors (1-3). Recent studies, however, report that thyroid hormone can also act directly at the hypothalamic level (4-8) and we have found that hyperthyroidism induced by thyroxine treatment decreased both in vivo and in vitro hypothalamic TRH release by 30-40% (9,10). Although several studies pointed to the importance of TRH for the increased TSH secretion in drug-induced primary hypothyroidism (9,11-16), hypothalamic release of TRH was not found to be increased (9,10). This could be due to differences in types and doses of the thyrostatic drugs used and, therefore, the severity of hypothyroidism elicited. Moreover, the duration of hypothyroidism may play an important role, since mRNA levels of the TRH precursor in the hypothalamus are only increased after long term hypothyroidism (4-6). Therefore, we have investigated the in vivo and in vitro hypothalamic TRH release in rats treated with different thyrostatic drugs for up to 3 weeks. Furthermore, the effect of electrolytic lesions in the hypothalamic paraventricular area (PVA) -previously shown to reduce hypothalamic TRH release by 85% (9)- on thyroid status in hypothyroid rats was investigated.

MATERIALS AND METHODS.

Animals. In all experiments male Wistar rats (200-300 g BW) were used. They were housed under controlled conditions (light on 05.00-19.00h; 20-24 C) and had free access to food and water.

Induction of hypothyroidism. Rats were treated for 1, 2 or 3 weeks with methimazole (MMI; Janssen Chimica, Beerse, Belgium; 0.05% or 0.1% in drinking water), propylthiouracil (PTU; Sigma Chemical Company, St. Louis, MO, USA; 0.1% in drinking water), or with a combination of MMI (0.1% in drinking water) and iopanoic acid (IOP; Sterling-Winthrop Group Ltd., Guildford, Surrey, England; 4 mg/100 g BW/d i.p.). The latter was dissolved in a solution of ethylene glycol, 0.1 N NaOH and 0.25 M NaCl (50:48:2 vol/vol/vol). Untreated rats served as euthyroid controls.

Static incubation of hypothalamic fragments. Rats were anaesthetized with ether and decapitated. Trunk blood was collected to determine serum levels of T3, T4 and TSH, and hypothalami were isolated as described previously (17). Also the pituitaries were removed, weighed and placed in tubes containing 2 ml methanol and homogenized with a glass grinder to estimate TRH content. Hypothalami were quartered and randomly distributed over a 24 wells dish, kept in melting ice, such that each well contained the equivalents of two hypothalami in ice-cold Krebs Ringer bicarbonate medium (pH 7.3) containing 118 mM NaCl, 5 mM KCl, 1.1 mM $MgSO_4.7H_2O$, 2.5 mM $CaCl_2.2H_2O$, 1.2 mM KH_2PO_4, 25 mM $NaHCO_3$, 10 mM glucose, 0.1% BSA and 0.5 g/l bacitracine to prevent enzymatic degradation of TRH. The medium was replaced with 0.5 ml warm medium (37 C) and the plate was placed on a slightly angled, slowly rotating disk in an incubator maintained at 37 C and gassed with 95% O_2/5% CO_2. After a 30 min preincubation period, the medium was replaced with fresh medium and TRH release was measured during three 1-hour intervals: during the first and second hour basal TRH release was estimated, while in the third hour period K^+-induced TRH release was determined. High K^+ medium contained 56 mM KCl instead of the equivalent amount of NaCl. After each hour the medium was collected and stored at -20 C until analysis. After incubation, the hypothalamic fragments were homogenized in 2 ml methanol with a glass grinder to measure TRH content (17). Release of TRH was expressed as a percentage of hypothalamic TRH content.

Collection of hypophysial portal blood. Rats were anaesthetized with urethane (1.1-1.2 g/kg BW i.p.) and fitted with a polythene cannula into the right femoral artery. Then the hypophysial stalk was exposed as described previously (17). Before the stalk was sectioned 500 IU heparin was injected into the femoral cannula and a blood sample was taken from this cannula to measure serum T3, T4 and TSH levels. After sectioning the hypophysial stalk, blood was collected with less than 20 seconds delay into methanol. In this way possible effects of thyroid hormone-dependent alterations in plasma TRH degradation (18) on TRH levels in hypophysial portal blood are minimized.

Electrolytic lesions in the PVA. Rats were anaesthetized with ether and placed in a stereotaxic apparatus to make bilateral electrolytic lesions in the PVA as described previously (9). In short, a lesion electrode was

lowered into the PVA (coordinates: 6.4 anterior, 3.5 dorsal and 0.5 lateral) (19) and anodal current (1 mA) was then applied for 30 sec. Control rats were subjected to similar surgery, but no current was applied. After surgery rats were treated for 3 weeks with MMI 0.05% in drinking water and peripheral blood was collected from the orbital plexus, while the rats were lightly anaesthetized with ether, to measure levels of T3, T4 and TSH. The animals were subsequently killed by decapitation and their brains quickly removed and immersed in Bouin's fixative. The site of the lesions was determined microscopically in 10 μm sections of the hypothalamus stained with hematoxylin and eosin. All lesions turned out to be in or just caudal to the paraventricular nuclei (9).

Hormone determinations. TRH was determined using a previously described RIA (20). All samples of each experiment were run in one assay. The sensitivity (defined as the amount of hormone that reduces binding of tracer to 90% of that occurring in the absence of unlabeled hormone) is 1-2 pg per tube, and the intra-assay coefficient of variation is 4%. TSH was determined by RIA using materials and protocols supplied by the NIADDK. Standard RIA procedures were used to determine serum T3 and T4.

Statistical analysis. Results are presented as means±SEM. Analysis of variance followed by Duncan's multiple range tests or Student's t test for comparison of two means were used to establish significant differences. Differences were considered to be significant at $p \leq 0.05$.

RESULTS.

Table 1 shows the effect of 1 to 3 weeks treatment with MMI on thyroid function and TRH release into hypophysial portal blood. The effect of MMI on serum T3 and T4 levels is maximal after 1 week, whereas serum TSH continues to increase until 3 weeks. Release of TRH into hypophysial stalk blood did not vary throughout the 3 week period.

The effects of 1 to 3 weeks treatment with MMI or PTU on thyroid function and on in vivo hypothalamic TRH release are summarized in Table 2. Treatment with PTU results in lower levels of serum T3 and higher levels of serum TSH after 1 and 2 weeks compared to MMI treated rats. After 3 weeks treatment, however, these differences are not significant anymore. In vivo

hypothalamic TRH release after 3 weeks is significantly higher in PTU-treated rats than in MMI-treated rats. The concentration of TRH in hypophysial portal blood, however, is not increased by PTU treatment.

Table 1: Serum T3, T4 and TSH levels and TRH release into hypophysial portal blood in rats treated with methimazole (MMI).

	T3 nmol/l	T4 nmol/l	TSH ng RP-2/ml	In vivo TRH release pg/h	pg/ml
control	1.22±0.08	57±4	1.8±0.2	950± 71	1845±219
1 week MMI	0.56±0.15a	7±1a	12.5±2.5a	924± 73	1986±178
2 weeks MMI	0.43±0.03a	5±1a	22.4±1.7ab	956±110	2430±531
3 weeks MMI	0.40±0.03a	4±1a	41.6±5.3abc	956± 73	2171±194

MMI: 0.05% in drinking water. Untreated rats served as controls (n=8; $^{a}p \leq 0.05$ vs control, $^{b}p \leq 0.05$ vs 1 week MMI, $^{c}p \leq 0.05$ vs 2 weeks MMI).

Table 2: Serum T3, T4 and TSH levels (n=20-34) and TRH release into hypophysial portal blood (n=14) in rats treated for up to 3 weeks with methimazole (MMI) or propylthiouracil (PTU).

		T3 nmol/l	T4 nmol/l	TSH ng RP-2/ml	In vivo TRH release pg/h	pg/ml
0 weeks	MMI	1.85±0.06	82±3	1.1±0.1	ND	ND
	PTU	1.83±0.04	84±2	1.0±0.1	ND	ND
1 week	MMI	0.82±0.04	22±1	6.8±0.7	ND	ND
	PTU	0.54±0.04*	20±2	9.0±0.5*	ND	ND
2 weeks	MMI	0.76±0.04	12±1	12.4±0.9	ND	ND
	PTU	0.62±0.04*	12±1	14.4±0.7*	ND	ND
3 weeks	MMI	0.54±0.03	7±1	19.8±1.7	828±67	1501±170
	PTU	0.50±0.02	7±1	20.4±1.3	1166±81*	1820±182

MMI/PTU: 0.1% in drinking water (*$p \leq 0.05$ vs MMI (t test), ND not determined).

Treatment with a combination of MMI and IOP results in slightly higher levels of serum T4 compared to rats treated with MMI alone (Table 3). There were no consistent differences in serum T3 and TSH levels between both groups, although the combination of MMI and IOP tends to lower T3 levels more acutely after 1 week treatment. In vivo TRH release is slightly but not significantly increased in rats treated with both MMI and IOP.

Basal secretion of TRH from hypothalamic fragments is not influenced by the different treatments (Table 4). It is shown that 56 mM K^{+} increases TRH release 3-4 fold without significant differences among groups. Content

of TRH in the hypothalamus is neither influenced by the different treatments. Treatment with MMI or with a combination of MMI and IOP lowers pituitary TRH content with 46% and 36%, respectively (Figure 1A).

Table 3: Serum T3, T4 and TSH levels (n=12-20) and TRH release into hypophysial portal blood (n=6-7) in rats treated for up to 3 weeks with methimazole (MMI) or with a combination of MMI and iopanoic acid (IOP).

	T3 nmol/l	T4 nmol/l	TSH ng RP-2/ml	In vivo TRH release pg/h	pg/ml
0 weeks MMI	1.85±0.06	82±3	1.1±0.1	ND	ND
MMI+IOP	1.74±0.04	84±2	0.9±0.1	ND	ND
1 week MMI	0.92±0.06	25±1	8.0±1.0	ND	ND
MMI+IOP	0.70±0.04*	31±2*	10.1±0.8	ND	ND
2 weeks MMI	0.67±0.03	15±1	14.5±1.2	ND	ND
MMI+IOP	0.72±0.02	18±1*	18.7±0.8*	ND	ND
3 weeks MMI	0.55±0.04	9±1	30.0±2.5	742± 98	1410±260
MMI+IOP	0.55±0.04	12±1*	25.2±1.8	1221±245	1759±382

MMI: 0.1% in drinking water, IOP: 4 mg/100 g BW/d i.p. (*$p \leq 0.05$ vs MMI (t test), ND not determined).

Table 4: Basal and 56 mM K^+-induced TRH release from hypothalamic fragments of rats treated for 3 weeks with methimazole (MMI) or propylthiouracil (PTU), or with a combination of MMI and iopanoic acid (IOP).

	In vitro TRH release (%/h)			Hypothalamic TRH content
	Basal I	Basal II	56 mM K^+	ng/hypothalamus
controls	0.39±0.05	0.50±0.08	1.64±0.15	14.8±0.4
MMI	0.46±0.09	0.52±0.05	1.55±0.13	12.2±0.7
PTU	0.44±0.06	0.45±0.06	1.63±0.11	12.7±0.7
MMI+IOP	0.42±0.03	0.47±0.08	1.72±0.18	11.9±0.4

MMI/PTU: 0.1% in drinking water, IOP 4 mg/100 g BW/d i.p. Basal release was measured in the first (I) and second (II) hour period, 56 mM K^+-induced release in the third hour period. Values are expressed as % of the hypothalamic TRH content. Hypothalamic fragments of untreated rats served as controls (n=5-7).

In Figure 1B the effect of electrolytic lesions in the PVA on plasma levels of TSH is shown. TSH levels in rats treated with MMI for 3 weeks after the placement of the lesions are reduced with 30% compared to intact hypothyroid rats. Levels of T3 and T4 remained unchanged (T3: 0.43±0.04 vs 0.45±0.03 nmol/l; T4: 8±1 vs 7±1 nmol/l; intact vs lesioned rats).

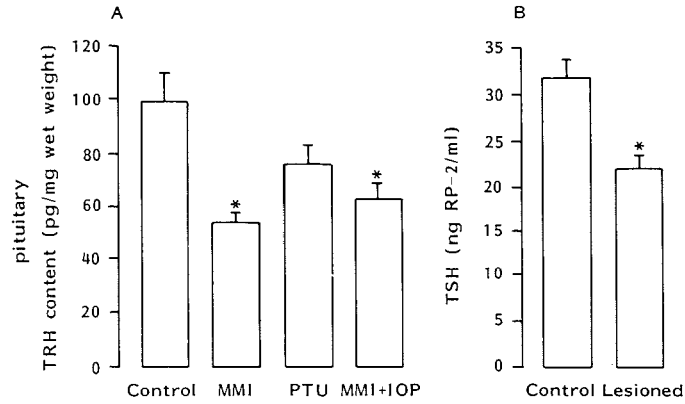

Figure 1: A. Pituitary content of TRH of rats treated with methimazole (MMI) or propylthiouracil (PTU) (0.1% in drinking water) or with a combination of MMI and iopanoic acid (IOP; 4 mg/100 g BW/d i.p.). Pituitaries of untreated rats served as controls. **B.** Serum TSH levels in rats with bilateral electrolytic lesions in the PVA. Sham-operated rats served as controls. Rats received 0.05% MMI in drinking water for 3 weeks after surgery (n=5-8; *$p \leq 0.05$ vs control).

DISCUSSION.

Previous studies reported that TRH is important for TSH secretion in euthyroid rats (9,11-15). However, hypothalamic lesions (12-16) or administration of anti-TRH antibodies (11) do not prevent the increased TSH secretion in hypothyroid animals. In this study we found that TSH levels in MMI-treated rats were reduced by 30% in rats bearing bilateral electrolytic lesions in the PVA. In euthyroid rats TRH release into hypophysial portal blood was reduced by 85% twelve days after lesioning the PVA (9) and TSH levels were decreased by approximately 70% (9), which is in agreement with other reports (11-15). Although we have no data on TRH release in hypothyroid rats three weeks after lesioning the PVA, the small reduction of TSH in these animals suggests that TRH plays only a minor role in TSH secretion during hypothyroidism, in contrast to its role in euthyroidism (9,11-15).

Recent studies provide more and more evidence that thyroid hormone may act at the hypothalamic level. Synthesis of TRH in the paraventricular nucleus (4-6), transport of TRH along pathways to the median eminence (7) and TRH content in the median eminence (8) are all influenced by thyroid status. In a previous study we found that TRH release into portal blood was reduced by 30-40% in hyperthyroid rats, whereas mild MMI-induced

hypothyroidism had no effect (9). These findings were confirmed in in vitro experiments in which TRH release was measured from hypothalamic fragments during static incubation (10). The lack of effect of hypothyroidism on hypothalamic TRH release could be due to the fact that rats were treated with MMI for only 1 week whereas the effect of hypothyroidism on proTRH mRNA levels is seen after prolonged treatment with thyrostatic drugs (4-6). Therefore, in this study rats were treated for up to 3 weeks with different thyrostatic drugs, which produce hypothyroid states differing in onset and severity.

Prolonged treatment with MMI increased TSH levels 20-fold without any significant effect on TRH release into portal blood. Because of the small reduction in circulating levels of T3 in MMI-treated animals, we treated rats with PTU. This drug does not only block thyroidal T4 production, as does MMI, it also inhibits type I deiodinase activity, thereby reducing peripheral T4 to T3 conversion (21). Indeed, in PTU-treated rats there was a more acute and drastic reduction in T3 levels and increment in TSH levels after 1 and 2 weeks treatment compared to MMI-treated rats. Although after 3 weeks treatment these differences are not significant anymore, in vivo TRH release is increased by 40% at this time. The physiological relevance of this finding, however, is questionable since the concentration of TRH in hypophysial portal blood is not significantly changed due to an increased stalk blood flow. An increased portal plasma flow in PTU-treated rats has also been reported by Eckland et al. (22), but this was not seen in our previous study following MMI treatment (9).

Treatment with a combination of MMI and IOP showed the same tendency. IOP inhibits both type I and type II deiodinase, thereby reducing peripheral and central T4 to T3 conversion (23,24). Combined treatment with these drugs reduced T4 levels less than with MMI alone. Again, levels of T3 were more acutely reduced after 1 week in the MMI plus IOP treatment group. Although in vivo TRH release tended to be somewhat higher in the MMI plus IOP-treated animals, this difference was not significant compared to the MMI-treated rats.

The results suggest that TRH release tends to rise only if serum T3 is drastically reduced. Recent studies showed that mRNA levels of preproTRH in the paraventricular nucleus are diminished by systemic (4,5) or local (25) administration of T3. Thus, local conversion of T4 by the type II deiodinase may not be involved with the feedback of thyroid hormone at the

hypothalamic level. This is compatible with the finding of Riskind et al. who showed that type II deiodinase activity is absent in the paraventricular nucleus (26).

The effect of hypothyroidism on TRH release in vivo observed in our studies is small and could not be confirmed in other studies (27-30) nor in our in vitro experiments (this study, 10). This seems to contradict studies that show a clear increase in mRNA levels and content of preproTRH in the paraventricular nucleus of hypothyroid rats (4-6). However, since a recent study showed that hypothalamic content of TRH-Gly, the direct precursor of TRH, is not increased in hypothyroidism (31), and since we could not find an increased hypothalamic release of TRH, posttranslational processess probably counteract the increased gene transcription in hypothyroidism.

Despite the fact that hypothalamic TRH release is at the most only slightly increased in hypothyroidism, the amount of TRH reaching its receptor on the thyrotroph may be augmented because of an increased blood flow through the pituitary stalk (22) and a decreased enzymatic plasma degradation of TRH (18) that is seen in hypothyroidism. Surprisingly, however, TRH content in the pituitary is decreased in hypothyroid rats and this is in agreement with observations by Childs et al. (32). Hypophysial TRH can be derived from three sources. The major part resides in the posterior pituitary, and this TRH probably stems from parvocellular cellbodies in the magnocellular division of the paraventricular nucleus (33,34). In the anterior pituitary, TRH stems from exogenous (i.e. hypothalamic), TRH-receptor bound hormone on the thyrotroph and lactotroph (35), or from in situ processing of prepro-TRH in plurihormonal cells (36). Because of vascular connections between anterior and posterior pituitary (37) it is impossible to speculate on the exact source of hypophysial TRH and the physiological meaning of changes in its concentration remains obscure. Interestingly however, treatment with PTU -that was shown to increase hypothalamic TRH release- seems to prevent the decrease in pituitary TRH content.

In conclusion, hypothalamic TRH release tends to be increased in hypothyroidism of rapid onset in which circulating levels of T3 are acutely reduced. However, this effect is only minimal. This indicates that feedback of thyroid hormone on hypothalamic TRH release is small in hypothyroid rats. Therefore, thyroid status seems to affect TSH secretion mainly by a direct action at the pituitary level.

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C.

EFFECT OF STARVATION AND REFEEDING ON THYROID FUNCTION AND ON IN VITRO RELEASE OF HYPOTHALAMIC TRH IN THE RAT.

ABSTRACT.

The effects of starvation and refeeding on thyroid function were studied in young (5-6 weeks old) and older (more than 12 weeks old) male and female Wistar rats. Serum levels of T4 and T3 are reduced in all rats during starvation for up to 4 days. Subsequent refeeding restores serum T3 and T4 in young female rats after 2 days, whereas in old male rats serum T4 remains significantly lower. In young female rats starvation lowers TSH and PRL levels and refeeding increases TSH levels to above control levels, while PRL levels are restored to normal. In male rats serum TSH does not respond to starvation, although refeeding tends to increase TSH levels to above control values. In all rats the dialyzable fraction of T4 in serum is increased during starvation and restored during refeeding. The serum free T4 (FT4) levels, however, decrease most quickly in young female rats, whereas only prolonged fasting decreases the FT4 in male rats, which is still apparent during refeeding. Plasma levels of glucose fall during fasting and increase to normal levels again during refeeding, whereas levels of free fatty acids rise during starvation and decrease to normal during refeeding.

Since the effects of starvation and refeeding on thyroid function appear especially in young female rats, these rats were used to study fasting- and refeeding-associated changes in hypothalamic TRH release in a static incubation system. Basal and 56 mM K^+-induced TRH secretion from hypothalamic fragments were not significantly altered during starvation or refeeding. Also hypothalamic TRH content and levels of TRH in peripheral blood were not influenced by nutritional status. Content of TRH in the pituitary, however, increased during starvation. In conclusion, the most consistent and significant effects of starvation and refeeding on thyroid function are seen in young female Wistar rats. These changes are not associated with an altered in vitro hypothalamic TRH release.

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INTRODUCTION.

Starvation has pronounced effects on thyroid function (1-5). Circulating plasma levels of T3 and T4 are reduced concomitant with unchanged or decreased TSH levels in food-deprived rats, and these changes are rapidly reversed with refeeding (6-8). It is believed that these changes are associated with an altered secretion of TRH from the hypothalamus (4-6). Indeed, TRH tests in starved rats show an enhanced TSH response (1,4,5), resembling the situation in hypothalamic hypothyroidism. Moreover, the refeeding-induced TSH rise can be abolished with an antiserum against TRH (6). Studies in which hypothalamic TRH release is measured directly, however, are not available. Therefore, the aim of this study was to measure in vitro TRH secretion from hypothalamic fragments derived from starved and refed rats. In previous studies we found that in vitro TRH release is a fair estimate of in vivo TRH release (9,10). Furthermore, since the serum binding of thyroid hormone during starvation in the rat has received little attention, we also measured free hormone concentrations and plasma levels of free fatty acids, which are known to influence serum binding of thyroid hormone (11,12).

MATERIALS AND METHODS.

Starvation and refeeding. In all experiments Wistar rats were used. Since gender and age may influence control of TSH secretion (13-16), 5-6 weeks old female and male rats (young rats) or more than 12 weeks old male rats (old rats) were used. Groups of rats were totally deprived of food for 1, 2, 3 or 4 days, or starved for 4 days and consequently refed for 1, 2 or 3 days. During the entire experimental period rats had free access to water. Body weights were monitored and trunk blood was collected after decapitation under ether anesthesia to measure plasma levels of T4, T3, TSH, free T4 (FT4) and free fatty acids (FFA). Rats which had free access to pelleted food served as controls. Blood collection took place in the afternoon.

Static incubation of hypothalamic fragments. Young female rats were starved for 1, 2 or 3 days, or starved for 3 days and subsequently refed for 1 or 2 days. Every day body weights and condition of the animals were monitored.

Control rats had free access to food. Between 10 and 11 am, rats were decapitated and hypothalami were isolated as described previously (17). Trunk blood was collected to determine levels of glucose, T3, T4, TSH and PRL. To measure concentrations of TRH in peripheral blood, trunk blood of half of the animals was collected into methanol. Also the pituitaries were removed, weighed and placed into methanol-containing tubes to estimate TRH content. Hypothalami were incubated in Krebs Ringer bicarbonate medium as described in **Chapter III (Section B; Materials and Methods).** TRH release was measured during two 1-hour intervals: during the first hour basal TRH release was estimated, while in the second hour period K^+-induced TRH release was determined. After incubation, the hypothalamic fragments and pituitaries were homogenized in 2 ml methanol with a glass grinder to measure TRH content. For this purpose the tubes were centrifuged and the supernatants were dried under a stream of nitrogen and dissolved in 0.5 ml Krebs Ringer bicarbonate medium. Release of TRH was expressed as a percentage of hypothalamic TRH content. The trunk blood collected into methanol-containing tubes was processed similarly. The residue of blood after the extraction with methanol was weighed to estimate the volume of blood collected into methanol (17).

Hormone determinations. Levels of TSH and PRL were measured by RIA using materials and protocols supplied by the NIADDK and TSH-RP-2 and PRL-RP-1 were used as standard. Concentrations of T3 and T4 were determined in unextracted serum by specific RIAs. Levels of TRH were measured using a previously described RIA (18). Detection limits of the assays, defined as the amount of hormone that reduces binding of tracer to 90% of that occurring in the absence of unlabeled hormone, were 0.4 ng/ml RP-2 TSH, 2 nmol/l T4, 0.2 nmol/l T3 and 1-2 pg/tube TRH. Intra- and interassay coefficients of variation for the assays were between 3 and 12%. Samples from each experiment were run in duplicate in the same assay. Blood glucose was determined with test strips (Haemo-Glukotest, Boehringer Mannheim, Germany) and a reflectance photometer (Reflolux, idem). Serum FFA levels were measured using an in vitro enzymatic colorimetric method supplied by Wako Chemicals GmBH (Neuss, West Germany). The dialyzable fraction of FT4 (DFT4) was measured by equilibrium dialysis. The FT4 was subsequently calculated as the product of the total T4 and DFT4.

Statistical analysis. All data are presented as means±SEM. Analysis of variance followed by Duncan's multiple range tests was used to establish significant differences. Differences were considered statistically significant at $p \leq 0.05$.

RESULTS.

Starvation and refeeding. The changes in serum levels of T4, T3, TSH, DFT4 and FT4, and FFA during starvation and refeeding in young male and female and old male rats are shown in **Tables 1, 2 and 3,** respectively. The loss in body weight after 4 days of food-deprivation was 30% in young female and male rats, whereas the weight gain in the corresponding normally fed controls was 20 and 45 %, respectively. Old male rats lost approximately 15% body weight after 4 days of fasting. In all animals refeeding restored body weights after 2 (female rats) or 3 days (male rats).

In general, serum levels of T4 and T3 fall during starvation and rise during refeeding. In young female rats the fall in T4 and T3 levels is progressive and highest after 3 days starvation. Only female rats show a significant reduction in serum TSH levels during fasting **(Table 2)**. Levels of TSH in food-deprived male rats do not change **(Tables 1 and 3)**. Refeeding restores T4 and T3 to control levels of normally fed rats after 2-3 days in young male rats. Serum levels of T3 increase even to above control levels during refeeding of young male rats and also TSH levels rise significantly to above control values during refeeding of young male rats **(Table 1)**; in old male rats, however, serum T4 stays significantly lower during refeeding.

In all rats DFT4 increases during fasting and returns to normal during refeeding. Serum FT4 decreases in parallel with serum total T4 in female rats **(Table 2)**. In young male rats it is significantly reduced only after 4 days of fasting and restored to normal after 3 days of refeeding **(Table 1)**. Refeeding does not restore serum FT4 in old male rats.

Serum levels of FFA increase during fasting. In male rats these changes are not statistically significant. However, refeeding reduces FFA to below control values **(Table 1)**. In female rats serum FFA rises significantly after 2 days of fasting **(Table 2)**.

Table 1: Serum levels of T4, T3, TSH, dialyzable fraction of T4 (DFT4), free T4 (FT4) and free fatty acids (FFA) in 5-6 weeks old male rats during starvation or refeeding.

	n	T4 nmol/l	T3 nmol/l	TSH ng RP-2/ml	DFT4 %	FT4pmol/l	FFA mmol/l
controls	6	58±3	1.09±0.02	0.98±0.09	0.025±0.001	14.6±0.6	0.94±0.07
starvation 2d	6	20±5*	0.43±0.07*	0.80±0.08	0.047±0.002*	10.0±3.0	1.13±0.07
3d	5	28±4*	0.39±0.07*	0.91±0.10	0.046±0.002*	13.3±2.5	1.17±0.18
4d	5	23±6*	0.43±0.11*	1.06±0.14	0.039±0.004*	8.3±2.0*	0.88±0.13
refeeding 1d	4	19±6*	0.53±0.16*	0.97±0.02	0.038±0.003*	9.2±1.1*	0.56±0.13*
2d	5	37±5*	1.58±0.09*	1.14±0.09	0.021±0.002	7.8±1.2*	0.65±0.03*
3d	12	65±3	1.54±0.06*	1.74±0.19*	0.020±0.001	12.8±0.5	0.56±0.03*

Rats were starved for 2, 3 or 4 days, or refed for 1, 2 or 3 days after 4 days fasting. Normally fed rats were used as controls (*$p \leq 0.05$ vs controls).

Table 2: Serum levels of T4, T3, TSH, dialyzable fraction of T4 (DFT4), free T4 (FT4) and free fatty acids (FFA) in 5-6 weeks old female rats during starvation.

	T4 nmol/l	T3 nmol/l	TSH ng RP-2/ml	DFT4 %	FT4 pmol/l	FFA mmol/l
controls	53±3	1.40±0.08	0.89±0.07	0.028±0.001	14.8±1.1	0.80±0.06
starvation 1d	26±3a	0.66±0.07a	0.59±0.03a	0.039±0.002a	9.8±0.7a	0.76±0.05
2d	20±3a	0.45±0.05ab	0.57±0.06a	0.045±0.002a	8.8±0.9ab	1.12±0.07a
3d	15±3ab	0.15±0.05abc	0.66±0.07a	0.045±0.002a	6.8±1.4ab	0.90±0.11
4d	15±3ab	0.25±0.07abd	0.73±0.09	0.054±0.009a	7.2±0.9ab	0.78±0.06

Rats were starved for 1, 2, 3 or 4 days. Normally fed rats were used as controls (n=6; $^{a}p \leq 0.05$ vs controls, $^{b}p \leq 0.05$ vs starvation 1d, $^{c}p \leq 0.05$ vs starvation 2d, $^{d}p \leq 0.05$ vs starvation 3d, ND not determined).

Table 3: Serum levels of T4, T3, TSH, dialyzable fraction of T4 (DFT4) and free T4 (FT4) in more than 12 weeks old male rats during starvation or refeeding.

	T4 nmol/l	T3 nmol/l	TSH ng RP-2/ml	DFT4 %	FT4 pmol/l
controls	38±5	1.05±0.07	1.10±0.14	0.027±0.002	10.6±1.6
starvation 1d	23±2a	0.72±0.05a	0.84±0.06	0.030±0.002	7.2±0.7
2d	18±2a	0.64±0.07a	0.92±0.07	0.032±0.002	5.8±0.8a
3d	20±3a	0.52±0.05a	0.98±0.09	0.036±0.002ab	7.6±1.6
4d	22±3a	0.59±0.05a	1.11±0.10	0.034±0.002a	7.5±1.1
refeeding 1d	20±2a	1.23±0.12	0.78±0.06	0.032±0.001	6.3±0.8a
2d	24±3a	0.96±0.07	1.01±0.10	0.024±0.001	5.7±0.4a
3d	26±5a	1.17±0.05	0.95±0.11	0.022±0.002	6.7±1.2a

Rats were starved for 1, 2, 3 or 4 days, or refed for 1, 2 or 3 days after 4 days fasting. Normally fed rats were used as controls (n=6-8; $^{a}p \leq 0.05$ vs controls, $^{b}p \leq 0.05$ vs starvation 1d).

Static incubation of hypothalamic fragments. Table 4 shows the in vitro hypothalamic TRH release from starved and refed female rats as well as TRH levels in hypothalami, pituitaries and peripheral blood. No statistically significant changes occur in in vitro hypothalamic TRH release during starvation or refeeding. 56 mM K^{+} stimulates TRH secretion 3-fold without

significant differences among groups. The hypothalamic TRH content or levels of TRH in peripheral blood are not influenced by starvation nor refeeding. However, the hypophysial TRH content increases during starvation and this increase is still apparent during refeeding. In **Table 5** plasma levels of glucose, T3, T4, TSH and PRL of the female rats that were used in this experiment are given. Plasma glucose falls during fasting, being lowest after 1 d, and is restored after refeeding. Glucose levels after one day of refeeding are higher than in the normally fed controls. Again levels of T3 and T4 fall during starvation and are restored again after 2 days of refeeding. Plasma TSH tends to fall during fasting. However, these changes are not significant. Refeeding increases TSH levels to above control values in the normally fed rats. Also PRL levels fall during fasting and return to normal after refeeding.

Table 4: Basal and 56 mM KCl-induced (K^+) hypothalamic TRH release per hour expressed as percentage of hypothalamic TRH content, and levels of TRH in hypothalami (hypoth.), pituitaries (pituit.) and peripheral blood of starved and refed rats.

	TRH secretion in vitro		TRH levels in		
	basal (%/h)	K^+ (%/h)	hypoth. (ng)	pituit. (pg/mg)	blood (pg/ml)
controls	1.70±0.50	5.88±0.81	8.1±0.6	66±2	7.0±0.9
starvation 1d	1.46±0.26	6.06±1.10	8.6±0.5	77±5	7.0±1.0
2d	1.52±0.20	5.75±1.03	8.4±0.6	92±5*	6.4±0.9
3d	1.57±0.26	4.73±0.62	8.5±0.7	96±4*	6.3±1.0
refeeding 1d	1.90±0.47	4.56±0.43	8.4±0.7	100±6*	6.8±0.9
2d	1.76±0.29	4.39±0.43	8.5±0.6	93±6*	9.5±1.3

Female rats were starved for 1, 2 or 3 days or refed for 1 or 2 days after 3 days fasting. Normally fed rats were used as controls (n=16; *$p \leq 0.05$ vs controls).

Table 5: Plasma levels of glucose, PRL, T3, T4 and TSH of starved and refed rats.

	glucose mmol/l	PRL ng/ml	T3 nmol/l	T4 nmol/l	TSH ng RP-2/ml
controls	8.3±0.3	106±19	1.65±0.05	60±4	1.97±0.30
starvation 1d	6.0±0.3a	110±24	1.27±0.06a	51±5	1.39±0.18
2d	6.8±0.2a	50±14a	0.85±0.07ab	26±2ab	1.30±0.19
3d	7.2±0.2a	41±15a	0.61±0.04abc	26±2ab	1.46±0.17
refeeding 1d	9.7±0.3a	63±15	1.25±0.06a	46±3a	4.29±0.44a
2d	8.9±0.3	54±10	1.52±0.06	53±3	3.28±0.38a

As in Table 4 (n=15-16; [a]$p \leq 0.05$ vs controls, [b]$p \leq 0.05$ vs starvation 1d, [c]$p \leq 0.05$ vs starvation 2d).

DISCUSSION.

In rats food-deprivation reduces serum levels of T3 and T4, while serum TSH decreases or does not change (this study, Refs. 1-5). It should

be mentioned that basal TSH levels in young female rats differ greatly between experiments (cf. Table 2 and Table 5: 0.89±0.07 vs 1.97±0.30 ng/ml). These changes seem to be due to diurnal variations in serum TSH (19,20), since the experiments were done on separate times of the day (see Materials and Methods). The fact that serum TSH does not change or is even reduced in the face of low serum levels of T3 and T4 points to a central cause of the fasting-associated changes in thyroid function. The influence of starvation on serum TSH appeared especially in young female rats, in which TSH levels tend to decrease. A recent study pointed to the influence of gender on the starvation-induced changes in serum TSH in the rat (13). In this study Cohen et al. reported that serum TSH does not decline in female rats, whereas in male rats levels decrease by approximately 50% during fasting. These findings seem to contradict our study, in which the starvation-induced TSH decline appears especially in female rats. These paradoxical findings may be ascribed to some differences between both studies: Cohen et al. used 2-3 months old Sprague Dawley rats, whereas in our study 5-6 weeks old Wistar rats were starved. Several studies have stressed the differential responsiveness of Wistar and Sprague Dawley rats to common stressors such as cold (21) or fasting (2). Moreover, the rat's age (14,15) may influence the TSH response to food-deprivation. Indeed, starvation-induced weight loss was most severe in young animals, and only moderate in old male rats, which did not show a fasting-induced TSH decline.

In the rat the effects of starvation and refeeding on thyroid function seem to be associated with an altered hypothalamic release of TRH (4-6). Several arguments are in favour of this hypothesis. Firstly, in the food-deprived rat the amount of TSH secreted in response to TRH is enhanced (1,4,5) and this may point to a hypothalamic origin of the fasting-induced TSH decline. Secondly, during refeeding TSH secretion in both young male and female rats is stimulated to above control levels in normally fed rats (this study) and this has previously been shown also in the bull (7) and in the rat after short-term refeeding (6). The increase in plasma TSH during refeeding can be abolished by anti-TRH antiserum (6). Finally, we found that fasting depresses plasma PRL levels, confirming previous studies (3,5). Also the PRL response to TRH is enhanced in starved rats (3,5). Since TRH is claimed to be a PRL-releasing factor (22), the fasting-associated PRL reduction may be mediated by TRH.

Because in this study the effects of starvation and refeeding on body weight and thyroid function were most consistently and significantly seen in young female rats, these rats were further used to study hypothalamic TRH release. However, in vitro TRH release from hypothalamic fragments is not influenced by starvation or refeeding. Several mechanisms may be responsible for this finding. Firstly, other hypothalamic factors such as somatostatin or dopamine are known to influence TSH secretion (23) and thus might be more important than TRH for the regulation of TSH release during fasting. It has been shown that hypothalamic somatostatin secretion is enhanced in hypoglycemia (24). Moreover, peripheral plasma levels of somatostatin increase during starvation (25) and antiserum to somatostatin enhances basal and TRH-stimulated TSH release in starved rats (26). Therefore somatostatin might play an important role in the fasting-induced reduction of TSH secretion. The role of dopamine in the fasting-induced TSH decline is ambiguous (26,27). Secondly, some studies suggest that inhibition of TSH secretion occurs at the level of the pituitary, through an increased sensitivity of the thyrotroph to T3 (28), or inhibition by corticosterone (23,29,30), plasma levels of which are increased during starvation (6,31,32). However, there is no evidence for an increased feedback of T3 at the pituitary level in starved rats since intracellular T4 to T3 conversion and the number of nuclear T3 receptors are decreased during starvation (33-35). Thirdly, during starvation complex metabolic changes occur in the body causing profound alterations in the plasma composition which are not maintained in our in vitro system. Indeed, plasma levels of glucose fall during fasting and increase to normal again during refeeding, while the reverse situation is shown with regard to FFA (this study). It is known that the effect of fasting on thyroid function depends primarily on dietary carbohydrate reduction (36,37) and refeeding with glucose alone can restore thyroid status completely (6,37,38). Experiments are now undertaken to investigate the influence of the glucose concentration on in vitro TRH release and to determine in vivo TRH release into hypophysial portal blood of starved rats.

Content of TRH in the hypothalamus is unchanged during fasting (this study, Ref. 1). However, hypophysial TRH content increases during starvation and this increase is still apparent during refeeding. Several studies reported that the major part of hypophysial TRH resides in the posterior pituitary (39,40). It has further been suggested that this TRH is

of hypothalamic origin, derived from parvocellular TRH-containing neurons in the magnocellular division of the paraventricular nucleus (39) and is secreted by the same mechanisms as TRH located in the median eminence (41). Moreover, because of vascular connections between anterior and posterior pituitary (42) it may influence secretion of anterior pituitary hormones. The physiological meaning of the starvation-induced increase in hypophysial TRH, however, remains speculative.

Little attention has been paid to changes in the free hormone concentrations of T4 and T3 in the starved rat (1,43-47). Serum FT4 has been shown to be unchanged (43,44), increased (45) or decreased (this study, Refs. 1 and 47) during starvation, whereas the free T4 fraction in serum is shown to increase (this study). It is believed that the increased levels of ketones or FFA during starvation cause displacement of bound T4 from binding proteins (11,12). Moreover, levels of TBPA decrease during starvation (45). Again, the fasting-induced changes in free hormone concentrations are most significant in female rats (this study). The decrease in serum FT4 levels in male rats is only apparent after prolonged fasting. Refeeding seems to restore plasma binding of T4 more quickly than total T4 levels. Therefore, serum FT4 levels are reduced in refed male rats. Serum total T3, the free T3 fraction, and serum FT3 all decrease during fasting (1,46). In rats fed a low protein-high carbohydrate diet the FT3 fraction decreases and the serum FT3 is normal or reduced, whereas total T3 increases (48-51). It is suggested that these changes stem from an increased production of a T3-binding globulin (46-48). These changes are most significant in young rats (50).

It should be stressed that the effects of starvation on thyroid function in man and rat are different. In the rat serum T3, T4, TSH and FT4 decrease, while blood concentrations of TRH fall (52) or remain unchanged (this study) and TSH sensitivity to TRH is increased. Also plasma PRL levels decrease, while the PRL response to TRH is enhanced in starved rats (3,5). In man, serum T3 and TSH decrease, while serum T4 remains unchanged and FT4 and rT3 increase (53-56). Moreover, TSH sensitivity to TRH is decreased (27,57,58) and plasma TRH is normal (54). Plasma PRL levels are not affected by starvation, nor is the PRL response to TRH (27,59). Thus, it seems that in the fasting human TSH reduction occurs at the pituitary level, presumably mediated by the increase in FT4 (54). With regard to peripheral blood concentrations of TRH in rat and man, it should be

mentioned that this parameter is considered a poor estimate of hypothalamic TRH release (60,61).

In conclusion, starvation and refeeding have pronounced effects on thyroid function and these effects appear especially in young female rats. Although a hypothalamic cause of these alterations has been suggested, in vitro TRH secretion is not changed in starved or refed rats.

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D.

EFFECT OF STREPTOZOTOCIN-INDUCED DIABETES MELLITUS ON THYROID FUNCTION AND ON HYPOTHALAMIC TRH RELEASE IN THE RAT.

ABSTRACT.

One and two weeks after induction of diabetes mellitus (DM) by streptozotocin (STZ) in male Wistar rats serum levels of T3 and T4 decreased by 30-40% vs. untreated animals. The dialyzable fraction of T4 increased concomitantly and, consequently, free T4 levels were similar in control and diabetic animals. Levels of TSH and PRL were reduced by 30% after 2 weeks. To investigate if the DM-associated hypothyroidism and hypoprolactinemia resulted from a decreased TRH release, hypothalamic fragments of rats treated with STZ two weeks previously were incubated for 1 h in basal (i.e. with 5 mM K^+) and depolarizing (i.e. with 56 mM K^+) medium with 10 mM glucose. It was shown that basal TRH secretion was reduced by 37%, while K^+-induced TRH release was decreased by 14% vs. control values of untreated rats. Hypothalamic TRH content and levels of TRH immunoreactivity in peripheral blood were not influenced by STZ treatment.

To investigate if in vitro secretion of TRH by hypothalamic fragments was also modulated by glucose concentrations in the incubation medium, hypothalami of untreated rats were incubated in medium with 0, 10 or 30 mM glucose. Increasing the medium glucose concentration from 10 mM to 30 mM did not influence TRH secretion. However, in the absence of glucose basal TRH release was increased two-fold.

In conclusion, STZ-induced DM results in hypothyroidism and hypoprolactinemia, associated with a decreased hypothalamic secretion of TRH. Since increasing the medium glucose concentration from 10 mM to 30 mM does not reduce TRH release from hypothalami of untreated rats, the effect of STZ-DM on TRH secretion does not seem to depend on a direct impairment of TRH release by high levels of glucose.

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INTRODUCTION.

In the rat streptozotocin-induced diabetes mellitus (STZ-DM) and hyperglycemia are associated with thyroid hormone abnormalities suggestive of tertiary (i.e. centrally mediated) hypothyroidism (1-5). Circulating levels of T3, T4 and TSH are all decreased in experimentally induced diabetes, and it seems that these changes are caused by a reduced hypothalamic release of TRH, since hypothalamic TRH content and systemic plasma immunoreactive TRH (IR-TRH) are decreased in diabetic rats (2-4). Indeed, experimentally induced diabetes mellitus has pronounced effects on the morphology of the mediobasal hypothalamus, causing anatomical lesions in this area (6-8). Therefore, it is suggested that the neuroendocrine impairments that frequently occur in STZ-DM are caused by detrimental effects of diabetes on hypothalamic functions. Furthermore, hypothalamic content of neuropeptides other than TRH, such as LHRH (6), somatostatine (9) and neuropeptide Y (9,10), has been shown to change in diabetic animals. Few studies, however, report on the functional release of hypothalamic hormones in diabetic rats (6,11). Therefore, in this study we investigated the effects of STZ-DM on thyroid function and on in vitro hypothalamic TRH release.

MATERIALS AND METHODS.

In all experiments male Wistar rats (200-300 g) were used. They were kept in a temperature-controlled room (20-22 C) with a light cycle of 14-h light and 10-h darkness. Rats had free access to food and tap water. Diabetes mellitus was induced by injection of streptozotocin (65 mg/kg BW) in the external jugular vein or in the penile vein of ether-anesthetized rats. Untreated rats served as controls. After one and two weeks blood was collected from the orbital plexus under light ether anesthesia to measure serum levels of glucose, T3, T4, free T4, TSH and PRL. Also the body weights were monitored.

After the last blood collection rats were decapitated and their hypothalami were isolated as described previously (12). Trunk blood (2-3 ml) was collected into tubes containing 2 ml methanol to determine whole blood IR-TRH. Processing of these tubes and static incubation of the hypothalami in Krebs Ringer bicarbonate (KRB) is described in **Chapter III,**

sections B and C (Materials and Methods).

To investigate if the glucose concentration in the KRB influences TRH release, hypothalamic fragments of untreated rats were incubated in medium containing 0, 10 or 30 mM glucose. Osmolality was kept constant by equivalent changes in NaCl concentration.

Hormone determinations. Levels of T3 and T4 were measured by routine RIA procedures. Levels of TSH and PRL were measured by RIA using materials and protocols provided by the NIADDK with TSH-RP-2 and PRL-RP-1 as standards, respectively. The dialyzable fraction of T4 (DFT4) was measured by equilibrium dialysis, and the free T4 concentration (FT4) was subsequently calculated as the product of total T4 and DFT4. Concentrations of TRH were measured using a previously described RIA (13). Blood glucose was determined with test strips (Haemo-Glukotest, Boehringer Mannheim, Germany) and a reflectance photometer (Reflolux, idem). Detection limits of the RIAs, defined as the amount of hormone that reduces binding of tracer to 90% of that occurring in the absence of unlabeled hormone, were 0.4 ng/ml RP-2 TSH, 2 nmol/l T4, 0.2 nmol/l T3 and 1-2 pg TRH. Intra- and interassay coefficients of variation for the assays were between 3 and 12%. Samples from each experiment were run in duplicate in the same assay.

Statistical analysis. All data are presented as means±SEM. Analysis of variance followed by Duncan's multiple range tests, or the Student's t test for comparison of two means was used to establish significant differences. Differences were considered statistically significant at $p \leq 0.05$.

RESULTS.

The effect of STZ-DM on body weight, and serum levels of glucose, T3, T4, TSH and PRL after one and two weeks is shown in **Figure 1**. One week after injection of streptozotocin glucose levels rise to approximately 25 mmol/l. After two weeks these levels decrease slightly but significantly to approximately 20 mmol/l. It is also shown that in diabetic rats body weights remain constant, while untreated rats keep on growing. Therefore, relative weight loss amounts to 18% and 25% vs untreated rats one and two weeks after the induction of diabetes, respectively.

Serum T3 and T4 levels are reduced by 30-50% in one and two week diabetic animals, while levels of TSH are only reduced two weeks after streptozotocin treatment. Also in untreated rats, TSH levels are reduced after two weeks, but the reduction in diabetic rats is significantly greater and levels amount to 70% of those of control rats. In general, plasma PRL levels are high and variable but decrease significantly 2 weeks after STZ-DM to 28% of control levels in untreated rats, and to 18% of levels after 1 week STZ-DM.

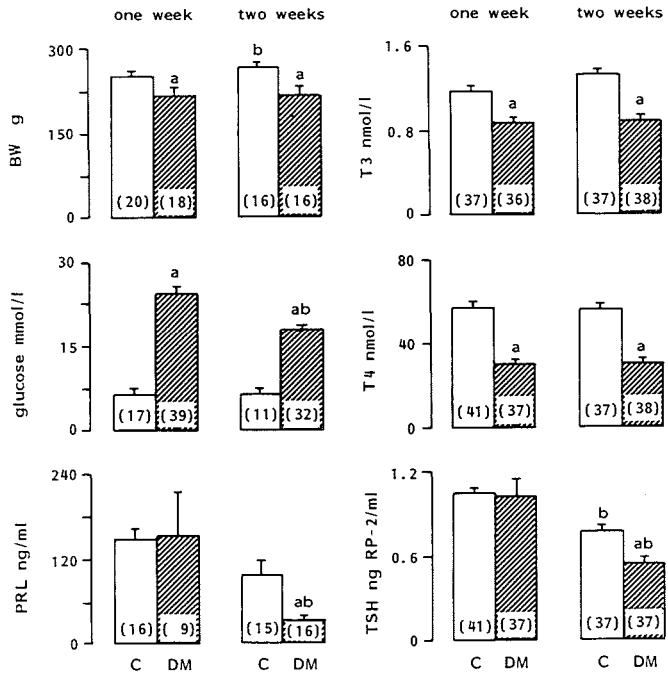

Figure 1: Body weights (BW), and levels of glucose, PRL, T3, T4, and TSH one and two weeks after streptozotocin-induced diabetes mellitus (DM, hatched bars). Untreated rats served as controls (C, white bars). (Number of animals in parenthesis; $^{a}p \leq 0.05$ vs control, $^{b}p \leq 0.05$ vs one week).

Table 1 shows that the dialyzable fraction of T4 (DFT4) is increased in experimental diabetes of 2 weeks duration. Consequently, free T4 (FT4) levels remain unchanged in STZ-DM, despite the fact that levels of total T4 are significantly reduced vs. control animals.

In **Table 2** the effects of diabetes mellitus on in vitro hypothalamic TRH release and on IR-TRH in peripheral blood are shown. Hypothalamic

fragments of diabetic rats secrete less TRH than those of untreated rats, both under basal and K^+-stimulated conditions. No significant differences could be established in hypothalamic TRH content or levels of TRH in peripheral blood between diabetic and control rats.

Table 1: Influence of diabetes mellitus on levels of T4, the dialyzable fraction of T4 and on the free T4 (FT4) levels.

	control	diabetes mellitus
T4 nmol/l	42±2	16±1*
DFT4 %	0.038±0.002	0.093±0.006*
FT4 pmol/l	15.6±1.2	14.3±1.0

Rats were treated with streptozotocin 2 weeks previously. Untreated rats served as controls (n=16; *$p \leq 0.05$ vs control).

Table 2: Levels of IR-TRH in peripheral blood (n=8), hypothalamic TRH content and basal (5 mM KCl) and K^+-induced (56 mM KCl) secretion of TRH from hypothalami (n=16) of diabetic rats.

	control	diabetes mellitus
TRH peripheral blood (pg/ml)	13.7±0.7	14.3±1.0
TRH hypothalamus (ng)	11.8±0.8	11.5±0.6
TRH secretion: 5 mM KCl (pg/h)	97±15	61± 5a
56 mM KCl (pg/h)	317±16b	273±11ab

Rats were treated with streptozotocin 2 weeks previously. Control values are from untreated rats ($^a p \leq 0.05$ vs control, $^b p \leq 0.05$ vs 5 mM KCl).

In Table 3 the effects of glucose concentrations on in vitro hypothalamic TRH release are shown. Results are expressed as percentage release of hypothalamic TRH content. It is shown that in the absence of glucose basal TRH release is significantly increased almost two-fold compared to incubations in which the medium contained 10 mM glucose. Increasing the medium glucose from 10 to 30 mM tends to decrease TRH secretion, but this difference is not statistically significant.

Table 3: Influence of glucose concentration on in vitro hypothalamic TRH secretion. Hypothalami of untreated rats were incubated with 0, 10 or 30 mM glucose.

	TRH release (%/h)	
	5 mM KCl	56 mM KCl
glucose: 0 mM	0.89±0.06a (16)	1.15±0.11b (8)
10 mM	0.46±0.03 (32)	1.09±0.06b (16)
30 mM	0.38±0.02 (8)	1.20±0.08b (8)

Results are the pooled data of 4 experiments of 8 incubations each and expressed as percentage of hypothalamic TRH content (number of incubations in parenthesis; $^{a}p \leq 0.05$ vs 10 mM glucose, $^{b}p \leq 0.05$ vs 5 mM KCl).

DISCUSSION.

Diabetes mellitus is frequently associated with neuroendocrine impairments, such as sexual dysfunction (14) and hypothyroidism (1-5, this study). Indeed, in the rat STZ-DM lowers plasma levels of FSH and LH (14), PRL (14, this study) and TSH (1-5, this study) and it is suggested that these changes are caused by an action of diabetes at the hypothalamic level. This study reports that hypothalamic TRH release is decreased by approximately 40% in STZ-DM and thus may play a causal role in the hypothyroidism and hypoprolactinemia (14) that may occur in diabetes mellitus and severe hyperglycemia induced by glucose infusions (5). Bestetti et al. have shown that STZ-DM affects the morphology of the mediobasal hypothalamus, causing functional and anatomical lesions in this area (6-8). Indeed, in vitro hypothalamic LHRH (6) and somatostatin (11) release has been shown to be decreased in diabetic rats.

Other studies have reported on alterations in hypothalamic-pituitary-thyroid regulation produced by diabetes (1-4). All studies report decreased levels of T3, T4 and TSH following administration of streptozotocin. In our study, TSH and PRL levels are decreased two weeks after induction of diabetes mellitus. Also in control animals reduction of TSH levels was observed, which may be due to the stress of repeated blood collection (15), but the reduction in diabetic animals was significantly greater. With regard to plasma PRL levels it should be mentioned that these values are high and variable, probably due to blood sampling stress.

Since the biological effect of thyroid hormone is controlled by its free fraction, serum TSH may be reduced because of increased free T4 levels. Indeed, the dialyzable fraction of T4 is increased. This effect overcomes, but does not counteract the low total T4 concentrations in diabetic rats and the free T4 levels are, consequently, not affected by STZ-DM. Therefore, changes in FT4 do not play a role in the diminished serum TSH in diabetic rats. Since binding of T4 to plasma proteins is affected by free fatty acids (FFA) (16), it is tempting to suggest that the increased DFT4 is caused by the increase in FFA that occurs in untreated diabetes mellitus. One might assume that if levels of free T3 (FT3) follow the same pattern as FT4 (i.e. unaffected by STZ-DM), rats are not hypothyroid. Although we did not measure FT3 levels, it is known that binding of T3 can be increased in conditions in which binding of T4 is decreased. This especially occurs in starved rats in which DFT4 is augmented probably because of an increase in FFA (17). The dialyzable fraction of T3, on the other hand, is decreased in the fasting rat, probably because of an increased binding of T3 to a thyronine-binding globulin (18). As it is, the fact that serum TSH levels are low despite normal levels of FT4 and low serum T3 concentrations, points to a centrally mediated reduction of TSH secretion.

This study shows evidence that STZ-DM is associated with decreased hypothalamic TRH secretion in vitro. During the preparation of this manuscript a paper by Bestetti et al. appeared confirming our results (19). It was shown that STZ-DM has characteristic functional and morphological patterns in the mediobasal hypothalamus impairing in vitro TRH secretion. Other studies used hypothalamic TRH content and plasma IR-TRH as parameters for hypothalamic TRH release (2-4). In our study hypothalamic TRH content is not changed two weeks after STZ treatment and this is in agreement with the litterature (2,3). It seems that only prolonged STZ-DM (i.e. of more than two weeks duration) decreases hypothalamic TRH content (2), since content of TRH was not found to be changed 3 or 14 d after STZ administration (2,3). However, hypothalamic content of TRH does not necessarily reflect release and is also dependent on synthesis and tissue degradation. Indeed, hypothalamic TRH content has been shown to be unchanged in conditions in which TRH release is clearly altered (20). Several studies report on plasma IR-TRH in STZ-DM. These were found to be normal (2, this study) or decreased (3) in diabetic rats. However, only part of TRH in the

systemic circulation is derived from the hypothalamus (21) and only part of plasma IR-TRH represents authentic TRH **(Chapter II, section B).** Therefore, no conclusion should be based on estimations of TRH levels in the systemic circulation with regard to hypothalamic TRH release.

Since in STZ-DM somatic growth is impaired, the effects of diabetes on hypothalamic TRH release may be caused by poor nutritional status, rather than by STZ-DM per se. However, 4 days of starvation leading to the same weight loss as STZ-DM of 2 weeks duration does not alter hypothalamic TRH release **(Chapter III, section C).** It should be stressed that during STZ-DM complex metabolic changes occur that profoundly change plasma concentrations of various nutrients and hormones. These changes are not reflected in the constant medium composition in our static incubation system. Indeed, all hypothalami -whether originating from untreated or STZ treated rats- were incubated in KRB containing 10 mM glucose and this could clearly blunt the effect of STZ-DM on hypothalamic secretion of TRH. Therefore, we tested the effect of altering glucose concentrations in our incubation system. Increasing the glucose concentration in the medium from 10 mM to 30 mM does not affect TRH release from hypothalami of normal rats. Therefore, acute hyperglycemia does not seem to affect hypothalamic TRH release, while STZ-DM of 2 weeks duration does. It is tempting then to suggest that the decreased hypothalamic TRH release in experimentally induced diabetes mellitus is caused by an anatomical lesion in the MBH.

Interestingly, in the absence of glucose TRH release is stimulated two-fold and this has also been shown with regard to somatostatin (22), LHRH (22) and CRF release (23). It is known that induction of intracellular glucopenia in the central nervous system whether by insulin (causing hypoglycemia) or 2-deoxy-d-glucose (causing hyperglycemia) (24) stimulates thyroid function (25,26). Again, this may stem from an increased hypothalamic TRH release (this study, 27).

In conclusion, hypothalamic TRH release is modulated by glucose concentrations. While glycopenia stimulates thyroid function (as shown in the litterature) and hypothalamic TRH release, secretion of TRH is reduced in STZ-DM and therefore may cause hypothyroidism and hypoprolactinemia.

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E.

EFFECT OF COLD EXPOSURE ON THE HYPOTHALAMIC RELEASE OF TRH, DOPAMINE AND ADRENALINE.

ABSTRACT

The present study was concerned with the effect of exposure to cold on the hypothalamic release of thyrotropin-releasing hormone (TRH), dopamine and adrenaline in male rats as estimated with push-pull perfusion of the mediobasal hypothalamus or anterior pituitary gland. Before cold exposure rats were kept at room temperature or adapted to 30 C for 3 weeks. In both groups of rats transfer to a room at 4 C induced an increase in plasma TSH within 30 min, but this TSH response was stronger in animals adapted to 30 C before exposure to cold. Also plasma PRL levels rose within 30 min after cold exposure.

The hypothalamic content of TRH and dopamine remained similar when rats, kept at room temperature, were placed in a room at 4 C. However, the hypothalamic adrenaline content gradually increased from 3.3±0.6 ng before cold-exposure to 9.5±1.3 ng at 6 h of low temperatures. In vivo hypothalamic release of TRH, dopamine and adrenaline did not change significantly in rats exposed to cold when compared with data of control animals not exposed to cold. In rats kept at 30 C, however, the amount of TRH in perfusate of the mediobasal hypothalamus increased 3-fold during the first 15 min of cold-exposure. However, no changes in TRH release were observed in such rats during perfusion of the pituitary gland. In conclusion, the cold-induced stimulation of the hypothalamo-pituitary-thyroid axis is enhanced in rats previously adapted to 30 C.

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INTRODUCTION

Exposure of rats to cold is known to enhance TSH release within 30 min (1-5). This cold-induced secretion of TSH is believed to be mediated by an augmented hypothalamic release of thyrotropin-releasing hormone (TRH), since the TSH response to low temperatures is diminished after passive immunization against TRH (6) or after destruction of the paraventricular nucleus (5), known to reduce the hypothalamic TRH release (7). Conflicting results have been reported about the effect of exposure to cold on hypothalamic TRH content (1,3,8-11) and about the amount of immunoreactive TRH in peripheral blood (12,13,14). In vivo release of TRH into hypophysial portal blood in anesthetized rats did not alter during hypothermia (15), whereas TRH release estimated with push-pull perfusion of the median eminence has been found to increase during cold exposure, with the peak occurring 30-60 min after transfer to the cold room (16-18).

Other hypothalamic factors may also be involved in the cold-induced secretion of TSH. For instance, dopamine and adrenaline have been found to alter the release of TSH by an action on the hypothalamus (18,19), or on the pituitary gland (20,21). In this study push-pull perfusion of the mediobasal hypothalamus (MBH) or anterior pituitary gland (AP) was used to determine the in vivo release of TRH, dopamine and adrenaline in male rats exposed to low temperature. Previous studies have provided evidence that push-pull perfusion is a valid method to estimate in vivo hypothalamic release of TRH, dopamine and adrenaline (22-24).

MATERIALS AND METHODS

Male Wistar rats (200-300 g BW) were used. They were housed under controlled conditions (lights on 0.500-19.00 h; 20-22 C or 30 C) and had free access to food and tap water.

Experiment 1. Male rats were transferred from room temperature (20-22 C) to a room maintained at 4 C and they were killed by decapitation 30, 60, 120, 240 or 360 min later. Rats transferred to a room maintained at 22 C served as controls (0 min). Trunk blood was collected in heparinized glass tubes and the hypothalamus, excluding the preoptic region, was excised within 2 min after decapitation. The tissue was placed into methanol and homogenized

with a glass grinder kept in melting ice. After centrifugation at 4 C the supernatant was divided into two parts. To one part $HClO_4$ was added to a final concentration of 0.1 M and this aliquot was used for the measurement of dopamine and adrenaline. The remainder was dried at 40 C under a stream of nitrogen, and the residue was taken up in RIA buffer and assayed for TRH. Plasma of the collected trunk blood was used to determine TSH, PRL, T3 and T4. All samples were kept at -20 C until they were assayed.

Experiment 2. Rats were placed in a stereotactic instrument with the upper incisor bar 3 mm above interaural line. A 7x7 mm stainless steel push-pull cannula with a 10 mm needle of 0.5 mm outer diameter (for details see ref. 22) and a removable stylette was placed in the MBH (coordinates according to de Groot (25): 5.2 mm anteroposterior, 0.2 mm mediolateral, ventrodorsal 0.2 mm above base of skull) and was fixed on the skull with screws and dental cement. After surgery the rats were caged individually and handled daily, and push-pull perfusion was performed two weeks later (22,23). Artificial cerebrospinal fluid (22) was delivered at a rate of 20 μl/min and perfusate was collected continuously into tubes kept on melting ice. The transit time from the tip of the inner cannula to the sample tubes was 5 min and 15 min samples were collected. The samples were divided into two aliquots: 200 μl for TRH estimation and 100 μl to measure dopamine and adrenaline. To the latter aliquot 10 μl $HClO_4$ was added. Samples were frozen immediately after collection and stored at -20 C.

Experimental animals were perfused for 30 min at room temperature and were then gently transfered to the cold room at 4 C, where they were perfused for another 120 min. During transfer perfusion was not interrupted. Controls were kept at room temperature during perfusion and were perfused for 150 min. Only those animals which were perfused for 150 min (51 out of 112 rats) were used for statistical analysis.

Experiment 3. Rats were placed in a room kept at 30 C. Three weeks later a blood sample was taken from the orbital plexus of the rats while they were lightly anesthetized with ether. Then, they were transferred to a cold room (4 C), and further blood samples were taken 30 and 360 min later. Plasma samples were stored at -20 C until assayed for TSH.

Experiment 4. Rats were implanted with a push-pull cannula either in the

MBH (see above) or the AP (coordinates: anteroposterior 3.0 mm, mediolateral 0.5 mm, ventrodorsal 0.7 mm above base of skull). After recovery from surgery, the rats were placed in a room kept at 30 C. Three weeks later, push-pull perfusion was started and after 30 min of sampling the rats were gently transferred to 4 C without interrupting perfusion. Push-pull perfusion continued for another 90-120 min. Perfusion samples (15-min samples) were frozen immediately after collection and stored at -20 C until assayed for TRH.

Hormone determinations. Plasma TSH was determined by RIA using materials and protocols of the NIADDK, with TSH-RP-2 as the standard. Plasma PRL was measured by RIA (26) with PRL-RP-1 as the standard. Levels of T3 and T4 were estimated by RIA. Due to limited amount of sample and low level of TRH, singular determinations were done in 200 μl by RIA (27). The detection limit (defined as the amount of hormone that reduces binding to 90 % of that occurring in the absence of unlabeled hormone) is 1-2 pg, and intra- and inter-assay coefficients of variation are between 4 and 10 %. Dopamine and adrenaline were determined with a high-pressure liquid chromatographic-electrochemical method (28, 29) using a Coulochem 5100A with conditioning cell 5021 and analytical cell 5011 (ESA, Bedford, MA, USA) as detector. The minimal detectable amount was 5-10 pg for dopamine and adrenaline (signal to noise ratio of 2).

Statistical analysis. Results are presented as means±SEM. For the statistical analysis, undetectable levels of TRH, dopamine and adrenaline in push-pull medium were assigned halve the value of the detection limit. The longitudinal data of TRH, dopamine and adrenaline obtained with the push-pull perfusion experiments were subjected to repeated measurements analysis of variance. The data of hormone levels in hypothalamic tissue and peripheral blood were analyzed with non-parametric tests. Groups were considered to be significant when the two-tailed probability was 5% or less.

RESULTS

The effect of acute cold exposure on peripheral and hypothalamic hormone levels in male rats is summarized in Table 1. The levels of TSH and

PRL had increased at 30 min of cold-exposure. The PRL levels returned gradually to prestimulation values, but TSH at 60 min of cold-exposure was similar to the value recorded at room temperature. Between 120 and 360 min, the levels of TSH were again higher than prestimulation values. The levels of T3 and T4 remained similar throughout the period of observation.

Table 1: Changes in hormone levels in plasma and hypothalamus in male rats transferred from room temperature (20-22 C) to 4 C.

	min of cold exposure					
	0	30	60	120	240	360
T3 nmol/l	1.9±0.1	1.9±0.1	2.1±0.1	2.2±0.2	2.7±0.2*	2.2±0.1
T4 nmol/l	68±4	71±3	70±4	69±3	79±4	72±5
TSH ng/ml	1.5±0.1	2.4±0.2*	1.9±0.2	2.6±0.3*	3.2±0.3*	3.2±0.2*
PRL ng/ml	36±8	69±18*	55±8	45±8	31±5	42±7
TRH ng	8.4±0.5	8.3±0.7	8.3±0.6	8.4±0.6	9.3±0.7	8.6±0.7
DA ng	4.7±0.4	4.5±0.2	4.5±0.4	4.0±0.4	4.6±0.3	6.2±1.0
A ng	3.3±0.6	4.8±0.9	5.9±0.8	6.1±1.0*	8.7±1.6*	9.5±1.3*

At various times after transfer to the cold room the plasma levels of T3, T4, TSH and PRL, and the levels of TRH, dopamine (DA) and adrenaline (A) in the hypothalamus were determined (n=12; *$p \leq 0.05$ vs 0 min).

The hypothalamic content of TRH and dopamine did not change, but the hypothalamic levels of adrenaline gradually increased during exposure to low temperatures.

The response of TSH and thyroid hormones to cold exposure in rats kept for 3 weeks at 30 C is given in **Table 2.** In these rats, the cold-induced release of TSH was more pronounced than in rats kept at room temperature.

Table 2: Changes in plasma levels of TSH, T3 and T4 in male rats transferred from 30 to 4 C.

	min of cold exposure		
	0	30	360
T3 nmol/l	1.0±0.1	1.0±0.1	1.4±0.1*
T4 nmol/l	39±3	37±2	61±5*
TSH ng/ml	1.6±0.1	4.5±0.9*	1.8±0.3

n=6; *$p \leq 0.05$ vs 0 min

The changes in in vivo hypothalamic release of TRH, dopamine and adrenaline as estimated with push-pull perfusion of the MBH of control rats maintained at room temperature and rats exposed to a temperature of 4 C are presented in **Figure 1.** Mean prestimulation values of TRH, dopamine and

adrenaline were 6, 225 and 265 pg/15 min, respectively. No significant changes in the release of TRH, dopamine and adrenaline in perfusate of the MBH were observed. Similar results were obtained in another 28 rats perfused for a period shorter than 150 min (data not shown).

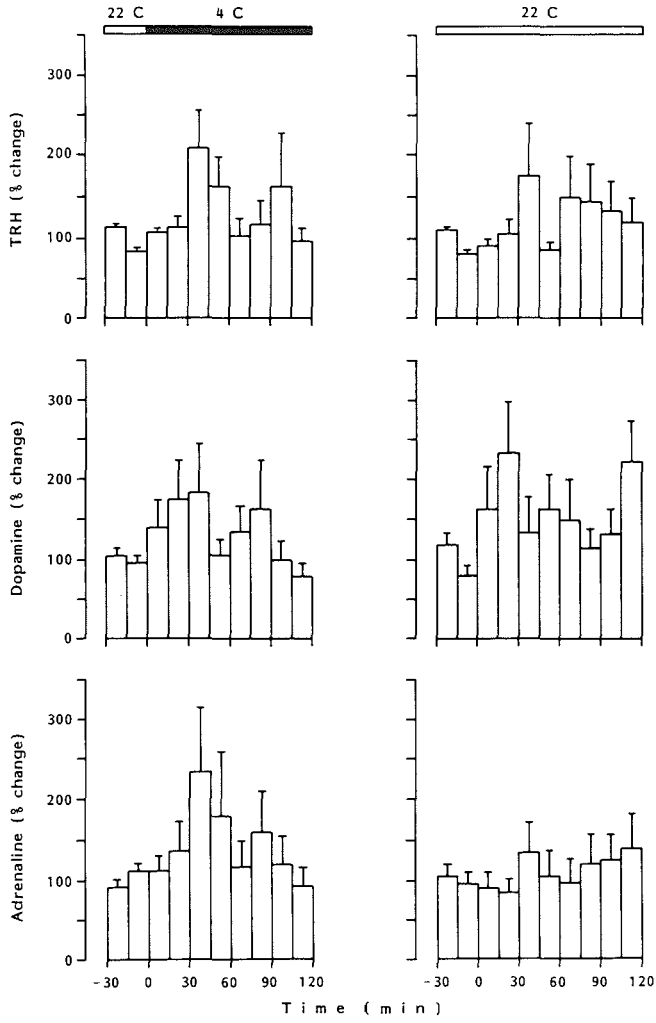

Figure 1: Effect of transfer from 20-22 C to 4 C on the in vivo release of TRH, dopamine and adrenaline estimated with push-pull perfusion of the hypothalamus in male rats. As controls, males not exposed to low temperatures were used. Because of the large variance in prestimulation values, data are presented as a percentage of the mean of the first two samples for each animal (n=11-12).

The basal release of TRH into the medium perfused through the MBH or AP was 5-6 pg/min in rats previously kept at 30 C (**Figure 2**). Transfer of these male rats from 30 C to 4 C increased the amount of TRH in push-pull perfusate of the MBH, but not in that of the AP, during the first 15 min of cold-exposure.

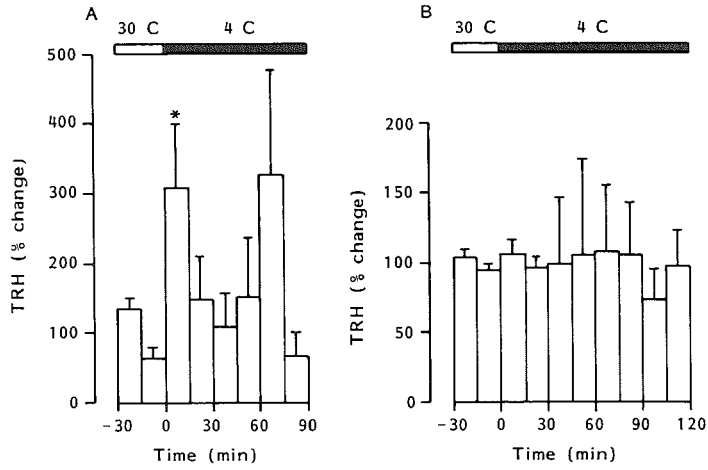

Figure 2: Effect of transfer from 30 C to 4 C on the in vivo release of TRH as estimated with push-pull perfusion of the mediobasal hypothalamus **(A)** or anterior pituitary gland **(B)** (n=6-7; *$p \leq 0.05$ vs 0-30 min).

DISCUSSION

The present study was concerned with the effect of cold exposure on the hypothalamic release of TRH, dopamine and adrenaline. In the literature, acute cold exposure has been reported to increase serum TSH within 30 min, while plasma T3 and T4 concentrations are increased within 1-2 h (1-5). In our study, transfer to a room kept at 4 C resulted in a small but significant increase in the plasma levels of TSH after 30 min in male rats previously kept at room temperature. Plasma T3 increased only after 4 h. Levels of T4 did not change during the 6 h cold exposure. In rats kept at 30 C for 3 weeks, exposure to 4 C induced a much greater increase in plasma TSH, and both plasma T3 and T4 rose significantly after 6h of cold exposure. It should be stressed, however, that basal and cold-induced levels of thyroid hormone in rats acclimatized at 30 C are lower than in rats previously kept at room temperature. Therefore, it is obvious

that the cold-induced rise in plasma T3 and T4 in rats previously acclimatized to 30 C is mainly due to the low thyroid hormone levels at 30 C rather than to the high levels at 4 C. It seems that acclimatization to 30 C enhances the cold-induced rise in plasma levels of T3, T4 and TSH.

Various studies report that the cold-induced rise in plasma TSH levels is TRH-dependent. Antiserum to TRH (6) completely abolishes the cold-induced TSH increase as do hypothalamic deafferentation (2) and paraventricular area lesions (5). Few studies report on in vivo release of TRH during cold. Guillaume et al. could not find an increased hypothalamic TRH release into hypophysial portal blood (15). However, this technique may have some serious drawbacks such as surgical stress and anesthesia that might affect the neuroendocrine response to cold. Evidence of a cold-associated rise in hypothalamic TRH release in freely moving, unanesthetized rats as determined with push-pull perfusion of the median eminence was provided before by Arancibia et al. (16-18). They observed a small but significant rise in hypothalamic TRH release 40 min after cold transfer (16). Unfortunately, it is not known to what extent thyroid function is activated under the conditions used in these studies, since plasma levels of T3, T4 and TSH were not reported. In our study, hypothalamic TRH release was significantly increased within 15 min after cold transfer, but only in rats that were previously acclimatized to 30 C. Indeed, in these rats the cold-induced activation of thyroid function was more pronounced than in rats acclimatized to room temperature. In rats transferred from room temperature to 4 C hypothalamic TRH release did not increase.

In a previous study we found that electrical stimulation of the paraventricular area raises TRH levels in push-pull perfusates of the MBH approximately to the same extent as in perfusates of the AP (24). Therefore, we concluded that push-pull perfusion of the AP can also be used to monitor hypothalamic TRH release. In the present study, however, perfusion of the AP did not reveal a cold-induced TRH rise, whereas perfusion of the MBH did. Until now, we do not know the reason for this discrepancy.

Interestingly, cold exposure profoundly augments hypothalamic content of adrenaline after 2-6 h. Hypothalamic content of TRH did not change in our animals, but studies that report on hypothalamic TRH content as an estimate of hypothalamic TRH release during various conditions

provide conflicting results (8-11). The rise in adrenaline content supports the concept that the neuroendocrine response to cold is mediated by catecholamines (30). Indeed, the cold-induced hypothalamic TRH release was found to be abolished by α-adrenergic antagonists (18) and by deafferentation of the MBH resulting in degeneration of noradrenergic nerve endings (30,31). However, in the present study hypothalamic release of adrenaline was not significantly affected by cold, although it tended to increase. Unfortunately, we do not have data on cold-associated hypothalamic adrenaline release in rats previously acclimatized to 30 C.

Plasma levels of PRL were found to increase 30 min after cold transfer, then slowly returning to basal values. These findings are in excellent agreement with other studies in which cold acutely stimulated PRL secretion within 5 min in the rat (3,32). Other workers, however, observed reduced PRL levels during cold in the rat (33,34) or in man (35). Since plasma PRL levels rapidly respond to stress (32,34,36,37) it is extremely important to control for animal handling and transfer. Therefore, the cold-associated PRL rise may be due to a nonspecific stress stimulus, rather than to a specific neuroendocrine response to cold. With regard to the cold-associated stimulation of thyroid function, this response seems specific, since stress is known to reduce rather than to increase TSH levels (37).

Release of PRL is thought to be controlled by PRL inhibiting and releasing factors (for review see Ref. 38). It is now established that dopamine is a PRL inhibiting factor, whereas the role of TRH as a physiological PRL releasing factor remains unsettled (23). Although it is tempting to conclude that the cold-induced PRL secretion is secundary to the augmented hypothalamic release of TRH, it has been reported that the effect of cold on PRL is not abolished by TRH antiserum (39). Moreover, in the present study both hypothalamic content and release of TRH and dopamine were not affected by cold. Presumably, vasoactive intestinal polypeptide (VIP) may also mediate PRL secretion during cold (40).

In conclusion, the cold-induced activation of the hypothalamo-pituitary-thyroid axis is enhanced in rats previously acclimatized to 30 C. In these rats, cold exposure increases hypothalamic TRH release within 15 min. The cold-associated rise in plasma PRL levels may be due to transfer stress, rather than to a specific neuroendocrine mechanism.

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CHAPTER IV.

THE ROLE OF TRH IN THE REGULATION OF PRL SECRETION.

Neuroendocrinology 48: 93–96 (1988)

A.

Effect of Suckling on the in vivo Release of Thyrotropin-Releasing Hormone, Dopamine and Adrenaline in the Lactating Rat

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Key Words. Dopamine · Prolactin · Thyrotropin-releasing hormone · Adrenaline · Suckling · Hypothalamus · Push-pull perfusion · Lactation

Abstract. The present study was concerned with the effect of suckling on the hypothalamic release of thyrotropin-releasing hormone (TRH), dopamine and adrenaline in lactating rats as estimated by push-pull perfusion of the median eminence-arcuate nucleus area. The push-pull cannula was implanted on day 15 of pregnancy. This surgery did not interfere with pregnancy, time of delivery or lactation. Push-pull perfusion was performed on day 8 or 14 of lactation and 30 out of 42 perfusions were successful. On the day of perfusion mothers and young were separated. Six hours later push-pull perfusion was begun and 6 samples at 15-min intervals were collected. In control animals, not allowed to nurse pups during perfusion, the release of TRH, dopamine and adrenaline did not change during the 90-min period. In experimental animals, reunited with their litter after 30 min of perfusion, the hypothalamic release of adrenaline did not change. However, both on day 8 and 14 suckling induced a 50% decrease in the release of dopamine ($p \leq 0.025$) which lasted for 15–30 min. Suckling on day 14 did not affect the concentration of TRH in the perfusate, but on day 8 the TRH output gradually decreased for 45 min after the onset of suckling.

Various hypothalamic hormones seem involved in the control of prolactin secretion [for reviews, see ref. 5, 14]. Suckling induces a rapid increase in the release of prolactin in lactating rats [2, 7, 23]. A similar response of prolactin can be achieved in anesthetized lactating rats by electrical stimulation of an isolated mammary nerve [4, 6, 16–19]. This stimulus led to higher hypophysial stalk plasma levels of thyrotropin-releasing hormone (TRH) and lowered those of dopamine for a short period of time [4, 6, 17–19]. This transient decline of dopamine release enhances the sensitivity of the pituitary gland to prolactin-releasing factors like TRH [6–10, 19].

Although mammary nerve stimulation probably can be viewed as a simulated suckling stimulus [4, 6, 16], it is not certain that the changes in TRH and dopamine release cited above represent physiological events. Firstly, the procedure to collect hypophysial stalk blood likely interferes with normal physiological hormone responses, since anesthetics are used and the connection between hypothalamus and pituitary gland is interrupted. Secondly, most if not all mammary nerves are stimulated by the pups during normal suckling whereas with electrical stimulation only one mammary nerve is excitated. Push-pull perfusion of the median eminence-arcuate nucleus area seems to overcome these problems [24], since this technique measures the release of hypothalamic hormones in conscious rats nursing their pups. Therefore, we used this technique to measure the in vivo release of TRH, dopamine and adrenaline before and during suckling from the mediobasal hypothalamus of lactating rats.

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Materials and Methods

Animals

Locally bred (RxU) F_1 hybrid rats, weighing 200–250 g, were used. They were housed under controlled conditions (lights on 05.00–19.00 h; 20–24 °C) and had free access to food and water.

Experimental Procedures

Female rats were caged with male rats of proven fertility. After mating, as established by the presence of a vaginal plug, they were caged individually and a push-pull cannula was implanted 15 days later in the mediobasal hypothalamus as described previously [24].

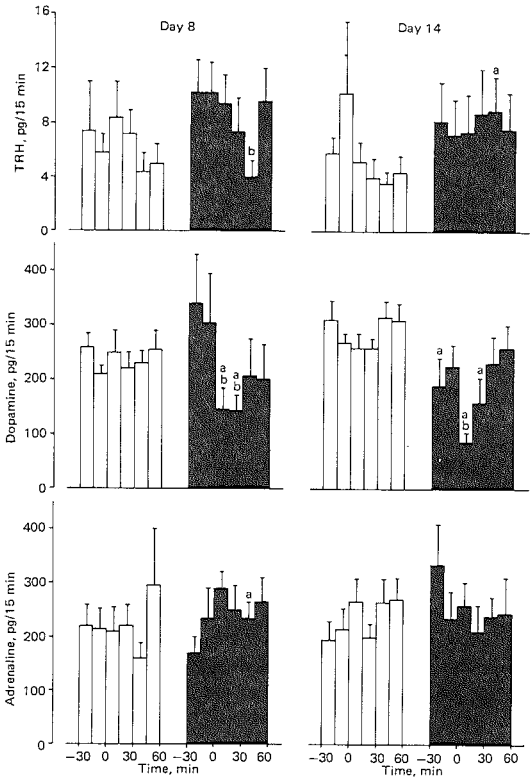

Fig. 1. In vivo TRH, dopamine and adrenaline output from the medial basal hypothalamus of lactating rats at 15-min intervals during a period of 90 min. The open bars represent control rats which were not allowed to nurse their young during perfusion of the median eminence-arcuate nucleus area. The black bars indicate the data from rats reunited with their pups (at 0 min) after 6 h separation. The results are given as means ± SEM of 9–10 rats per group. a = Significant differences between controls and experimental rats; b = values significantly different from those before introduction of the pups (time –30 to 0 min).

After cannulation rats were handled daily and rats showing signs of infection, continued weight loss or any other impairment were not used for the perfusion experiments. The surgery did not affect the time of delivery (day of parturition is day 1 of lactation) and normal lactation. Litters were reduced to 8–10 young on day 2. The mothers were used for perfusion on day 8 or 14 of lactation, after 6 h of separation from their litter. Artificial cerebrospinal fluid [24] was delivered at a rate of 20 µl/min to the push cannula and perfusate was collected continuously into 1.5-ml tubes kept in melting ice. Every 15 min a new sample was begun; routinely the first fraction was disregarded. After 30 min of perfusion, mother and pups were reunited and perfusion continued for another 60 min. Control animals were perfused for 90 min without nursing pups. Data were not used when during perfusion bubbles appeared in the pull tubing, indicating occlusion at the tip of the cannula (8 out of 42) or when the mother did not nurse the litter (1 animal). Each sample was divided into two aliquots: 200 µl for TRH and 100 µl for measurement of dopamine. To the latter aliquot, 10 µl 1.0 *M* $HClO_4$ was added. All samples were stored at –18 °C immediately after collection until analyzed. After weaning the young on day 22 of lactation, the mother was killed by decapitation, the push-pull cannula removed and the brain placed in Bouin-Hollande fixative. Serial 10-µm sections were cut along the plane of the cannula trace and stained with cresyl violet. If the cannula placement proved to be outside the median eminence-arcuate nucleus area the data were disregarded (3 out of 42).

Hormone Determinations

Due to the limited amount of sample and the low level of TRH, singular determinations were done in 200-µl samples using a previously described radioimmunoassay [22]. The detection limit (defined as the amount of hormone that reduces binding to 90% of that occurring in the absence of unlabeled hormone) of this assay is 2–3 pg, and the intra- and interassay coefficients of variation are between 4 and 10%. All samples were run in one assay. Dopamine and adrenaline were determined with a high-pressure liquid chromatographic-electrochemical method [6, 24]. The minimal detectable amount was 15 pg for dopamine and 10 pg for adrenaline (signal to noise ratio of 2).

Statistical Analysis

Results are presented as means ± SEM. Analysis of variance followed by Duncan's multiple range tests were used to establish significant differences. Differences were considered to be significant at $p \leq 0.05$.

Results

The results are presented in figure 1. Both on day 8 and 14 the in vivo release of TRH, dopamine and adrenaline did not change significantly during the period of observation in animals which were not allowed to nurse their litter. When compared to the period before introduction of the pups (–30 to 0 min), suckling on day 8 of lactation led to a gradual decrease in the release of TRH between 0 and 45 min, but was without effect on the TRH output on day 14 of lactation. Suckling caused a decrease in dopamine release for 15–30 min both on day 8 and on day 14. Adrenaline output did not change significantly, although suckling on day 8 of lactation tended to increase its secretion.

Discussion

Push-pull perfusion of the median eminence-arcuate nucleus area was used to study the effect of suckling on the

hypothalamic release of TRH, dopamine and adrenaline. Successful perfusion was achieved in approximately 70% of the rats which is in agreement with a previous report [15]. Our study also showed that cannulation and push-pull perfusion do not interfere with pregnancy, parturition and lactation.

Suckling is known to stimulate prolactin release within 15 min [2, 7, 23]. In the present study it was found that hypothalamic dopamine release decreased very rapidly after the onset of suckling both on day 8 and 14 of lactation. This transient decline lasted for 15–30 min. These results are in excellent agreement with those obtained with electrical stimulation of an isolated mammary nerve of urethane-anesthetized lactating rats: in these studies a transient decline of dopamine was found in hypophysial stalk plasma [4, 6, 20] and in the median eminence [17, 18].

It is believed that prolactin release induced by suckling consists of 2 phases: a fast depletion-transformation phase controlled by dopamine freeing intracellular prolactin to be secreted, and a steady release phase controlled by prolactin-releasing factors which induce the secretion of hypophysial prolactin [5–10, 18, 19]. Only after initiating the depletion phase by a short period of suckling [7–10] or by lowering dopamine levels artificially by α-methyl-*p*-tyrosine [6] can prolactin-releasing factors stimulate prolactin release. Although this working hypothesis on the control of prolactin secretion induced by suckling was derived from indirect evidence [7–10] or from anesthetized rats [4, 6, 17, 18], our present results strongly reaffirm that dopamine is important for suckling-induced prolactin release.

The hypothalamic release of adrenaline was found to remain primarily constant during the period of observation, although suckling on day 8 of lactation tended to increase its release. Adrenaline concentration is high in certain nuclei of the hypothalamus [11], particularly in the paraventricular nucleus where it may be involved in the control of oxytocin secretion. It is found in low concentrations in the arcuate nucleus [11]. The role of adrenaline in prolactin release is uncertain: it was found to have no [12], biphasic [13] or inhibiting [21] effects on prolactin secretion in vivo.

In a previous study [6] in urethane-anesthetized rats we found that the mean release of TRH into hypophysial portal blood increased by 65% after electrical stimulation of an isolated mammary nerve. In the present study, however, no significant increase in TRH output was observed in the conscious, lactating rats during suckling. In fact, on day 8 of lactation we observed a gradual decline of TRH levels in the perfusate of the medial basal hypothalamus. This finding may imply that the observed increase in TRH after mammary nerve stimulation in anesthetized animals [6] represents a nonphysiological phenomenon or that the measurement of TRH in push-pull perfusates of the median eminence-arcuate nucleus area is not a suitable means to monitor hypothalamic release of TRH.

Evidence has been presented [24] that push-pull perfusion is a valid method to study the release of dopamine, as indicated by the good correlation between dopamine levels in the perfusates and those in hypophysial portal blood. This is supported by the present study demonstrating a suckling-induced decrease in dopamine output similar to what has previously been observed in hypophysial portal blood of anesthetized rats [6]. However, basal TRH release as measured with push-pull perfusion (15–50 pg/h) is much lower than what would be expected from extrapolation of the release of TRH into hypophysial portal blood [ref. 6: 1,000–2,500 pg/h], even when it is taken into account that only approximately 20% of the median eminence-arcuate nucleus is perfused during push-pull perfusion [24]. Degradation of TRH in the push-pull perfusate cannot be responsible for these low levels of TRH since the recovery of synthetic TRH added to the perfusion medium was found to be at least 95% following perfusion [24; J.M.M. Rondeel, unpubl. results].

One could hypothesize that if very little of synthetic TRH added to the perfusate diffuses into the medial basal hypothalamus, neither will endogenous TRH in medial basal hypothalamus diffuse into the perfusate. Consequently, measured release of TRH will be low. Arancibia et al. [1] used the technique of push-pull perfusion of the median eminence and found a small, short-lasting increase of TRH output from the medial basal hypothalamus during cold stimulation, thereby confirming the importance of TRH in cold-induced release of TSH and the validity of the push-pull perfusion for the study of hypothalamic TRH release. However, also in the latter study the levels of TRH in the push-pull perfusate were low compared with the levels of TRH found in hypophysial portal blood [6].

An increased hypothalamic release of luteinizing-hormone-releasing hormone has been found after castration using push-pull perfusion of the anterior pituitary gland but not during perfusion of the medial basal hypothalamus [3]. The method of push-pull perfusion of the anterior pituitary gland was also used to study basal hypothalamic release of TRH but the results were not different from those obtained during perfusion of the medial basal hypothalamus [6–10 pg TRH/15 min; J.M.M. Rondeel, unpubl. results]. Thus, the low basal TRH release determined by push-pull perfusion as well as the lack of stimulation by a suckling stimulus contrast with our previous findings of TRH in hypophysial portal blood. Possible explanations for this discrepancy are that (1) TRH released into hypophysial portal blood does not diffuse into the tissue compartment that is perfused (membrane impermeability), and (2) insertion of the push-pull cannula may disrupt nerve fibers projecting from the TRH-producing cell bodies in the paraventricular area to the median eminence.

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Hypothyroidism May Account for Reduced Prolactin Secretion in Lactating Rats Bearing Paraventricular Area Lesions

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ABSTRACT. Lesions in the paraventricular area (PVA) of lactating rats have been found to inhibit PRL release. We have examined whether this reduced PRL release is due to hypothyroidism resulting from destruction of the PVA. Rats were made hypothyroid by thyroidectomy on day 15 of pregnancy or by methimazole treatment from the day of parturition. Electrolytic lesions were placed bilaterally in the PVA on day 15 of pregnancy. The following variables were studied: weight gain of the pups, nursing behavior, thyroid status, and release of PRL. The treatments did not affect the time the mothers spent with the pups but reduced the daily weight gain of the pups. Rats with PVA lesions had reduced PRL and TSH levels during lactation compared with controls. Suckling-induced PRL release after 6 h of separation of mothers and pups was less in PVA-lesioned rats than in controls, but T_4-treatment did overcome this blunted response in rats with lesions. Levels of T_3 and T_4 in PVA-lesioned rats were lower than those in controls. In rats made hypothyroid by thyroidectomy or treatment with methimazole, PRL levels were lower and TSH levels higher than those in euthyroid mothers on days 8, 15, and 22 of lactation. Suckling after 6 h of separation of pups and mothers raised PRL levels both in control and methimazole-treated rats, but in the latter animals the response was blunted. It is suggested that the reduced PRL release in lactating rats with PVA lesions could be due to hypothyroidism resulting from these lesions. (*Endocrinology* **125:** 0612–0617, 1989)

MULTIPLE hypothalamic hormones appear to be involved in the control of PRL release during lactation. Dopamine has been identified as a major inhibiting factor of PRL release (for reviews see Refs. 1–4), and also GnRH-associated peptide may function as an inhibitor of PRL release (5). Various hypothalamic substances have been implicated as PRL-releasing factor, including TRH, polypeptide histidine isoleucine, and vasoactive intestinal peptide (6–14). In addition, factors with PRL-releasing activity have been isolated from the posterior pituitary (15, 16) and the liver (17).

The paraventricular nuclei synthesize a number of hypothalamic PRL-releasing factors, and destruction of these nuclei interferes with normal lactation (18) and PRL release (19, 20). Such lesions, however, also induce hypothyroidism by reducing the TRH content in median eminence (21) and hypophysial portal blood (22), and it has been reported that hypothyroidism increases dopamine release into hypophysial stalk blood (22, 23). Therefore, the abnormal PRL response in lactating rats with lesions in the paraventricular area (PVA) could be a consequence of the hypothyroid status of these animals. This was tested in this study by determining the effect of hypothyroidism on PRL release in lactating rats.

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Materials and Methods

Animals

Locally bred female (RxU) F_1 hooded rats, weighing 200–280 g, were used. They were housed in temperature-controlled rooms (22–24 C) with a light cycle of 14-h light and 10-h darkness, and had free access to food and water. The female rats were mated by males of proven fertility. Near the end of pregnancy the females were housed individually, and only those dams that gave birth to at least 8 pups were included in the experiments. The litters were adjusted to 10 pups on day 2 of lactation (day 1 is day of parturition). The weight of each litter was recorded daily. Since experimental interference could affect the growth of the pups, each day the litters were exchanged between control and experimental animals. Furthermore, mother-young interaction was determined with an automatic device (24) that records the time a mother spent on the nest with the pups. This method provides a good estimate for the time the mother nurses the pups up to day 16 of lactation (W. J. de Greef, unpublished data).

Experimental protocols

Hypothyroidism, litter growth, nursing behavior, and PRL release. Hypothyroidism was induced with methimazole, thyroidectomy, or thyroidectomy combined with methimazole treatment. Methimazole was added to drinking water (0.05%, wt/vol) and given from day 1 of lactation. Untreated lactating rats were used as controls. Thyroidectomy was carried out on day 15 of pregnancy, and ether was used as anesthetic; care was taken not to damage the nervus recurrens or parathyroids. Control rats underwent a similar operation, but the thyroid glands were not removed. The operations had no effect on time of delivery or on litter size. Thyroidectomized and sham-thyroidectomized rats received calcium chloride (1%, wt/vol) in their drinking water. In one experiment blood from the orbital plexus of lightly ether-anesthetized mothers was taken on days 8, 15, and 22 of lactation, and approximately 1.5 ml blood was obtained within 1 min after removal of the rat from its cage. In another experiment, lactating rats were anesthetized with ether on day 11, and a polyethylene cannula was placed into the jugular vein (7). Two days later mothers and pups were separated, and 6 h later a blood sample (1.0 ml) was taken from the jugular vein cannula. Then mother and litter were reunited, and all mothers were suckled by the pups within 5 min. Additional blood samples (0.6 ml) were taken at 15, 30, and 60 min after reintroduction of the pups. Sera were stored at −20 C until measurement of PRL, TSH, T_3, and T_4.

PVA lesions, litter growth, nursing behavior, and PRL release. On days 15–16 of pregnancy, rats were anesthetized with ether and placed in a stereotaxic apparatus to make bilateral electrolytic lesions in the PVA (22). The stainless steel electrode tip was placed 6.4 mm anterior and 3.5 mm dorsal from the interaural line, and 0.7 mm from the midline (25), and anodal current (1 mA) was applied for 20 sec. Control pregnant rats were subjected to similar surgery, but no current was passed through the electrode. These operations had no effect on time of delivery or litter size, but some rats refused to nurse their pups and were excluded from the experiments. Some rats with PVA lesions, received twice daily T_4 (0.5 μg sc/100 g BW) on days 1–16 of lactation. In one experiment blood samples were taken from the orbital plexus on days 8 and 15 of lactation, whereas in another experiment blood was sampled on day 13 of lactation from a jugular vein cannula at 0, 15, 30, and 60 min of suckling after separation of mothers and pups for 6 h (see above for details). At day 22 of lactation the mothers were killed by decapitation, trunk blood was collected, and the brains were immersed in Bouin's fixative. The part including the paraventricular nuclei was embedded in paraffin wax, and serial 10-μm sections were stained with hematoxylin and eosin. The sections were studied microscopically to determine the site and extent of the lesions. Only rats in which the lesions were correctly placed were included (in or just caudal to the paraventricular nuclei; see Ref. 22). The sera were stored at −20 C until assayed for PRL, TSH, T_3, and T_4.

Hormone determinations

Levels of PRL were determined by RIA (26) using NIADDK reference preparation (RP)-1 as standard. Levels of TSH were measured by RIA using materials and protocols supplied by the NIADDK, and TSH-RP-2 was used as standard. Concentrations of T_3 and T_4 were estimated in unextracted serum by specific RIAs. Detection limits of the assays, defined as the amount of hormone that reduces binding to 90% of that occurring in the absence of unlabeled hormone, were 2.5 ng/ml PRL, 0.4 ng/ml TSH, 2 nmol/liter T_4, and 0.2 nmol/liter T_3. Intra- and interassay coefficients of variation for the assays were between 3 and 12%. Samples from each experiment were run in duplicate in the same assay.

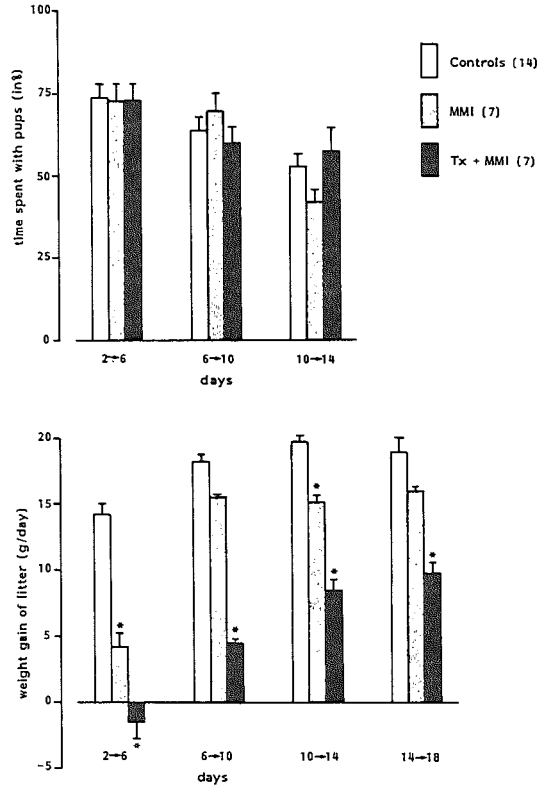

FIG. 1. Lactation behavior in rats made hypothyroid with methimazole (MMI) or thyroidectomy and methimazole (Tx + MMI). Untreated lactating rats served as controls. Observations were made on the time the mothers spent with the litter and the weight gain of the litter. The results are presented as means ± SEM, and the number of rats per group is in *parentheses*. *, $P < 0.05$ compared with controls.

Statistical analysis

Results are given as means ± SEM. Results were subjected to analysis of variance. When significant overall effects were obtained, comparisons between groups were made using Duncan's multiple range tests. Differences were considered to be significant at $P < 0.05$.

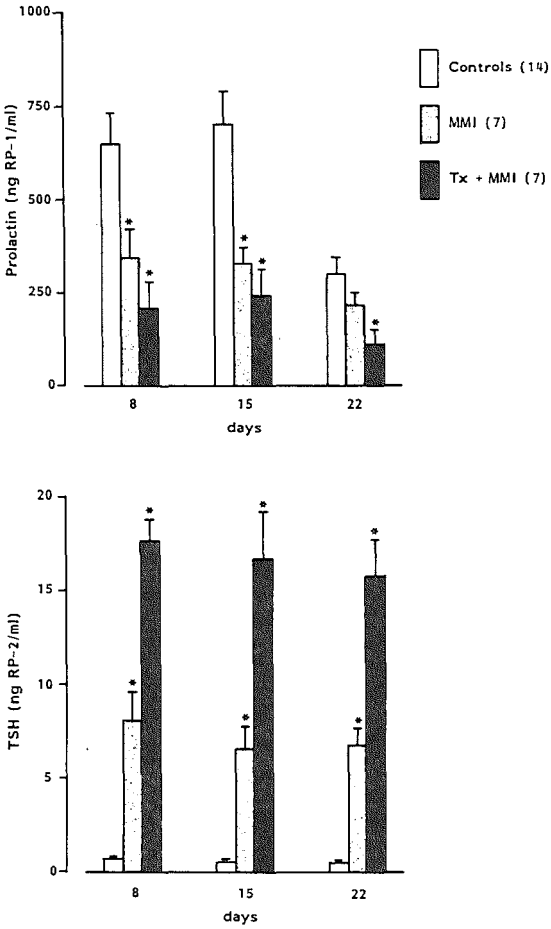

FIG. 2. Serum levels (means ± SEM) of PRL and TSH during lactation in untreated control rats and in rats made hypothyroid with methimazole (MMI) or thyroidectomy and methimazole (Tx + MMI). Number of rats in *parentheses*. *, $P < 0.05$ compared with controls.

Results

Hypothyroidism, litter growth, nursing behavior, and PRL release

Litters nursed by euthyroid rats gained more weight than those reared by hypothyroid mothers, although the time the mothers spent with the pups was not altered by the treatments (Fig. 1). Mean levels of TSH in control rats were below 1 ng RP-2/ml on days 8, 15, and 22 of lactation but were higher in hypothyroid rats (Fig. 2). Serum levels of PRL were lower in methimazole-treated intact or thyroidectomized mothers than in euthyroid mothers (Fig. 2). Thyroidectomized mothers not treated

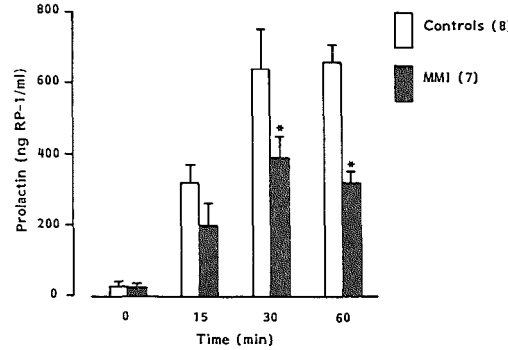

FIG. 3. Suckling-induced release of PRL on day 13 of lactation after separation of mothers and pups for 6 h in lactating rats made hypothyroid with methimazole (MMI) and untreated lactating control rats. The results are given as means ± SEM, and the number of rats is in *parentheses*. *, $P < 0.05$ compared with controls.

with methimazole (n = 5) had also lower PRL levels than controls (n = 6) during lactation (day 8, 408 ± 81 *vs.* 654 ± 96; day 15, 456 ± 35 *vs.* 688 ± 101; day 22, 238 ± 88 *vs.* 306 ± 87 ng RP-1/ml).

Suckling after separation for 6 h raised PRL levels both in control and methimazole-treated rats, but in the latter animals the response was significantly less (Fig. 3). Levels of T_3 (nanomoles per liter), T_4 (nanomoles per liter), and TSH (nanograms per ml) just before reintroduction of the pups were 1.27 ± 0.14, 13.4 ± 2.1, and 1.16 ± 0.24 in controls and 0.41 ± 0.05, 5.3 ± 0.9, and 9.7 ± 1.22 in methimazole-treated rats.

PVA lesions, litter growth, nursing behavior, and PRL releases

The daily weight gain of pups nursed by mothers with bilateral PVA lesions was less than that of sham-lesioned rats during the first week of lactation. Treatment with a substitution dose of T_4 prevented the effect of the lesions on litter growth (Fig. 4). The time the mothers spent with the pups was not affected by the treatments (Fig. 4). Animals with lesions placed in the PVA had reduced serum levels of PRL and TSH when compared with controls (Fig. 5). Suckling-induced PRL release after temporary separation of pups from the mothers was less in rats with PVA lesions than in controls, but treatment with T_4 did overcome this blunted response (Fig. 6). Levels of T_3, T_4, and TSH just before reintroduction of the pups are presented in Table 1.

Discussion

The present study confirms previous findings by Kiss *et al.* (19) that ablation of the PVA reduced, but did not prevent, PRL release in lactating rats. Treatment with

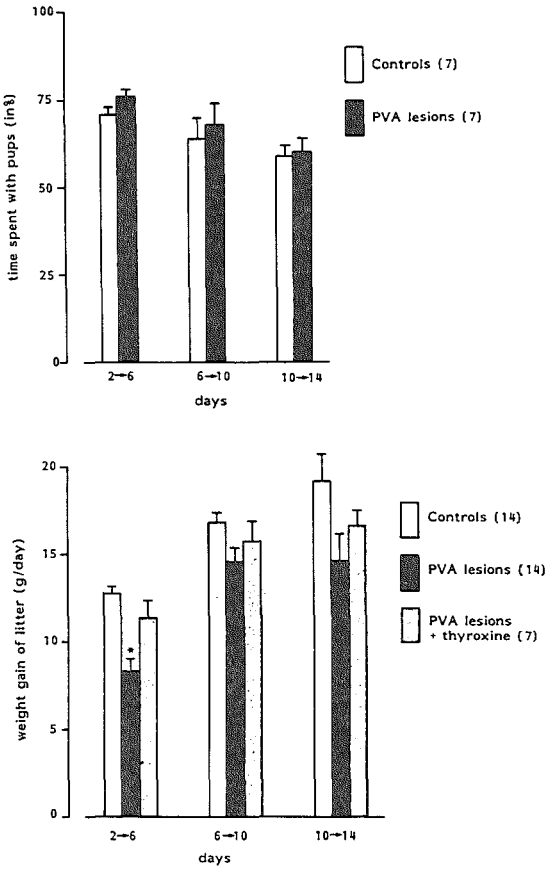

FIG. 4. Lactation behavior in rats with PVA lesions or sham lesions. The time the mothers spent with pups and weight gain of the litter were recorded. Some rats with PVA lesions had been treated twice daily with T_4 (0.5 μg/100 g BW) from day 1 of lactation. Results are presented as means ± SEM, and the number of rats is given in *parentheses*. *, $P < 0.05$ compared with controls.

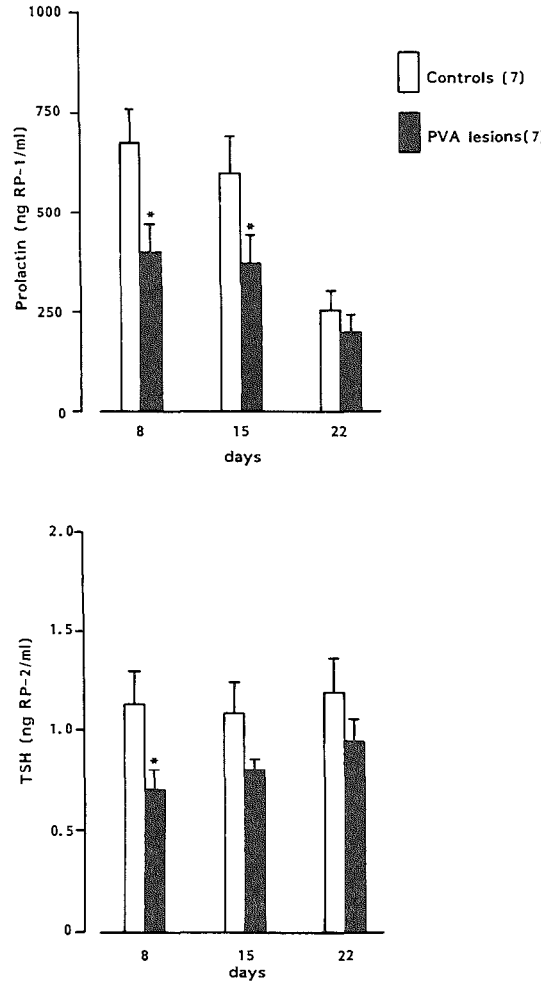

FIG. 5. Serum PRL and TSH levels (means ± SEM) on days 8, 15, and 22 of lactation in rats bearing bilateral PVA lesions and in sham-operated control lactating rats. Number of rats is in *parentheses*. *, $P < 0.05$ compared with controls

a substitution dose of T_4 did overcome the effect of the PVA lesions on suckling-induced PRL release. Furthermore, hypothyroidism appeared to lower PRL levels during lactation and to diminish suckling-induced PRL release. Thus, these findings suggest that the reduced PRL release in rats with PVA lesions (Refs. 19 and 20 and present study) could be due to the hypothyroid status of these animals rather than preventing the release of putative PRL-releasing factors from the paraventricular nuclei into hypophysial stalk blood. Our study, however, does not exclude that diminished release of PRL-releasing factors induced by PVA lesions may also account for the reduced PRL secretion during lactation in rats bearing PVA lesions.

It has been reported that the release of dopamine into hypophysial portal blood is enhanced in hypothyroid rats and in rats bearing bilateral lesions in the PVA (22, 23). Since dopamine is a major factor for the inhibition of PRL release (1–4), this increased dopamine secretion into hypophysial portal blood could be the cause of the reduced PRL levels in hypothyroid lactating rats.

An important prerequisite for the study of PRL release in lactating rats is that the experimental conditions do not interfere with normal suckling and nursing behavior. Therefore, the weight gain of the litters and the time

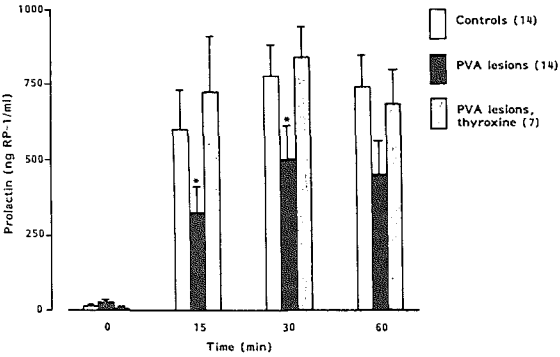

FIG. 6. Suckling-induced release of PRL on day 13 of lactation in rats with PVA lesions or sham lesions. Mothers and pups had been separated for 6 h. A number of rats with PVA lesions were treated daily with 1 μg T_4/100 g BW from day 1 of lactation. Number of rats per group in parentheses. *, $P < 0.05$ compared with controls.

TABLE 1. Levels of T_3, T_4, and TSH before introduction of the pups on day 13 of lactation in rats with PVA lesions or sham-lesions

Treatment	T_3 (nmol/liter)	T_4 (nmol/liter)	TSH (ng RP-2/ml)
Sham-operation (14)	1.30 ± 0.11	15.9 ± 3.4	0.97 ± 0.11
PVA-lesions (14)	0.74 ± 0.06[a]	7.9 ± 0.8[a]	0.76 ± 0.04[a]
PVA-lesions, thyroxine (7)	1.13 ± 0.20	28.8 ± 1.7[a]	0.67 ± 0.04[a]

Mothers and pups had been separated for 6 h. A number of rats with PVA lesions were treated daily with 1 μg T_4/100 g BW from day 1 of lactation. Number of rats is in parentheses.

[a] $P < 0.05$ compared with controls.

that the mothers spent with the pups were recorded in the present study. The latter variable was not affected by the experimental conditions, which suggests that nursing behavior was unaltered. However, litters of hypothyroid rats gained less weight than euthyroid rats, illustrating that thyroid hormones are essential for normal lactation (27). Also litters nursed by PVA-lesioned mothers gained less weight than those nursed by sham-lesioned mothers, although the difference was less pronounced than in rats made hypothyroid by thyroidectomy or methimazole treatment. This may be due to the severity of hypothyroidism induced by the various experimental treatments (Ref. 22 and present study).

The effect of PVA lesions on PRL release during lactation could thus be explained by the hypothyroid status of these animals. Although the levels of T_4 and T_3 are 50% lower in PVA-lesioned than in sham-lesioned rats, T_4 levels are already low in control lactating rats (10–20 nmol/liter; Ref. 14 and present study) compared with nonlactating rats (40–60 nmol/liter; Ref. 22 and our unpublished data). Similar observations have been made previously (28, 29) and could be explained by loss of thyroid hormones in milk (29, 30).

In conclusion, evidence has been presented that the diminished PRL release in lactating rats after placement of electrolytic PVA lesions can be explained by the hypothyroidism associated with lesioning the PVA. It is suggested that the increased secretion of dopamine into hypophysial portal blood related to hypothyroidism (22, 23) is part of the mechanism involved in the reduced PRL release in animals with bilateral PVA lesions.

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The authors wish to thank Mr. H. van de Giessen for construction of the automatic device for recording mother-young interactions during lactation, and the NIADDK for materials used in the RIAs. The assistance of Mr. W. Klootwijk and Mr. P. Uyterlinden is greatly appreciated.

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SUMMARY.

The tripeptide thyrotropin-releasing hormone (TRH) has diverse endocrine and non-endocrine functions and is ubiquitously located in and outside the central nervous system. This diversity stems from its two distinct functions: on the one hand TRH serves a neuromodulating role, affecting several organ systems; on the other hand TRH acts as a classical hypothalamic releasing hormone, affecting the hypophysial release of TSH and PRL. The latter effect is dealt with in this thesis. The endocrine effects of TRH are dependent on its bioavailability at its receptor on the thyrotroph and lactotroph. Numerous processes may influence this bioavailability, such as synthesis of TRH, its secretion and transport via axones and blood vessels to the pituitary gland and its degradation in plasma or tissue. However, regulation of the hypothalamic secretion of TRH seems a major mechanism in the control of the endocrine effects of TRH. This thesis therefore deals with the regulation of hypothalamic TRH release in relation to its two main endocrine functions, i.e. stimulation of TSH and PRL secretion. In general, three questions were asked: **1)** how can the hypothalamic release of TRH reliably be measured? **2)** what is the role of TRH in the regulation of TSH secretion? **3)** what is the role of TRH in the regulation of PRL secretion?

Numerous in vivo and in vitro techniques have been used to determine hypothalamic TRH release. In **Chapter II** the in vivo measurement of hypothalamic TRH release is discussed. Many investigators measured concentrations of immunoreactive TRH (IR-TRH) in peripheral blood as an estimate of hypothalamic TRH release. Indeed, we found that conditions known to affect plasma TSH levels (such as starvation, hyperthyroidism and hypothalamic lesions) also affect IR-TRH concentrations in peripheral blood **(Chapter IIB)**. However, levels of IR-TRH in peripheral blood are 1) extremely low, 2) only partly derived from the hypothalamus, and 3) only partly identical to authentic TRH. Moreover, it is possible that changes in peripheral blood levels of IR-TRH are due to an altered metabolic clearance of TRH in plasma, rather than to an altered hypothalamic TRH release per se.

Two techniques specifically measure the hypothalamic release of TRH: the collection of hypophysial portal blood, and push-pull perfusion of the

median eminence or the anterior pituitary (Chapter IIA). The stressful and damaging surgery and the necessity of anesthesia in the former technique may influence physiological homeostatic mechanisms. During push-pull perfusion, however, hypothalamic TRH release is measured in freely moving, unanesthetized rats and, therefore, the disadvantages of the collection of stalk blood are overcome. Electrical stimulation of the paraventricular area in the hypothalamus rises TRH levels in push-pull perfusates of the median eminence or the anterior pituitary to the same extent as levels in portal blood. However, TRH levels in push-pull perfusates are very low and variable, whereas TRH concentrations in stalk blood are high and more readily affected by conditions such as suckling. We do not know what causes these discrepancies between both techniques, and until now no gold standard technique seems available for the measurement of hypothalamic TRH release.

In general, therefore, the drawbacks of these techniques should be taken into consideration if data are to be interpreted.

In **Chapter III** the role of TRH in the regulation of TSH secretion is discussed. Numerous conditions affect plasma TSH levels. The best known are altered thyroid status, and the body's adaptive response to disease ("the low T3 syndrome") and to cold.

Thyroid status may affect plasma TSH levels partly by influencing hypothalamic TRH release. Indeed, we showed that hypothalamic TRH release into stalk blood in vivo and from hypothalamic fragments in vitro is reduced by 30-45% in hyperthyroidism **(Chapter IIIA,B)**. In primary hypothyroidism, however, hypothalamic TRH release is not affected, whether determined in vivo by collecting hypophysial portal blood, or in vitro from hypothalamic fragments. Only in hypothyroidism induced by propylthiouracil could we find a minimally elevated hypothalamic TRH release in vivo. The physiological meaning of this finding, however, remains speculative. Bilateral electrolytic lesions in the paraventricular area reduce plasma TSH levels with 70% in euthyroid rats, but only with 30% in hypothyroid rats. Therefore, we may conclude that TRH plays a minor role in the hypersecretion of TSH during primary hypothyroidism. Moreover, the main feedback of thyroid hormone is exerted at the pituitary level.

The low T3 syndrome is seen in non-thyroidal illness and is characterized by low circulating levels of thyroid hormone. It is considered an adaptive hypothyroidism by which the body responds to disease

in order to conserve energy. Since plasma TSH levels are inappropriately low in relation to the low levels of thyroid hormone, central mechanisms may play a role in the origin of this syndrome. Starvation in the rat is considered a model for the low T3 syndrome. It has pronounced effects on plasma levels of T3, T4 and TSH, which are all reduced, concomitant with decreased PRL levels **(Chapter IIIC)**. Also plasma binding of T4 is decreased in starvation presumably by the increased levels of free fatty acids during fasting. Interestingly, these changes are reversed during refeeding. Plasma TSH levels even rise above normal values during refeeding. Despite these profound changes in plasma T3, T4 and TSH, in vitro hypothalamic secretion of TRH is not affected by starvation or refeeding.

Also in streptozotocin-induced diabetes mellitus hypothyroidism is seen, and plasma PRL levels are reduced **(Chapter IIID)**. In vitro hypothalamic release of TRH is significantly reduced in diabetic rats. The effect of diabetes mellitus on TRH secretion seems specific, since weight loss or increasing the in vitro glucose concentration from 10 to 30 mM do not affect hypothalamic TRH release. Interestingly, in the absence of glucose hypothalamic TRH release is increased two-fold. Therefore, glucose levels play an important role in the regulation of hypothalamic TRH release.

Whereas the low T3 syndrome represents an adaptive hypothyroidism by which the body responds to disease, stimulation of thyroid function during cold exposure may represent an adaptive hyperthyroidism. We showed that the cold-associated rise in plasma TSH is enhanced if rats are previously acclimatized to 30 C **(Chapter IIIE)**. In these rats hypothalamic TRH release as determined with push-pull perfusion of the median eminence increases significantly during the first 15 min of cold exposure.

In conclusion, hypothalamic release of TRH is barely affected by thyroid status. In hypothyroidism TRH does not seem of substantial importance for the hypersecretion of TSH. Moreover, in hyperthyroidism hypothalamic TRH release is only moderately reduced. Therefore, the effect of altered thyroid status on plasma TSH is mainly due to a direct action of thyroid hormone at the pituitary level. With regard to the low T3 syndrome and cold exposure there are indications that TRH plays a role in the body's adaptive response to disease (at least in streptozotocin-induced diabetes mellitus in the rat) and to cold.

Chapter IV deals with the role of TRH in the regulation of PRL

secretion. Whereas dopamine is considered an established PRL inhibiting factor (PIF), the role of TRH in the suckling-induced PRL secretion is less clear. Some studies suggest that TRH is a physiological PRL releasing factor (PRF) and that hypothalamic TRH release into hypohysial stalk blood is increased during a simulated suckling stimulus of anesthetized rats. Suckling did, however, not increase TRH release during push-pull perfusion of the median eminence in freely moving, conscious rats **(Chapter IVA)**. The release of dopamine, however, was reduced transiently within 15-30 min after the beginning of suckling, thus confirming its role as a PIF.

Lesions in the paraventricular area effectively reduce the hypothalamic release of TRH and cause tertiary hypothyroidism. The PRL response to suckling is also reduced in lesioned rats **(Chapter IVB)**. Substitution with thyroxine in order to restore the hypothyroidism also reverses the blunted PRL response to suckling. Therefore, the reduced PRL release in lactating rats with hypothalamic lesions seems due to hypothyroidism, rather than to a reduced hypothalamic TRH release.

In conclusion, these studies do not confirm the role of TRH as a physiological PRF.

SAMENVATTING.

Het tripeptide "thyrotropin-releasing hormone" (TRH) heeft verscheidene hormonale en niet-hormonale eigenschappen en wordt zowel in als buiten het centrale zenuwstelsel aangetroffen. Deze diversiteit wordt toegeschreven aan twee belangrijke eigenschappen van TRH: aan de ene kant heeft TRH een neuromodulerende rol en beïnvloedt het verschillende orgaansystemen; aan de andere kant is TRH het klassieke hypothalame "releasing hormone" van de hypofyse hormonen TSH en PRL. Deze laatste functie is het onderwerp van dit proefschrift. Het endocriene effect van TRH wordt bepaald door diens beschikbaarheid aan de receptor op de thyreotrofe en lactotrofe cel van de hypofyse. Deze beschikbaarheid wordt bepaald door de aanmaak van TRH, diens afgifte en transport via zenuwvezels en bloedvaten naar de hypofyse en door de afbraak van TRH in bloed en in weefsel. Regulatie van de hypothalame afgifte van TRH lijkt echter het belangrijkste mechanisme waarmee het lichaam de endocriene effecten van TRH kan besturen. Dit proefschrift beschrijft hoe het lichaam de hypothalame afgifte van TRH reguleert onder omstandigheden waarin de TSH en de PRL afgifte veranderen. Drie vragen kwamen aan bod:

1) hoe kan de hypothalame TRH afgifte betrouwbaar gemeten worden?
2) welke rol speelt TRH in de regulatie van de TSH afgifte?
3) welke rol speelt TRH in de regulatie van de PRL afgifte?

Verscheidene in vivo en in vitro technieken worden gebruikt om de hypothalame TRH afgifte te meten. In **Hoofdstuk II** worden de meest gebruikte in vivo technieken besproken. Veel onderzoekers hebben immunoreactief TRH (IR-TRH) gemeten in perifeer bloed als maat voor de hypothalame TRH secretie. Wij vonden dat onder omstandigheden waarin TSH spiegels in bloed veranderen (zoals vasten, hyperthyreoidie en hypothalame lesies) ook de perifere bloed spiegels van IR-TRH veranderd zijn **(Hoofdstuk IIB)**. De gemeten concentraties van IR-TRH in perifeer bloed zijn echter erg laag en niet geheel afkomstig van de hypothalamus. Bovendien komt slechts een klein gedeelte van dit IR-TRH overeen met authentiek TRH en kunnen deze veranderingen in IR-TRH ook toegeschreven worden aan een veranderde metabole klaring van TRH.

Push-pull perfusie van de eminentia mediana of de hypofysevoorkwab en het verzamelen van hypofysesteelbloed maken het mogelijk specifiek de

hypothalame TRH afgifte te meten (**Hoofdstuk IIA**). Voor de laatste - chirurgische- techniek is narcose noodzakelijk, terwijl tijdens push-pull perfusie de hypothalame TRH afgifte gemeten wordt in onverdoofde, vrij bewegende ratten. Electrische stimulatie van het paraventriculaire gebied in de hypothalamus stimuleert de TRH afgifte tijdens push-pull perfusie in dezelfde orde van grootte als in hypofysesteelbloed. De concentraties die in push-pull perfusaten worden gemeten variëren en zijn vele malen kleiner dan die in hypofysesteelbloed. Tot nog toe hebben wij geen verklaring voor deze discrepantie en is er geen gouden standaard techniek om de hypothalame TRH afgifte te meten. Over het algemeen moeten de genoemde nadelen ernstig in acht genomen worden bij de interpretatie van gegevens.

In **Hoofdstuk III** wordt de rol van TRH in de regulatie van de TSH afgifte besproken. Talloze omstandigheden beïnvloeden de TSH spiegels in perifeer bloed. De meest bekende zijn schildklierstatus, en de reactie van het lichaam op ziekte (het "lage T3 syndroom") en koude.

De schildklierstatus beïnvloedt de perifere TSH spiegels deels door een effect op de hypothalame TRH afgifte. Wij vonden dat de TRH afgifte aan hypofysesteelbloed in vivo en door hypothalamusfragmenten in vitro verlaagd is met 30-45% in hyperthyreote ratten (**Hoofdstuk IIA,B**). In primaire hypothyreoidie echter blijft de TRH secretie onveranderd, zowel in vivo als in vitro. Alleen in hypothyreoidie geïnduceerd door propylthiouracil is de in vivo TRH afgifte licht verhoogd, maar de fysiologische implicatie van deze vondst blijft speculatief. Bilaterale electrische lesies in het paraventriculaire gebied reduceren de plasma TSH spiegels met 70% in normale ratten, maar met slechts 30% in hypothyreote ratten. We mogen daarom concluderen dat TRH geen rol van belang speelt voor het ontstaan van de hypersecretie van TSH tijdens een primaire hypothyreoidie. De schildklierstatus lijkt veel meer via een directe terugkoppeling van schildklierhormoon op hypofysair niveau de TSH spiegels te beïnvloeden.

Het lage T3 syndroom wordt gezien bij patiënten met uiteenlopende ziekten die de schildklier niet betreffen en wordt gekarakteriseerd door lage circulerende spiegels van schildklierhormoon. Dit syndroom wordt gezien als een gunstig adaptatie mechanisme van het lichaam als reactie op ziekte om daarmee energie te conserveren (een zgn. adaptieve hypothyreoidie). Aangezien plasma TSH spiegels relatief te laag zijn met betrekking tot de lage circulerende plasma concentraties van

schildklierhormoon, zouden centrale mechanismen een rol kunnen spelen bij het ontstaan van dit syndroom. De vastende rat staat model voor het lage T3 syndroom. Tijdens vasten dalen plasma spiegels van T3, T4 en TSH. Ook de PRL spiegels zijn gedaald **(Hoofdstuk IIIC)**. De plasma binding van T4 wordt eveneens door voedselonthouding beïnvloed, waarschijnlijk door een toename van de vrije vetzuur-concentraties. Al deze veranderingen herstellen zich weer door hervoeden. Het plasma TSH stijgt zelfs tot boven zijn uitgangspositie. Ondanks deze verregaande effecten op plasma T3, T4 en TSH verandert de in vitro afgifte van TRH niet tijdens vasten of hervoeden.
Ook bij diabetes mellitus, geïnduceerd door streptozotocine, wordt een hypothyreoidie aangetroffen, en zijn de plasma PRL spiegels verlaagd **(Hoofdstuk IIID)**. De in vitro afgifte van TRH is significant verlaagd in diabetische ratten. Dit effect lijkt specifiek, aangezien gewichtsverlies of verhoging van de in vitro glucose concentratie van 10 naar 30 mM geen effect hebben op de hypothalame TRH secretie in normale ratten. In de afwezigheid van glucose echter neemt de in vitro TRH afgifte tweevoudig toe, zodat glucose een belangrijke rol blijkt te spelen in de regulatie van de hypothalame TRH afgifte.

De schildklierfunctie wordt gestimuleerd door koude en dit mechanisme kan gezien worden als een adaptieve hyperthyreoidie. De door koude opgewekte stijging van plasma TSH is versterkt wanneer ratten eerst geacclimatiseerd worden bij 30 °C **(Hoofdstuk IIIE)**. Koude induceert in deze ratten ook een toegenomen TRH afgifte gedurende de eerste 15 minuten, zoals gemeten tijdens push-pull perfusie van de eminentia mediana.

Concluderend kunnen we zeggen dat de TRH afgifte nauwelijks door de schildklierstatus beïnvloed wordt. Tijdens hypothyreodie lijkt TRH van minder belang voor het ontstaan van de hypersecretie van TSH. Bovendien is de hypothalame TRH afgifte tijdens hyperthyreoide slechts in geringe mate verlaagd. Daarom lijkt het effect van een veranderde schildklierstatus op de TSH spiegels vooral tot stand te komen door een direct effect van schildklierhormoon op hypofysair niveau. Met betrekking tot het lage T3 syndroom en koude blootstelling lijkt TRH echter wel een belangrijke rol te spelen: veranderingen in plasma TSH tijdens ziekte (in ieder geval bij experimenteel geïnduceerde diabetes mellitus in de rat) en koude kunnen ten dele aan een veranderde TRH afgifte worden toegeschreven.

In **Hoofdstuk IV** wordt de rol van TRH in de regulatie van de PRL

afgifte besproken. Terwijl dopamine een bewezen PRL inhiberende factor (PIF) is, is de rol die TRH speelt voor de afgifte van PRL tijdens zogen nog onduidelijk. Enkele studies suggereren dat TRH een fysiologische "PRL releasing factor" (PRF) is, aangezien de hypothalame TRH afgifte aan hypofysesteelbloed toeneemt tijdens een artificiële zoogstimulus in verdoofde ratten. Zogen beïnvloedt de hypothalame TRH afgifte echter niet tijdens push-pull perfusie van de eminentia mediana van onverdoofde, vrij bewegende moederratten **(Hoofdstuk IVA).** Zogen vermindert binnen 15-30 minuten kortstondig de afgifte van dopamine, waarmee nogmaals de rol van dopamine als PIF bevestigd wordt.

Lesies in het paraventriculaire gebied verlagen de hypothalame TRH afgifte en veroorzaken daardoor een tertiaire hypothyreoidie. Ook de door zogen gestimuleerde PRL afgifte is verminderd door deze ingreep **(Hoofdstuk IVB).** Substitutie met schildklierhormoon ter herstel van de hypothyreoidie herstelt ook de PRL afgifte. De verminderde PRL afgifte in lacterende ratten met een hypothalame lesie lijkt dus veeleer een gevolg van de hypothyreoidie en niet van een verminderde hypothalame TRH afgifte.

Deze studies bevestigen de rol van TRH als fysiologische PRF dus niet.

DANKWOORD.

Velen ben ik dank verschuldigd bij de totstandkoming van dit proefschrift.

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Fred Bonthuis van Inwendige Geneeskunde I heeft meegeholpen met de diabetes experimenten.

Rogier Heide was het laatste jaar als medisch student betrokken bij het onderzoek betreffende vasten en diabetes mellitus.

Hans van Toor heeft de bepalingen van het vrije T4 en de vrije vetzuren gedaan en had altijd tijd om mee te helpen bij de vele TSH

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CURRICULUM VITAE.

De schrijver van dit proefschrift werd geboren op 29 oktober 1960 te Heerlen. Aldaar volgde hij de middelbare schoolopleiding aan het St. Bernardinuscollege. In 1979 behaalde hij het Gymnasium-B diploma en startte hij met de studie Geneeskunde aan de Rijksuniversiteit Limburg te Maastricht. Het artsexamen werd op 22 juli 1985 verkregen. Vanaf januari 1986 tot januari 1990 was hij als assistent in opleiding (AIO) verbonden aan de afdelingen Inwendige Geneeskunde III en Fysiologie II (Endocrinologie, Groei en Voortplanting) van de Erasmus Universiteit Rotterdam, alwaar het in dit proefschrift beschreven werk werd verricht. Momenteel is hij werkzaam op de afdeling Inwendige Geneeskunde van het Medisch Spectrum Twente te Enschede.