Impaired thyroid function in murine toxoplasmosis

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SUMMARY

A decline in serum thyroxine (T_4) occurs in Nya:NYLAR female mice infected with *Toxoplasma gondii*. To ascertain whether the hypothyroxinaemia might be the result of primary thyroid dysfunction, 2 parameters of thyrofollicular cell function were monitored to determine (a) if the cell surface membrane receptors for thyroid-stimulating hormone (TSH) were operative, and (b) whether the cyclic adenosine monophosphate (cAMP)-dependent cascade of intracellular events leading to the release of T_4 was responsive to exogenous cAMP. Our results indicated that both parameters were intact and functional in the infected-mouse thyrocytes. However, the elicited T_4 responses were distinctly diminished in magnitude, reflecting a lack of readily available thyroidal T_4 reserves. Because the continuing synthesis, storage, and release of T_4 is dependent on the pulsatile stimulation of the thyroid by TSH, we suggest that the depletion of T_4 reserves is likely due to perturbation of the pulsatile release of TSH from the pituitary, rather than to primary thyroid malfunction.

Key words: *Toxoplasma gondii*, hypothyroxinaemia, thyroid, thyroid-stimulating hormone (TSH), thyroxine reserves.

INTRODUCTION

Female Nya:NYLAR albino mice, infected with the Cornell (CS) strain of *Toxoplasma gondii*, develop a progressive wasting syndrome terminating in death within 6 months post-infection (p.i.). Early in the infection, prior to the emergence of the first prodromal signs of wasting, indications of endocrine dysfunction appear. During the first month p.i., the infected mice evince anaemia, adrenal hypertrophy, atrophy of the thymus, ovaries, and uterus, cessation of cycling, anovulation, and a precipitous decline in serum thyroxine (T_4) levels (Stahl & Turek, 1988). Because of the important central role of the thyroid in maintaining normal metabolic homeostasis (Davis, 1991), the aetiology of the hypothyroxinaemia was considered an opportune topic for further study.

A preliminary histological assessment of the thyroids of infected mice failed to disclose evidence of parasitic or inflammatory changes. The lack of overt thyroid pathology suggested that a physiological impediment may have developed within the hypothalamus–pituitary–thyroid (HPT) axis, interfering with normal thyroid function. To ascertain the locus of this hypothetical impediment, a systematic evaluation of the responsiveness of components of the HPT axis to stimulation was initiated. Our working hypothesis assumed that the defect was located in the thyrocyte and that (a) either the cell membrane surface receptors for thyroid stimulating hormone (TSH) were blocked and unresponsive, or (b) the internal second messenger system involving the formation and transport of cyclic adenosine monophosphate (cAMP) to the nucleus was impaired. To test the validity of these premises, the first series of experiments were to determine whether the infected-mouse thyroid was capable of responding to TSH i.e. would TSH bind to the cell membrane receptors and trigger the cascade of biochemical reactions leading to the formation of cAMP and the subsequent release of T_4? Since most of the known effects of TSH on the thyrocyte can be duplicated by injections of cAMP (Pastan, 1966), a second series of experiments, designed to circumvent the surface TSH receptors, was performed to determine if exogenous cAMP, administered in the form of dibutyryl cAMP (dbcAMP) to facilitate effective cellular penetration, would stimulate the thyroid.

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MATERIALS AND METHODS

Mice

Female Nya:NYLAR mice were obtained from the Griffin Laboratory, New York State Department of Health, Albany, N.Y. Details concerning the origin and genetic characteristics of the NYLAR strain have previously been described in detail (Benson & Abelseth, 1977). The mice were given standard lab chow and water *ad libitum*.

Toxoplasma

The Cornell (CS) strain of *T. gondii* was maintained in the cyst stage in the brains of chronically infected NYLAR mice. To obtain cysts, infected stock mice were killed by cervical dislocation and their brains emulsified in saline. After performing cyst counts,

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the volume of emulsion was diluted with saline to yield the standard inoculum of 8 cysts/0·5 ml aliquots. The infected brain emulsion was divided into 2 parts; the first pool of emulsion was used directly to infect the experimental mice, the second pool (for control purposes) was snap-frozen and thawed 3 times in succession, a procedure which has been shown to destroy the viability of the toxoplasmas but which theoretically should not affect any viral contaminants (Stahl & Turek, 1988). The control inocula were equal in volume and dilution to the infecting inoculum, and all inoculations were given by i.p. injection.

Reagents

Bovine thyroid stimulating hormone (TSH), N^6,O^2-dibutyryl 3′,5′-cyclic adenosine monophosphate (dbcAMP), and theophylline were purchased from Sigma Chemical Co. (St Louis, MO, USA). The thyroid culture medium, used in the *in vitro* experiments, consisted of 1 part of 0·1% ascorbic acid in water and 9 parts of Krebs-Ringer bicarbonate buffer, containing HEPES (20 mM; pH 7·4), 1 mg/ml glucose, and 5 mg/ml of bovine serum albumin (BSA).

Onset and duration of the post-infection hypothyroxinaemia

To illustrate the rapid fall in T_4 levels, a group of 10 mice were infected with *T. gondii* and bled at prescribed intervals during the succeeding 4 months. Ten normal mice were bled to establish a control baseline. Drops of blood were spotted on filter paper sheets and, after drying, small, uniformly-sized circles in the spots were punched out, the dried capillary blood eluted, and assessed for T_4 by radioimmunoassay (Larsen & Broskin, 1975).

Release of T_4 in response to TSH and dbcAMP

Groups of 10 female mice, chronically infected for 3, 6 and 16 weeks, were tested. Uninfected littermates served as controls. Some groups were inoculated intravenously (i.v.) with TSH (2 mU/0·2 ml). Two other groups of 10 mice, infected for 3 and 4 weeks, were inoculated i.p. with a dbcAMP-theophylline solution (2·5 and 3·0 μg/0·2 ml). The theophylline, a cyclic phosphodiesterase inhibitor, was added to delay the enzymatic inactivation of the dbcAMP (Bastomsky & McKenzie, 1967). At hourly intervals thereafter, several drops of blood were spotted on filter paper sheets and processed as described above.

Thyroidal cAMP response to TSH

Groups of mice were lightly anaesthetized with ether and injected i.v. with 0·2 ml of solution containing 100 mU of TSH. The diluent for the TSH was 0·9% NaCl containing 0·1% BSA. At intervals ranging from 15 to 60 min, after inoculation of the TSH, the mice were bled and killed. Very quickly, the thyroid glands were carefully dissected free and placed in glass homogenizers containing 0·5 ml of hot buffer (50 mM Tris–HCl, 4 mM EDTA, pH 7·4, 97 °C), which were immersed in a boiling water bath for 3 min. The thyroids were then homogenized and the tubes reimmersed in the boiling water bath for an additional 3 min. After cooling, the homogenates were centrifuged for 5 min at 15000 ***g***, after which the supernates were removed and stored at −20 °C. cAMP was measured in the supernates by a competitive binding assay, using a cAMP assay kit (The Radiochemical Center, Amersham, England).

The removal of the thyroid and its insertion into the hot buffer usually was accomplished within 30 sec. The reason for the rapid dissection was based on a report by Gafni, Sirkis & Gross (1975), who showed that a delay greater than 60 sec after death resulted in a drop of cAMP levels. Therefore, the weighing of the thyroid was omitted, and cAMP calculated as picomoles per thyroid.

Colloid droplet formation in response to TSH

Two groups of 4 mice, infected with *T. gondii* for 1 and 4 months, and a third group of 4 normal mice, were injected i.v. with 2 mU of TSH (0·2 ml of a solution containing 10 mU TSH/ml). One h later, the mice were anaesthetized and killed by exsanguination, then perfused with cold, 10% buffered formalin. The thyroids were removed, fixed for at least 48 h in formalin, and processed for histological assessment. Sections, 5 μm in thickness, were stained with periodic acid-Schiff reagent (PAS) and lightly counterstained with haematoxylin. The readily visualized colloid droplets were PAS-positive (bright red) cytoplasmic inclusions, representing colloid engulfed by follicle cells (Pastan & Wollman, 1967). Beginning with a follicle selected at random, and progressing to adjoining follicles, follicle cells and intracellular PAS-positive colloid droplets were counted simultaneously until a total of 15 follicles had been scanned. Follicles with poorly stained luminal colloid were excluded. Two different areas per section of thyroid were examined, yielding 8 determinations for each of the 3 groups of mice. Data were expressed as mean ± S.D. of the number of droplets per 100 thyrofollicular cells.

Release of T_4 in response to TSH and dbcAMP (in vitro)

Three weeks p.i., 40 infected mice were lightly anaesthetized with ether and bled from the retro-orbital sinus. The thyroid glands, attached to the trachea, were removed, placed in small capped tubes

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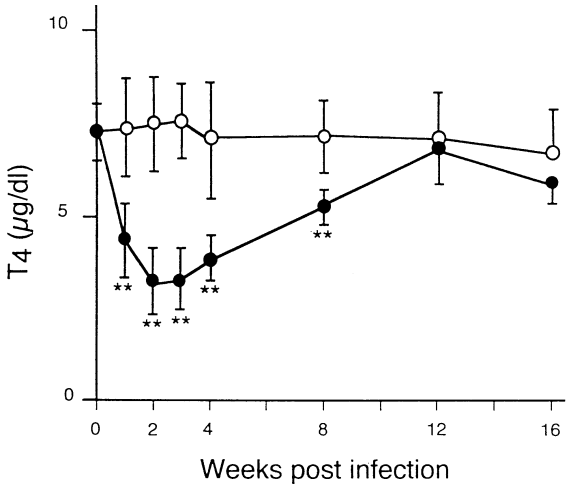


Fig. 1. Temporal changes in serum T_4 levels in Nya:NYLAR female mice infected with *Toxoplasma gondii*. Values are presented as mean ± S.D. Controls (○), $n = 10$); infected (●, $n = 10$). $^{**}P < 0{\cdot}01$ when compared with control group.

containing 1 ml of incubation medium, and pre-incubated for 1 h (Williams, 1972). Following the pre-incubation, the used medium was replaced with 1 ml of fresh medium containing graded amounts of TSH or dbcAMP-theophylline (Tonoue, Tong & Stolc, 1970). The thyroid lobes, under an atmosphere of 95 % O_2–5 % CO_2, were further incubated for 3 h at 37 °C in a shaking water bath. The tubes were gassed 30 min prior to use, at the beginning of incubation, and at 30 min intervals thereafter. After the 3 h of incubation, the incubation medium was transferred to clean vials, frozen, and later assayed for T_4 by radioimmunoassay.

Statistics

Significance of differences between groups was determined by Student's *t*-test. $P < 0{\cdot}05$ was considered significant.

RESULTS

Post-infection hypothyroxinaemia

The rapid onset of hypothyroxinaemia in Nya:NYLAR mice following infection with *Toxoplasma gondii* is shown in Fig. 1. Within the first week p.i., the T_4 levels in the infected mice dropped sharply and remained depressed for the next 2 months. During the succeeding weeks, the hypothyroxinaemic state gradually resolved and by the end of the third month p.i., T_4 levels were within the normal range.

T_4 response to TSH and dbcAMP stimulation

Groups of mice, chronically infected for 3, 6 and 16 weeks, were challenged with TSH and the results

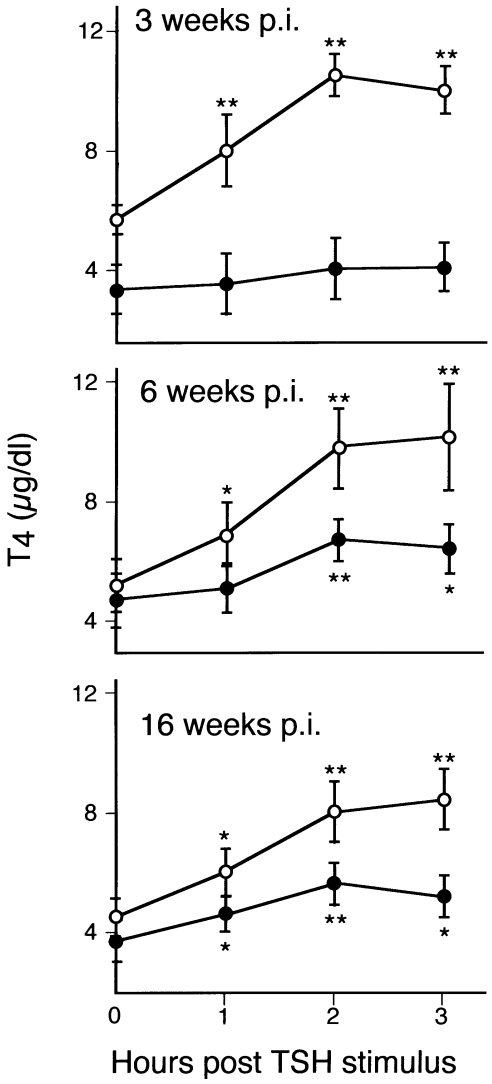


Fig. 2. TSH-induced changes in serum T_4 levels in groups of Nya:NYLAR female mice infected with *Toxoplasma gondii* for 3, 6 and 16 weeks; 10 mice/group. TSH: 2 mU/0·2 ml/i.v. Values are presented as mean ± S.D. Controls (○); infected (●). Single and double asterisks identify statistically significant differences ($P < 0{\cdot}05$ and $P < 0{\cdot}01$, respectively) between the hourly changes in T_4 levels and the 0-hour baseline value.

depicted graphically in Fig. 2. Following the TSH stimulus, the normal controls responded quickly with rising T_4 titres during the first 2 h. In contrast, the animals infected for 3 weeks responded poorly during the 3 h period of observation; the mice infected for 6 weeks showed a delayed response during the second h after challenge; and the mice infected for 16 weeks clearly responded during both the first and second h. In all cases, the T_4 responses of the infected mice were significantly lower than the controls, especially so in the mice infected for 3 weeks.

The T_4 response to dbcAMP was tested in 3 groups of 10 mice infected for 3 and 4 weeks. Because the results obtained at both time-intervals

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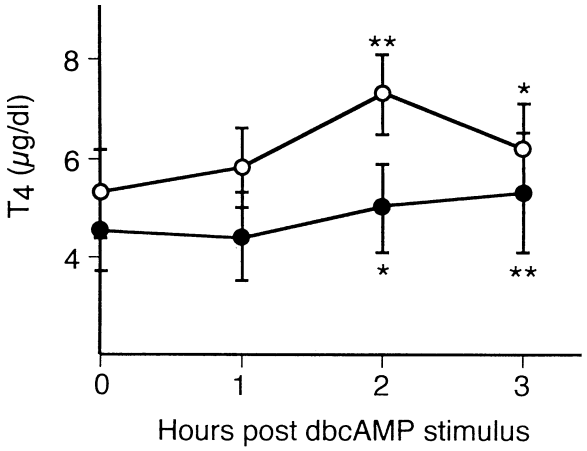


Fig. 3. DbcAMP-induced changes in serum T_4 levels in groups of Nya:NYLAR female mice infected with *Toxoplasma gondii* for 3 and 4 weeks. DbcAMP (2·5 mg) and theophylline (3·0 mg)/0·2 ml/i.p. Two experiments; data pooled, presented as mean ± S.D. Controls (○, $n = 30$); infected (●, $n = 30$). Single and double asterisks identify statistically significant differences ($P < 0·05$ and $P < 0·01$, respectively) between the hourly changes in T_4 levels and the 0-hour baseline value.

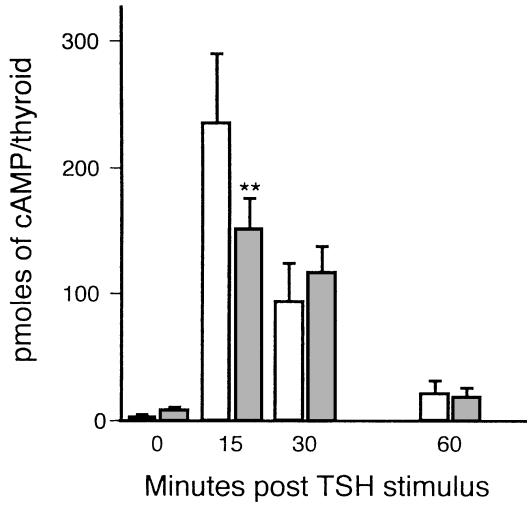


Fig. 4. Thyroidal cAMP responses to TSH after 15, 30 and 60 min in Nya:NYLAR female mice infected with *Toxoplasma gondii* for 3 weeks. TSH: 100 mU/0·2 ml/i.v. Results of 2 experiments pooled, presented as mean ± S.D. Controls (○); infected (●). $^{**}P < 0·01$ when compared with control group.

were comparable, the data were pooled and are presented in composite form in Fig. 3. In large measure, the T_4 response to dbcAMP approximated the response to TSH stimulation.

Thyroidal cAMP response to TSH

As shown in Fig. 4, the cAMP response to TSH peaked after 15 min, with the normal mice again exhibiting the stronger response. Thereafter, the cAMP levels dropped rapidly in both groups of

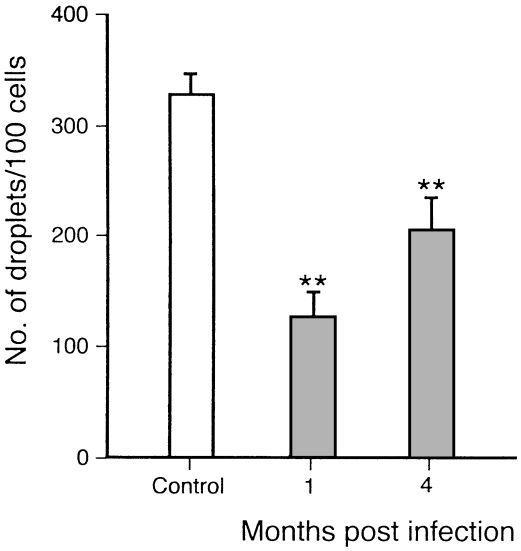


Fig. 5. Thyroidal colloid droplet response to TSH in Nya:NYLAR female mice infected with *Toxoplasma gondii* for 1 and 4 months. One h after inoculation of TSH (2 mU/0·2 ml/i.v.), thyroids were removed, fixed, sectioned at 5 μm, stained with periodic acid-Schiff (PAS), and examined microscopically at ×400. Four mice/group, 2 counts/thyroid. Values represent the number of droplets/100 follicle cells (mean ± S.D.). $^{**}P < 0·01$ when compared with control group.

mice. The increase in cAMP in the infected mice, although less pronounced than in the controls, nevertheless clearly signified a positive response, attesting to the integrity of the TSH surface receptors and to the subsequent biochemical reactions leading to the accumulation of cAMP.

Colloid droplet formation in response to TSH

Decreased numbers of colloid droplets appeared in the infected-mouse thyroids. At 1 month p.i., the number of droplets was approximately 30% of the number seen in the control thyroids, although by the 4th month p.i., the number had increased to 60% (Fig. 5). However, it is important to note the counts were performed 1 h after the TSH challenge. Based on the data depicted in Fig. 2, it is apparent that the reaction time of the infected-mouse thyroids to TSH stimulation is delayed, so the number of colloid droplets likely would continue to rise with additional time. Nevertheless, the appearance of droplets 1 h after the TSH stimulus denotes a positive response by the thyroids of the mice infected for 1 and for 4 months.

Release of T_4 in response to TSH and dbcAMP (in vitro)

In all cases, the T_4 response by the infected mice was greatly diminished in comparison with the uninfected controls (Fig. 6). Nevertheless, a definite response was obtained. Following incubation, the

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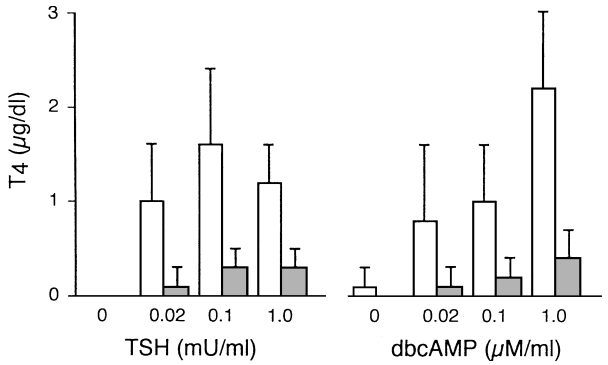


Fig. 6. *In vitro* release of T_4 from groups of mouse thyroids (5 thyroids/group) incubated for 3 h with graded doses of TSH or dbcAMP. *Toxoplasma gondii* infection for 3 weeks. Values are presented as mean ± S.D.

thyroids were fixed, processed, sectioned, and stained for colloid. Droplet formation was verified, but counts were not made.

DISCUSSION

Our experimental results indicate that the hypothyroxinaemia of the infected mice is not due to primary thyroid malfunction. This conclusion is based on the fact that the 2 parameters of thyroid function monitored were responsive to TSH and dbcAMP stimulation. To clarify the meaning of 'responsive', a brief review of the known effects of TSH on thyroid function should prove helpful. TSH binds to specific receptors located on the thyrocyte cell surface membrane, thereby activating adenyl cyclase within the membrane and catalysing the conversion of ATP to cAMP. The increase in cAMP levels within the cell initiates a series of reactions which lead to the enhanced resorption of follicular colloid by the thyrocyte, formation of colloid droplets, and release of thyroid hormones into the blood (Rapoport & Spaulding, 1996). The release of thyroid hormones in response to TSH, therefore, is the end result of a chain of events in which receptor binding, cAMP accumulation, and colloid droplet formation are early steps.

In the present study, the inoculation of infected mice with TSH or dbcAMP led to an accumulation of cAMP within the thyroid, an increase in colloid droplet formation, and the secretion of T_4. *In vitro*, the administration of TSH or dbcAMP to cultured thyroid glands resulted in the formation of colloid droplets and the release of T_4 into the incubation medium. Clearly, the cell surface receptors for TSH, the intramembrane events related to receptor–adenylate cyclase coupling, cAMP generation, and the intracellular cAMP second messenger system of the infected mouse thyrocytes were functional and responsive to stimulation. However, it was readily apparent that the magnitude of the elicited T_4 responses of the infected mice consistently failed to reach control levels. Because the responses to TSH and dbcAMP were only diminished, not absent, this finding is thought to reflect insufficient reserves of T_4 rather than primary thyroidal malfunction. For normal synthesis and secretion of thyroid hormones, the thyrofollicular cells need to be 'primed' by being intermittently stimulated in pulsatile fashion by the pituitary hormone TSH (Brabant *et al.* 1986; Greenspan *et al.* 1986). In turn, the synthesis and secretion of TSH by the pituitary thyrotope is dependent upon stimulation by thyrotropin-stimulating hormone (TRH), released from the hypothalamus in intermittent pulses (Brabant *et al.* 1991). If the pulsatile release of TRH is disturbed, this would affect the pulsatile release of TSH from the pituitary, leading to a depletion of both pituitary and thyroid hormone reserves and a diminished responsiveness of the HPT to exogenous stimulation.

We envision a physiological perturbation of the pulsatile release of hypothalamic TRH in the infected mice, resulting in inadequate pituitary and thyroid priming, depletion of trophic hormone reserves, a rapid decline in serum T_4 levels (hypothyroxinaemia), and diminished responsiveness of the thyroid to exogenous TSH and dbcAMP stimulation. The question of pituitary reserves of TSH and pituitary–thyroid responsiveness to TRH is addressed in the companion paper (Stahl & Kaneda, 1998).

Although a discrete locus for the thyroid dysfunction was not detected by the present series of experiments, primary malfunctioning of the thyroid has been effectively ruled out. Therefore, it is logical to proceed on the assumption that the impediment to normal thyroid function in the *T. gondii*-infected mice exists elsewhere in the HPT axis.

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REFERENCES

BASTOMSKY, C. H. & MCKENZIE, J. M. (1967). Cyclic AMP: a mediator of thyroid stimulation by thyrotropin. *American Journal of Physiology* **213**, 753–758.

BENSON, L. M. & ABELSETH, M. K. (1977). Investigation of the histocompatability of the Nya:NYLAR mouse colony by skin grafting. *Laboratory Animal Science* **27**, 333–335.

BRABANT, G., PRANK, K., HOANG-VU, C., HESCH, R. D. & VON ZUR MUHLEN, A. (1991). Hypothalamic regulation of pulsatile thyrotropin secretion. *Journal of Clinical Endocrinology and Metabolism* **72**, 145–150.

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BRABANT, G., RANFT, U., OCRAN, K., HESCH, R. D. & VON ZUR MUHLEN, A. (1986). Thyrotropin-an episodically secreted hormone. *Acta Endocrinologia* **112**, 315–322.

DAVIS, P. J. (1991). Cellular actions of thyroid hormones. In *Werner and Ingbar's The Thyroid : A Fundamental and Clinical Text*, 6th Edn (ed. Braverman, L. E. & Utiger, R. D.), pp. 190–203. J. B. Lippincott Co., Philadelphia.

GAFNI, M., SIRKIS, N. & GROSS, J. (1975). Inhibition of the response of mouse thyroid induced by chronic triiodothyronine treatment. *Endocrinology* **97**, 1256–1262.

GREENSPAN, S. L., KLIBANSKI, A., SCHOENFELD, D. & RIDGWAY, E. C. (1986). Pulsatile secretion of thyrotropin in man. *Journal of Clinical Endocrinology and Metabolism* **63**, 661–668.

LARSEN, P. R. & BROSKIN, K. (1975). Thyroxine (T_4) immunoassay using filter paper blood samples for screening of neonates for hypothyroidism. *Pediatric Research* **9**, 604–609.

PASTAN, I. (1966). The effect of dibutyryl cyclic 3′,5′-AMP on the thyroid. *Biochemical and Biophysical Research Communications* **25**, 14–16.

PASTAN, I. & WOLLMAN, S. H. (1967). Colloid droplet formation in dog thyroid *in vitro*. Induction by dibutyryl cyclic-AMP. *Journal of Cell Biology* **35**, 262–266.

RAPOPORT, B. & SPAULDING, S. W. (1996). Mechanism of action of thyrotropin and other thyroid growth factors. In *Werner and Ingbar's The Thyroid : A Fundamental and Clinical Text*, 7th Edn. (ed. Braverman, L. E. & Utiger, R. D.), pp. 207–219. Lippincott-Raven Publishers, Philadelphia.

STAHL, W. & KANEDA, Y. (1998). Aetiology of thyroidal dysfunction in murine toxoplasmosis. *Parasitology* **117**, 223–227.

STAHL, W. & TUREK, G. (1988). Chronic murine toxoplasmosis: clinicopathologic characterization of a progressive wasting syndrome. *Annals of Tropical Medicine and Parasitology* **82**, 35–48.

TONOUE, T., TONG, W. & STOLC, V. (1970). TSH and dibutyryl-cyclic-AMP stimulation of hormone release from rat thyroid glands *in vitro*. *Endocrinology* **86**, 271–277.

WILLIAMS, J. A. (1972). Cyclic AMP formation and thyroid secretion by incubated mouse thyroid lobes. *Endocrinology* **91**, 1411–1417.

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