Neonatal hyperthyroidism causes impairment in submandibular gland-nerve growth factor (SMG-NGF) ontogeny in mice

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Abstract. The effect of neonatal hyperthyroidism on submandibular gland (SMG) nerve growth factor (NGF) concentrations was studied in female Swiss-Webster mice. For this purpose, newborn female pups were treated with thyroxine (T4, 0.4 μg/g body weight/day) from birth through day 6, while their littermate pups were treated with similar volumes of vehicle. Animals were sacrificed on days 7, 15, 21, 31 and 71. Somatic growth (body weight and nose to rump length), serum T3 and T4 concentrations, and SMG and serum-NGF concentrations were measured. The data indicate that neonatal hyperthyroidism a) causes inhibition of somatic growth; b) induces alterations in normal ontogenic patterns of serum T4 and T3; c) impairs the developmental rise in SMG-NGF concentration and d) transiently increases serum-NGF levels.

The existence of a number of biologically active protein factors in adult mouse submandibular glands (SMG) has provoked an unique interest in the study of this gland (Barka 1980). One such factor that was discovered in extraordinarily high concentrations in this gland is the nerve growth factor (NGF) (Cohen 1960). This protein is essential for the survival and differentiation of sympathetic and sensory neurons of the peripheral nervous system (Levi-Montalcini & Angeletti 1968; Thoenen & Barde 1980). Mouse SMG is relatively immature from the time of birth to weaning and contains very low concentrations of NGF during this period (Walker et al. 1982). SMG undergoes rapid cytodifferentiation soon after weaning, during the time at which the animals change their food habits from liquid to solid diet (Jacoby 1959). The glandular concentrations of NGF, as well as other proteins, increase in parallel with histological changes (reviewed in Gresik 1980). Though SMG cytodifferentiation and synthesis of specific proteins presumably requires the activation of many genes, the factor or factors that trigger these transitions at a specific development period have not been clearly understood. Both hormones and the autonomic nervous system have been well recognized to influence growth and differentiation of SMG (reviewed in Pinkstaff 1980; Chretein 1977; Wells 1967). However, their interrelationship and their specific role(s) in the ontogeny of SMG-NGF expression is not clear.

SMG tissue in neonatal mice shows a precocious thyroxine (T4) sensitivity during the second week of life. T4 influences the morphological development of SMG (Aloe & Levi-Montalcini 1980) and also augments the levels of specific proteins such as NGF and epidermal growth factor (EGF) (Lakshmanan et al. 1984b, 1985b). These results demonstrate a well-coordinated sequence of events in which accelerated synthesis of specific proteins (NGF, EGF, etc.) is coupled with the formation of the specific intracellular structures involved in their storage (Aloe & Levi-Montalcini 1980). The present study examines the long-term effects of neonatal hyperthyroidism in female mice by measuring the SMG-NGF levels at the age of 31 and 71 days. In T4 treated animals, SMG-NGF levels were significantly lower indicating early hormone treatment causes impairment in the maturational increase SMG-NGF.
**Materials and Methods**

**Animals**

Experienced pregnant Swiss-Webster mice were purchased from Simonsen Laboratories (Gilroy, California). They were housed in a vivarium and given water and pelleted food ad libitum. Female pups born within 16 h were pooled and randomly distributed at 8 pups per mother. Within each litter, 4 pups were given injection of alkaline saline (control), while the remaining 4 were given T4 injections (0.4 µg/g body weight/day) sc on days 0–6. The T4 dose has previously established to augment SMG-NGF and EGF concentrations during the second week of life (Lakshmanan et al. 1984b, 1985b). Animals were periodically weighed and the time of both eruption and eyelid opening were recorded. All animals were changed daily to new cages. On day 21, the young were separated from their mothers and thereafter weanlings were maintained 4 per cage. Later, from day 26, they were housed 2 per cage until the time of sacrifice. (Studies in male littermate mice were abandoned due to an insufficient number. Experiments are in progress in male mice and the results will be reported later).

**Serum thyroid hormone measurements**

Groups of animals were sacrificed (by CO2 inhalation) on days 7, 15, 21, 31 and 71 for measurements of serum iodothyronines. In animals sacrificed on day 7, trunk blood was collected and for all other ages from the inferior vena cava. Blood was allowed to clot for 30 min at room temperature, then cooled in an ice water bath, centrifuged at 9000 g in a Beckman Microfuge B for 2 min and returned briefly to the bath before separation of serum. Samples were stored in microfuge tubes at −70°C. Serum T4 concentrations were measured using the methods of Chopra (1972) and serum T3 according to Chopra et al. (1972). The T4 and T3 specific antisera were purchased from Radoimmuno-assay System Laboratories, Inc., Carson, California. T4 cross-reaction in the T3 RIA was 0.02%.

**Fig. 1.**

Effect of T4 on somatic growth. Newborn female pups were treated with T4 or vehicle from days 0–6 as described in the text. A represents body weights with increasing age. B represents nose to rump length. Measurements on days indicated in the figure. Values are given as mean ± SEM. See text for other details.
Effect of T₄ on serum iodothyronines. Newborn female pups were treated with T₄ or vehicle from days 0–6 as described in the text. They were sacrificed on the days indicated and serum T₄ and T₃ were measured by specific RIA's. Values are given as ± SEM. See text for other details.

NGF analysis
NGF analysis in SMG and serum were performed in animals sacrificed on days 31 and 71. SMG tissues were homogenized in 10 volumes of 0.05 M phosphate buffered saline (PBS), pH 7.2, and the homogenates were centrifuged at 145,000 × g for 1 h. The supernatants were separated for measurement of protein and NGF. Protein was determined by the method of Lowry et al. (1951). NGF was measured using a specific double antibody liquid phase mouse βNGF-RIA (Lakshmanan, 1986). The RIA can detect 16–21 pg βNGF/tube. The antiserum was used at a final dilution of 1:350,000. βNGF used for immunization, iodination and standard was purified by the method of Mobley et al. (1976) with the modifications of Champman et al. (1979). [¹²⁵I] βNGF used in this study was iodinated using the chloramine T method (Greenwood et al. 1962) and labelled βNGF was purified on a CM-cellulose column using a buffer system similar to that described by Mobley et al. (1976). Specific activity of [¹²⁵I]βNGF determined by a self displacement assay range from 375–400 m/pg.

Statistical analysis
Body weights and nose to rump lengths of treated animals were compared with those of untreated animals at each age during development by a Student's two-tailed t-test. For all other parameters the differences between means were determined by a one way analysis of variance followed by Student's Newman Keuls test. P < 0.05 value was considered significant.

Results
Influence of T₄ treatment on somatic growth
The effects of T₄ treatment on somatic growth is shown in Fig. 1. Significant differences in body weights were observed between control and T₄ treated littermate female pups from day 9 onward, and the deficit in body weight was persistent throughout the experimental period (Fig. 1A). On day 71, the body weights of control and T₄-treated littersmates were (mean ± SEM) 34.8 ± 0.8 vs 27.4 ± 1.2 g, respectively (P < 0.01). Fig. 1B shows the body length (nose to rump length) at each age both for control and T₄ treated animals. A complete arrest of linear growth was manifested in hormone-treated animals between days 15–21. Although the linear growth resumed after weaning, the deficit in body length was persistent long
after T4 treatment had been discontinued. On day 71, the nose to rump length of control and T4-treated littermates were (mean ± SEM) 10.65 ± 0.05 vs 9.9 ± 0.1 cm, respectively (P < 0.01).

T4 treatment from days 0–6 accelerated both the time of tooth eruption and eyelid opening. In control animals, tooth eruption occurred on days 10 and 11 while this process was completed on days 7 and 8 in T4 treated animals. Eyelid opening was observed in control animals on days 13 and 14, whereas this process was accelerated to days 11 and 12 in T4 treated animals. The dose and duration of T4 treatment thus appeared to be sufficient to accelerate these developmental processes.

*Influence of T4 treatment on serum T4 and T3 ontogeny*

Developmental changes of serum T4 and T3 for control and neonatally T4 treated animals are shown in Fig. 2. Mean serum T4 (left panel) and T3 (right panel) were significantly elevated on day 7 in the hormone-treated group. Mean serum T4 and T3 levels were, however, significantly lower on day 15 in T4 treated animals compared to the control group. Neonatal T4 treatment completely abolished the normal developmental peak of serum T4 and T3 seen on day 15 in the control group. No significant differences were observed in mean serum T4 and T3 concentrations between control and T4 treated groups on days 21, 31 and 71.

*Influence of T4 treatment on SMG-NGF*

SMG-NGF concentrations on days 31 and 71 are shown in Fig. 3. In neonatally T4 treated animals, the glandular NGF concentrations were significantly lower at both times.
Influence of T₄ treatment on serum-NGF

Mean serum-NGF levels in control and T₄ treated animals are shown in Fig. 4. Serum-NGF levels were significantly elevated on day 31 but the levels were restored to control values by day 71.

Discussion

The data reported herein describe the long-term consequences of excess T₄ (0.4 μg/g body weight/day) treatment in female mice initiated during one specific period of development i.e., from days 0–6. Decreases in body weight were observed from day 9 onward while the linear growth was affected after day 15. The cause(s) for this decrease in somatic growth is not clear. Recent studies with similar T₄ dose and treatment protocol have revealed specific increases in EGF concentrations both in tissues (Lakshmanan et al. 1985a) and in urine (Perheentupa et al. 1984), indicating T₄ treatment augments either EGF availability or production in newborn animals. In this regard it is of interest to note that administration of EGF in mice during the neonatal period causes inhibition of somatic growth (Cohen 1965). The second possibility is that the growth retardation may be mediated by a deficiency in growth hormone (GH) levels. In this study, we did not measure GH levels. However, Pascual-Leone et al. (1976) have reported a permanent decrease in GH levels in rats treated with T₄ during the first week of life.

Neonatal T₄ treatment also abolished the T₄-ontogenetic peak which occurs around day 15 in control mice. The reason for this abnormality is not clear. Whether early T₄ treatment precociously lowered the ‘set point’ of hypothalamic-pituitary regulation is presently under investigation. In neonatal rats, excess T₄ treatment (several times higher than the T₄ dose employed in the present study) has previously shown to elicit a persistent defect in the hypothalamic pituitary-thyroid axis (Bakke et al. 1975). Examination of developmental profiles of serum T₃ also showed subnormal levels around day 15 in T₄ animals. Whether this decrease is secondary to the decreased production and secretion of T₄ or due to decreased peripheral conversion of T₄ into T₃ is presently being investigated. Though both serum T₄ and T₃ were restored to normal by day 21, the growth retardation persisted until day 71, the longest time employed in the present study. Both the time of tooth eruption and eyelid opening were advanced in T₄ treated animals. These effects, however, contrast the influences of T₄ treatment on somatic growth. The different responses indicate that various physiological processes may acquire thyroid hormone dependency during different stages of development.

Early T₄ treatment also caused significant reduction in SMG-NGF concentration on both days 31 and 71. In our previous study, using similar dose and treatment protocol, we observed no significant change in SMG-NGF concentration on days 7, 15 and 21 (Lakshmanan et al. 1984b). The decreased SMG-NGF levels in T₄ treated animals, therefore, indicate an impairment in the glandular maturation processes. It would be of great interest to study whether somatic growth and SMG-NGF levels show a catch-up later or are permanently affected.

The present findings also show increases in serum-NGF levels on day 31 in neonatally T₄ treated animals. However, by day 71, the serum-NGF levels returned to control levels. Very little is known about the origin and functions of NGF in circulation. In T₄ treated animals on day 31, decreases in SMG-NGF were accompanied by an increase in serum NGF, which may suggest that SMG-tissue may be the source. However, lack of similar findings in animals sacrificed on day 71 argues against the SMG tissues as the source. Whether tissues other than SMG have contributed to serum-NGF levels cannot be ruled out. The existence of NGF-mRNA in tissues other than SMG suggests that several tissues may be likely to synthesize NGF (Shelton & Reichardt 1984). Whether the release NGF into the circulation under normal or pathological conditions is not known.

In summary, neonatal hyperthyroidism elicits several adverse effects which include inhibition of somatic growth, alteration in normal ontogeny of serum T₄ and T₃, impairment in SMG-NGF maturation and increases in serum NGF levels. These results together with recent findings involving similar changes in SMG-EGF ontogeny and serum EGF levels (Lakshmanan et al. 1984a) suggest that neonatal hyperthyroidism may represent a disorder involving impairment in both NGF and EGF metabolism. Preliminary studies in male mice...
suggest that neonatal hyperthyroidism may elicit similar insults as in females, indicating that developmental regulatory mechanism(s) that govern the SMG-NGF and EGF levels during the postweaning stage may depend upon physiologic thyroid activation during the second week of life. The mechanisms of this dependence remain an interesting aspect for further studies.

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