Effect of thyroid hypofunction on the masseter motor innervation pattern in developing rats

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Abstract

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Introduction: The thyroid hormones have profound effects on the development of neuromuscular system. These hormones exert their influence on both muscle fibers and related motoneurons during development. The masseter is one of the most important muscles for mastication in mammals. We attempted to evaluate the effect of thyroid hormone deficiency on the morphological characteristics of masseteric motoneurons in the period of alteration from sucking to biting and chewing in the rat.

Material and Methods: To induce hypothyroidism, timed pregnant dams received 50 ppm antithyroid drug propylthiouracil (PTU) in their drinking water and PTU was administered to the pups during suckling period. Horseradish peroxidase (HRP) was injected into the masseter (0.5-5 µlit, 40%) of normal and prenatal hypothyroid pups on postnatal days of 1, 5, 13, and 21 (n=24). 24 to 48 hours later, following transcardial perfusion and fixation the brainstem blocks were cut to 50 µm thick sections. After TMB histochemical reaction the morphological characteristics of HRP labeled motoneurons and their HRP labeling intensity was evaluated. Student's t-test and two-way analysis of variance (ANOVA) were used for statistical analysis.

Results: No significant morphological differences were observed at the end of first week of life. On day 15, hypothyroid labeled masseteric motoneurons consisted of 70% small and 30% medium neurons versus 40% and 60% in normal pups respectively ($p<0.05$). At the time of weaning, the number of large motoneurons dropped to 30% of normal value ($p<0.001$) with few, short, and disoriented dendrites.

Conclusion: The alteration in particular patterns of masseteric motoneuron morphology and a severe delay in size transition could affect the development and plasticity of oral motor behavior under congenital hypothyroidism.

Keywords: hypothyroidism, horseradish peroxidase, masseteric motoneurons, masseter muscle, retrograde transport, rat pups

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Introduction

A pronounced shift in oromotor behavior occurs with the transition from sucking to chewing in humans and other mammals (1, 2). Previous neuroanatomical and biochemical investigations demonstrated that the development of skeletal muscles including the masseter is affected by both neuronal and thyroid hormonal effects (3-8). However, while details regarding anatomical, histochemical and functional changes have been demonstrated in skeletal muscles of rats with prenatal thyroid hypofunction, no conclusive morphometric data is reported in relation to their specific motoneurons. It is known that postnatal maturation of the central nervous system is critically dependent on thyroid hormone levels (9) and this might influence the neuromuscular system (10). Normally, the phenotypic properties of motoneurons and muscle fibers in the neuromuscular unit are matched (11-13). This effect is explained first as a result of an orthograde mechanism through the trophic factors secreted by different motoneuron types at the neuromuscular junction. The second explanation invokes a retrograde mechanism, so that once muscle fibers are differentiated into slow or fast types, they may modify properties of motoneurons via retrograde transport of substances (14, 15). Based on these hypotheses, Sickles et al. (16) and Bakels et al. (17) reported considerable alteration in the adult rats' soleus motoneuron morphology due to hyper- and hypothyroidism, respectively. In the case of masseter muscle,

neuroanatomical evidence related to the mechanisms of shifting from sucking to biting was first reported by Kubota et al. (18) in mice. Based on their observations, the differentiation of the trigeminal motoneurons related to biting is rapidly accelerated after birth. Miyata et al. (19) have reported morphometric alteration of superficial masseter motoneurons from sucking to chewing in normal rats so that the diameter of the largest motoneurons increases rapidly between 5th and 21st postnatal days. However, detailed morphometric data on the developing masseter innervation has been neglected in prenatal hypothyroid rats. Thus, in the present study, morphological features of the developing masseter motoneurons labeled by injection of HRP into the superficial masseter muscle were analyzed in normal and congenital hypothyroid rat offspring. HRP retrograde reaction product is observed as dark blue intracellular granules varying in quantity from motoneuron to motoneuron even in the same trigeminal motor nucleus (20). As hypothyroidism reduces neuronal growth, synaptogenesis, axonal transport velocity (21, 22), and neurotransmitter synthesis (14, 23), it was of especial interest to investigate the alteration in the HRP uptake and transport from the neuromuscular junction. In this regard, we attempted to evaluate the labeling quality of HRP backfilled masseteric motoneurons along with their size distribution profile under developmental hypothyroidism, which may lead to better understanding of the ontogenic changes in mastication.

Material and Methods

Animals

Timed pregnant Sprauge-Dawley rats (Pasteur's Institute, Tehran, Iran) were housed individually in plastic cages with free access to food and water. The animal room was maintained at constant temperature of 22-24ºC under a 12 hour light/ 12 hour dark cycle. The study was performed according to the guidelines for laboratory animal use and care set forth by the research council at Shaheed Beheshti University of Medical Sciences (Tehran, Iran). Prenatal hypothyroidism was induced by adding PTU (Sigma) to the drinking water of pregnant dams beginning at gestational day 16 to postnatal day 23.

For choosing the appropriate dose of PTU, we tested a range of 75 to 100 mg/liter of PTU, but the survival of the pups dropped sharply beyond the second postnatal week and so we finally used the 50 mg/liter concentration of the drug which induced a mild hypothyroidism and allowed us to have hypothyroid pups with a moderate rate of mortality until weaning.

It is note worthy that this concentration represents the same amount of PTU which is received by the pups during suckling period (24). Control dams received tap water. The litters were mostly culled to 8 pups on postnatal day 1 for each dam. In accordance with previous observations (24, 25), PTU-treated pups displayed skeletal and morphological deformities characteristic of hypothyroidism, including blunt snouts, unfolded ears, and rounded bodies compared to normal pups. Eye opening was also delayed for 2 days. In this study, PTU-treated pups were weighed at different times from birth to 23 days after birth (Fig 1). At the time of weaning, hypothyroid pups weighed 50% under normal weight.

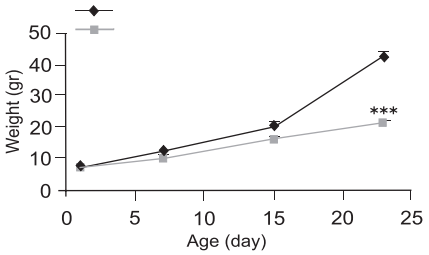

Fig 1: Body weight profile (means ± SEM) of hypothyroid offspring was significantly under normal rate from postnatal day 15 up to 23 (P< 0.001).

Intramasseter HRP injection

On the 1st, 5th, 13th and 21st days after birth, several male pups in each age group were anesthetized using i.p. injection of Ketamine (100mg/Kg) and Xylazine (5mg/Kg).

A small incision was made in the chick skin to expose the surface of the superficial masseter muscle.

Then, 1-5µlit of 40% HRP (type VI, Sigma) dissolved in sterile saline was slowly injected into the 2 to 5 points above as well as below the parotid duct in the left masseter using a Hamilton syringe as demonstrated by Kawagishi et al. (20). After each injection, the needle was left in situ for 1 minute to avoid backflow of the injected HRP, following which the needle was removed, the injection sites were cleaned with sterile saline, and the opening was sutured.

Histochemical procedure

After 24-48 h of survival time, the pups were deeply anesthetized and perfused transcardially with 20-50 ml saline (37°C) followed by 50-100 ml of fixative (1.25% glutaraldehyde and 1% paraformaldehyde in 0.1 M phosphate buffer at pH 7.4, 4°C).

Following perfusion and fixation, the lower brain stems were removed and post-fixed for 24 h.

The blocks of tissue were cut serially into 50 µm thick coronal sections using a vibratome. Then the sections were processed for HRP reaction using TMB method (26) and counterstained with 0.1% neutral red.

Microscopic examination

In each experimental group, three pups with the most reliable labeling in their trigeminal motor nucleus (Mo5) were chosen for microscopic study. From rostral to caudal part of Mo5, eight cross sections were selected per animal for each age group of normal and hypothyroid pups. The HRP labeled motoneurons showing a nucleolus or with visible primary dendrites were counted and semi-quantitatively divided into strong (S) and weak (W) intensities based on their HRP labeling profile. The cell body area of 500 HRP labeled neurons with both intensities were measured through cross sections using a computer based image analysis system (Olympus BX60, DP12, Olysia soft imaging system, Japan). To measure the soma areas, images of labeled cells were displayed on a monitor and their cell bodies peripheries in continuous with soma-dendritic transitional regions were outlined. Photomicrographs were arranged using CorelDRAW v.12.

Statistics

Differences between normal and hypothyroid groups were analyzed using two-tailed student's t-test. Two-way analysis of variance (ANOVA) was employed to assess the variation of soma size in relation to labeling intensity of masseteric motoneurons in different groups. The level of statistical significance was set at $p<0.05$. Values were expressed as mean ± SEM.

Results

Technical considerations

Masseter labeled motoneurons:

No contralateral neuronal labeling was observed. Unlabeled motoneurons were excluded from the study. The labeled motoneurons of normal and hypothyroid pups at days 1, 7, 15, and 23 are shown in Fig. 2. The number of labeled motoneurons in hypothyroid pups was not significantly different from those found in their control homologues (table 1).

Table 1: Mean numbers of total HRP labeled motoneurons subsequent to HRP injection in superficial masseter muscle

Pups	day 1	day 7	day 15	day 23
Normal	190±18.8	170±13.9	195±19.1	174±17.0
Hypothyroid	182±24.0	182±22.7	199±13.5	176± 7.8

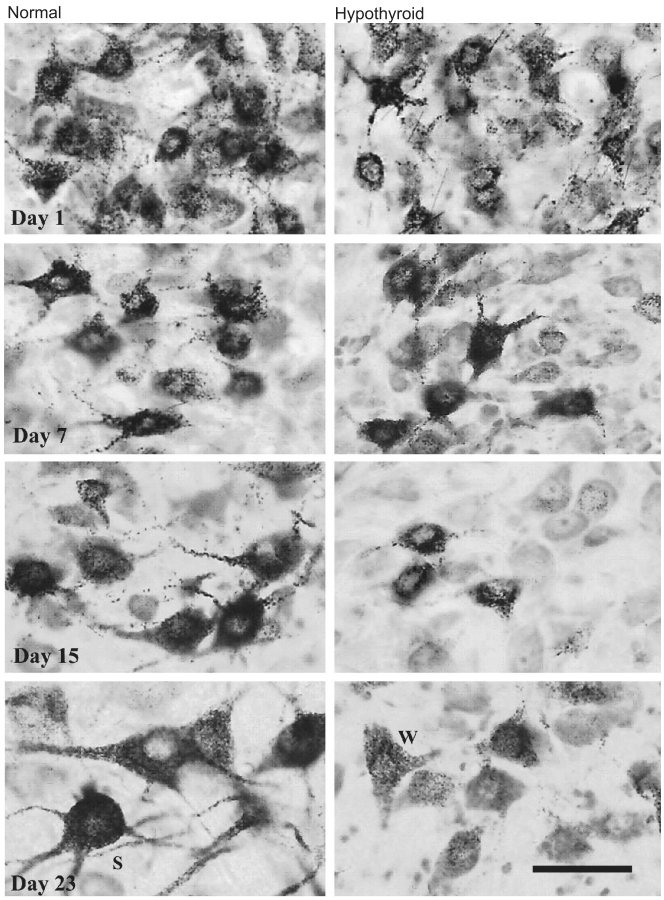

Fig 2: Photomicrographs showing superficial masseter HRP labeled motoneurons from day 1 to day 23 in normal and hypothyroid trigeminal motor nucleus. A Golgi like labeling appearance exhibits longer extension of primary dendrites in normal masseter motoneurons than those of their hypothyroid homologues at days 15 and 23. More number of labeled motoneurons in a frame indicates smaller size of motoneurons for hypothyroid pups at the same days of age. S; example of a strongly labeled motoneuron. W: example of a weakly labeled motoneuron. Scale bar = 50 microns.

In addition, the total number of strongly labeled motoneurons was higher than weakly labeled ones in both normal and hypothyroid pups during postnatal development. Indeed, the most obvious morphological changes in hypothyroid masseter motoneurons could be detected from day 15 to 23, in that the hypothyroid masseteric motoneurons showed less primary dendrites with shorter processes and slightly weaker HRP labeled soma compared to normal pups (Fig 2).
The correlative results between soma size and HRP labeling intensity of 500 measured motoneurons in each group are as follows:

Day 1
At day 1 after birth, a similar number of HRP-positive neurons was found in hypothyroid and in normal pups. The soma area of the labeled motoneurons ranged between 80 and 400 μm^2. Among them, the number of smaller motoneurons (soma area < 200μm^2) was about 2/3 of all the labeled cells. In addition, about 60% of them were strongly labeled (Table 2).

Day 7
One week after birth, the masseter motoneurons grew rapidly and about 4/5 of total labeled cells reached a soma area of 200 to 500 μm^2; the S/W ratio of neurons was about 2/1 in both normal and hypothyroid pups (Table 2).

Day 15
The medium motoneurons appeared at 15th postnatal day with a significantly lower value in hypothyroid pups ($p<0.001$, Table 2). In contrast, their small, weakly labeled motoneurons (<500μm^2) were higher than normal ($p<0.01$, Table 2).
Both the intensely and weakly labeled hypothyroid neurons displayed quite shorter processes in comparison to normal animals (Fig. 2).

Day 23
The most pronounced changes in soma area and labeling intensity were observed at weaning. Small motoneurons in normal pups comprised of less than 20% of all labeled motoneurons, whereas hypothyroid Mo5 contained twice more small motoneurons ($p<0.001$). The medium motoneurons had nearly equal quantity (~45% and 50%) in both normal and hypothyroid pups, respectively. While the number of large motoneurons reached to 40% of total labeled motoneurons in normal pups, the hypothyroid masseteric motor pool contained 15% of large cells ($p<0.001$) (see Table 2 for details). The HRP labeling was strong in larger motoneurons in normal pups, while it was strong in smaller motoneurons in hypothyroid pups. The Golgi-like labeling of primary dedritic processes were shortened remarkably in hypothyroid motoneurons (Fig. 2).

Discussion
Propylthiouracil (PTU) is frequently used to induce chemical hypothyroidism in laboratory animals. To induce prenatal mild hypothyroidism (29) in the present study, administration of 50 mg/liter PTU (mild thyroid depletion) resulted to 50% reduction in weight gain of weaned offspring. Similar to our results, other studies have shown that PTU caused a severe decline in body weight starting from second postnatal week (7, 24, 26, 27).
In rats, there is a significant rise up to peak serum T4 levels at 15 days followed by a slight decline to mature values (3). Sawin et al (26) showed that birth weights of hypothyroid animals were slightly lower than normal; this moderate retardation persisted until 15 days, after which hypothyroid animals stopped growing and became clinically cretinous. According to our results, there was no simultaneous significant difference in soma area and HRP intensity profile of masseteric labeled motoneurons between normal and hypothyroid pups by the first week of life.
The premature profile of masseter muscle begins to appear around the pre-weaning time (15 days) in rats. The diameter of muscle fibers enlarge progressively from slow to fast type to adapt to rapid functional changes from weaning to chewing motion (19).
To meet these muscle functional properties, 2 weeks after birth, the medium labeled motoneurons appeared at the expense of a reduction in the number of small motoneurons. Meanwhile, however, the number of medium motoneurons was about one third normal and the quantity of small motoneurons remained unchanged in hypothyroid pups. Accordingly, Gambke et al. (3) defined the maturation of motoneurons and the neuromuscular junctions from 15th day of postnatal life in rat.
At the time of weaning, the total number of small hypothyroid motoneurons was twice normal value, but still half of their pre-weaning time (day 15), However number of their medium size motoneurons was similar to normal pups. The developmental course of the feeding function includes 3 stages: suckling, suckling-chewing, and chewing-biting (28).
Based on our previous results (23) in a mild model of prenatal hypothyroidism, the pups seem to reach the pre-weaning stage of feeding behavior. However, having only 30% of normal value for large motoneurons, they were well behind in reaching their weaning period. It is known that when suckling of rats was continued on days 17-25 after birth, the development of the superficial masseter muscle was inhibited (30).

Thus, to obtain functional utility, the differential growth of muscle fibers is accompanied by changes in motoneuron soma and dendritic processes (31). In general, the metabolic and electrical properties of motoneurons are closely correlated to their soma size and also the number of target muscle fibers as a motor unit (11, 32, 33). Accordingly, alpha motoneurons have commonly been coarsely subdivided into “fast” and “slow” types (12). Consequently, regarding to size principle, the small motoneurons innervate slow-twitch muscle fibers, whereas large motoneurons innervate fast-twitch muscle fibers (34). Gojo et al. (35) showed that during the weaning period, the masseter muscle is composed predominantly of fast MHC isoforms in response to the post-weaning motion of chewing. In accordance with a recent report by Shida et al. (36), our results showed that the innervation pattern of weaned hypothyroid masseter muscle is similar to pre-weaning normal pups with a possible slower contraction (37) functioning in suckling-chewing stage.

Numerous histological and biochemical studies reported that the reduction of brain weight in hypothyroid rats is due to a decrease in cell size with the ultimate number of cells remaining unchanged (38). Consistent with previous data (23), our results revealed that hypothyroidism does not decrease the total number of masseteric motoneurons, whereas the appearance of large neurons is delayed, showing that the differentiation and maturation of masseter motoneurons are affected by hypothyroidism. Another important finding from our study involved changes in dendritic morphology resulted from neonatal hypothyroidism. The Golgi-like appearance of HRP-labeled motoneurons (39) clearly revealed thin, short and poorly ramified dendrites (see Fig 2) of hypothyroid masseteric motoneurons in comparison with normal pups. This effect of thyroid hormone deficiency on the growth and ramification of neuronal processes seems to be a general effect throughout the brain (9, 22, 39). Perturbations in neuronal process growth induced by developmental hypothyroidism are likely to contribute to the permanent impairments in synaptic transmission and plasticity (40). In particular, a significant increase in synaptic inputs to jaw- closer motoneurons from postnatal day 15 has been reported by Honma et al. (41, 42).

It has been also demonstrated that the combination of thyroid hormone and nerve growth factor is necessary to regulate the expression of cytoplasmic dynein, a protein that is involved in retrograde axonal transport (14). According to our results, weakly HRP-labeled motoneurons were insignificantly increased in hypothyroid pups compared to normal. Indeed from birth up to weaning time, the number of strongly HRP-labeled neurons was higher than weakly labeled neurons in both cases. This may suggest that the uptake and retrograde transport of an exogenous substance such as HRP is not sensitive to thyroid hormone deficiency. This finding could be due to different mechanisms in binding and transportation of different exogenous substances (43).

Conclusion

Any alteration in particular patterns of masseter motoneuron morphology such as size, dendritic architecture, dendritic orientation, and severe delay in size transition may affect the development and plasticity of oral motor behavior due to thyroid hypofunction.

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اثرکم کاری تیروئید برالگوی عصب دهی عضلهٔ جوندهٔ موش صحرائی در طول رشد پس از تولد

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چکیده

دریافت مقاله: ۸۴/۸/۱۵، پذیرش مقاله: ۸۴/۱۱/۳۰

* **هدف:** بررسی اثر کمبود هورمون تیرویید بر روی ویژگی‌های مورفولوژیک نورون‌های حرکتی عضلهٔ جونده در طول شیرخوارگی و نحوهٔ تغییر الگوی تغذیه از مکیدن به جویدن

* **مواد و روش‌ها:** موش‌های باردار از روز شانزدهم بارداری داروی پروپیل تیو اوراسیل (PTU) را با غلظت ۵۰ میلی‌گرم در لیتر به شکل محلول در آب آشامیدنی دریافت کردند و این تیمار تا روز بیست و سوم پس از تولد نوزادان ادامه یافت. در روزهای ۱، ۵، ۱۳ و ۲۱ پس از تولد، ۰/۵-۵ میکرولیترآنزیم (horseradish peroxidase: HRP) با غلظت ۴۰درصد به عضلهٔ جوندهٔ نوزادان نر هایپوتیرویید و نرمال تزریق شد (n= ۲۴). پس از گذشت ۴۸-۲۴ ساعت، نوزادان از طریق قلب پرفیوز شده و از بلوک‌های فیکس شدهٔ ناحیهٔ ساقهٔ مغز برش‌های ۵۰ میکرونی تهیه شد. فعالیت آنزیمی HRP با روش TMB آشکار و ویژگی‌های مورفولوژیک و شدت نشان‌دار شدن نورون‌های حرکتی عضلهٔ جونده مورد ارزیابی قرار گرفت. برای آنالیز آماری از آزمون‌های Student's t-test و ANOVA استفاده شد.

* **یافته‌ها:** تا روز هفتم پس از تولد تفاوت معنی‌داری در مورفولوژی نورون‌ها مشاهده نشد؛ اما در روز پانزدهم در گروه هایپوتیرویید ۷۰درصد نورون‌های نشان‌دار کوچک بوده و ۳۰درصد به اندازهٔ متوسط رسیدند در حالی که این نسبت در گروه کنترل ۶۰درصد کوچک و ۴۰درصد متوسط بود ($p<0.05$). در پایان شیرخوارگی تعداد نورون‌های بزرگ در گروه هایپوتیرویید به ۳۰درصد میزان نرمال رسید ($p<0.001$) و دندریت‌ها با تعداد کمتر و کوتاه‌تر مشاهده شدند.

* **نتیجه‌گیری:** هایپوتیرویید‌یسم مادرزادی با تغییر مورفولوژی نورون‌های حرکتی عضلهٔ جونده و ایجاد تاخیر چشم‌گیر در رسیدن آنها به اندازهٔ طبیعی می تواند شکل گیری و پلاستیسیتی رفتارهای حرکتی دهانی را تحت تاثیر قرار دهد.

کلیدواژگان: هایپوتیرویید‌یسم، horseradish peroxidase، نورون‌های حرکتی، عضلهٔ جونده، انتقال رتروگراد، نوزادان موش صحرایی