Effects of perinatal hypothyroidism on rat behavior and its relation with apoptosis of hippocampus neurons

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ABSTRACT. Thyroid hormone is an important factor for proper development of the mammalian brain. Perinatal hypothyroidism leads to long-term behavior and neuromotor competence alterations in humans and animals. Our study aimed to investigate the effects of perinatal hypothyroidism on behavior changes of rat pups and its relation with the apoptosis of hippocampus neurons. Behavior tests were taken to evaluate the effects caused by perinatal hypothyroidism. TUNEL staining was used to analyze the apoptosis of neurons on CA3 region of hippocampus. The study suggested that perinatal hypothyroidism affects behavior development, as well as leading to the decrease in spatial learning and memory capability. This condition can be improved with hormone substitute treatment. Furthermore, the changes of learning and memory capability are closely related to the increasing number of apoptotic neurons in the hippocampus.

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INTRODUCTION

Thyroid hormone (TH) is essential for growth and development of the mammalian brain. Insufficiency of TH results in structural and functional dysfunction of the brain in humans and experimental animals (1-3). In initial reports it was well documented that early deficiency of TH delays the maturation of innate reflexes, decreases behavior activity and impairs the ability to acquire some complex forms of learning (4-7). Darbra et al. (8) also observed that TH deficiency causes an increase of activity in animals in spite of a large period of rehabilitation. Their recent report concluded that perinatal hypothyroidism causes increased locomotor activities, decreased neuromotor competence and anxiety-related behavior (9). Severe hypothyroidism during the neonatal period leads to structural alterations, including hypomyelination and defects of cell migration and differentiation, with long-lasting, irreversible effects on behavior and performance (10).

The mechanisms by which TH regulates brain development remain to be elucidated. TH deficiency has 2 effects: neuron proliferation and neuron apoptosis. Early research focused more on the former (11-15), but few on TH regulation of the neurons apoptosis. Singh et al. (2) demonstrated that TH would inhibit the release of apoptotic molecules to prevent excess apoptosis during cerebellar development. Nothing has been reported yet on the research of perinatal hypothyroidism on apoptosis of hippocampus neurons. Hippocampus is a complex neural structure consisting of gray matter and located on the floor of each lateral ventricle, intimately involved in motivation and emotion as part of the limbic system, which also has a central role in the formation of memories (16). Our recent study indicated that perinatal hypothyroidism induces the apoptosis of hippocampus neurons *via* Bcl-2/Bax pathway (17). In the present study, we aimed to investigate the effects of perinatal hypothyroidism on locomotor activity, emotionality, behavior, learning and memory capability, so as to highlight their relationship with the neuron apoptosis of hippocampus.

MATERIALS AND METHODS

All procedures involving animals were carried out in accordance with the National Research Council's Guide for the Care and Use of Laboratory Animals and on approval of Animal Care and Use Committee of Zhejiang University.

Key-words: Perinatal hypothyroidism, thyroid hormone, brain, behavior, hippocampus.

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Experimental animals

Experimental Sprague-Dawley rats were used. Eighteen pregnant Sprague-Dawley rats were purchased from the experimental center of Zhejiang University. Propylthiouracil (50 mg/d, Nanhua Pharmaceuticals Co., Ltd. China) was administered through the oral gastric tube to the mother rats from gestational day 15 until weaning on post-natal day 21 (P21). The birth date was designated as post-natal day 1 (P1).

Sixty-four male hypothyroid pups on P1 were randomly assigned to the following two groups: (i) thyroid group (32 pups), and (ii) treatment group (32 pups); T_4 was administered peritoneally to the pups in this group at P1 until weaning on P21.

Each group was again subdivided into 2 groups: P21 group (no.=20), P50 group (no.=12). Behavior tests were performed on P21 and P50, as well as the control group containing 32 pups (P21: no.=20; P50: no.=12).

All the pups were housed in groups of 3-4 in each cage with free access to water and food. The temperature in the experimental animal center was maintained at 21 C±2 C under a 12 h light:12 h dark cycle (lights on at 08:00 h).

Body weight of pups were measured on P1, 21, 50. Pups in different groups were closely watched for clinical signs of daily changes .

Locomotor activity test

Inclined plane test

The inclined plane assesses an animal's ability to maintain its position on a board which is raised in 45° increments. Rats were placed at the bottom of the plane inclined at 45°, facing downward. A 180° turn made in less than one body length was scored as a negative geotactic reaction. We recorded the time it took for the head to turn upward instinctively.

Wire-hanging test

Holding the rat by the nape of the neck, its forelimbs were placed in contact with a horizontal bar with a diameter of 0.5 cm (50 cm away from the ground) until they grasped it. The animal was then released and the latency to fall was recorded as total hang time. This prehensile reflex test measuring the ability of the animal to remain suspended is presumed to indicate muscle strength (18). The test was performed 3 times and the mean data was recorded.

Tests on emotionality changes

Open-field test

To perform the test, a wooden, rectangular, light brown-colored open-field apparatus measuring 60×60×60 cm was used. The floor was divided into 36 rectangular squares. The following behavioral components were quantitatively examined in each animal for 5 min: ambulation (crossed squares): the number of floor squares entered by an animal; rearing: upright standing with hind paws while forepaws are free; defecation: number of fecal boli dropped by an animal in the open-field arena. The floor of the test apparatus was thoroughly cleaned after each test.

Tests on learning and memory capability

Passive avoidance test

This test was performed only on the P50 groups. The apparatus (SFK2 I) consists of a two-compartment box with a lighted compartment connected to a darkened one by a door. As soon as they entered the dark compartment pups received a punishing electrical shock (35 mV), going back to the lighted compartment passively. Staying in the lighted area for 5 min was considered to accomplish the learning process successfully. We recorded the times into the dark area during a period of 5 min. This test can indicate pups' recent learning capability. We then put the pups into the lighted compartment after 24 h and recorded the time when pups came from the lighted area to the dark one. It was designated as latency time for memory and can indicate the pups' recent memory capability.

Radial maze task

This task was carried out on the P50 pups. Pups were given free access to water and maintained at the 80-5% of their free feeding body weight. Experiments were performed between 10:00 h to 17:00 h daily.

The apparatus used was described in Chen et al.'s previous reports (19, 20). The radial maze had a center platform with a diameter of 30 cm and a height of 30 cm. Around the center platform stainless steel walls with a depth of 4.5 cm were positioned so as to produce 8 arms, radiating out to an open field. To familiarize the rats with the radial maze, prior to training, we gave them 2 days to grow accustomed. Food pellets (45 mg each, Bio-Serv, Frenchtown, NJ, USA) were scattered over the 4 legs (no. 1, 3, 5, and 6) and 3 or 4 rats were simultaneously placed in the radial maze and allowed to swim and take food freely for 10 min. After adaptation, all pups were trained with one trial per day. Pups were placed on the center platform that was closed off by a door. After 15 sec, the door was opened and the rat was allowed to choose any arm to obtain food pellets until all 4 pellets had been eaten or 5 min had elapsed. The number of entries into the never-baited arm was regarded as a reference memory error (RME), while re-entry into the arms with the pellet was regarded as a working memory error (WME).

Hormonal analysis

Blood samples were collected for evaluation of the TH. The rat pups were sacrificed for decapitation 21 and 50 days respectively after the behavioral tasks were accomplished. Blood was collected and centrifuged, and serum was frozen and stored at –20 C. Serum free T_3 (FT_3), free T_4 (FT_4) and TSH was determined with Chemiluminescent method according to the protocol provided by DPC Co., Ltd, USA.

TUNEL staining

All the pups were anesthetized with *Chlorali Hydras* (10%) and sacrificed for decapitation after the behavior tasks; 200 ml salide chloride and 4% paraformaldehyde were perfused into the heart consecutively. Brain tissue was removed rapidly and carefully from the pups and fixed with 4% paraformaldelhyde in phosphate buffer (pH=7.2), then imbedded in paraffin wax. We deparaffinized the brain samples slides with xylene and dehydrated them with ethanol. The endogenous peroxidase activity was blocked by incubation in acetic acid and blocking proteins were digested in proteinase k solution (20 μl/ml). TUNEL staining kit (BM, Germany) was used to detect the apoptotic cells according to the manufacture's protocol.

Apoptotic cells in the CA3 region of the hippocampus were observed under the microscope. The numbers of apoptotic neurons per field (1.0 mm^2) were recorded.

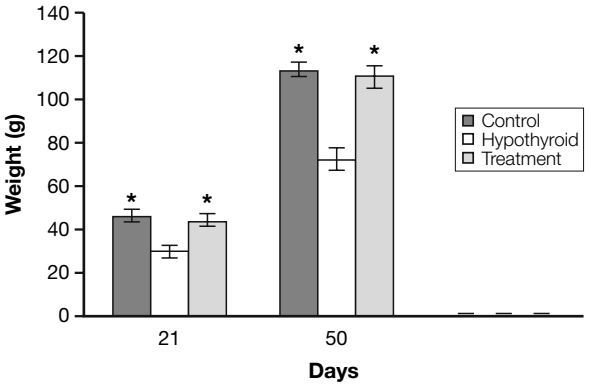

Fig. 1 - *Weight of pups in different groups. *:* p<*0.01 compared with the hypothyroid group. Data are mean±SD values.*

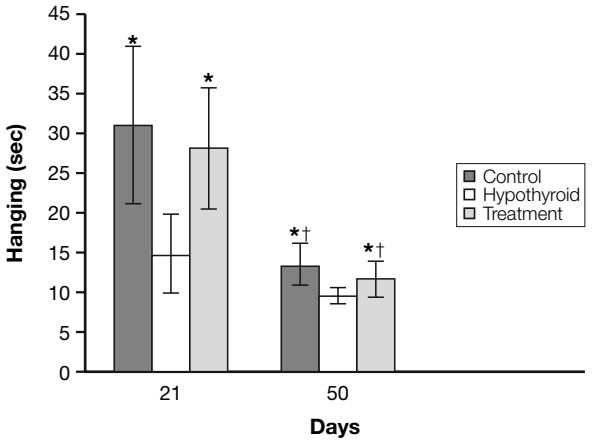

Fig. 2 - *Total hang time of the groups. *:* p<*0.01, compared with the hypothyroid group of the same age. †:* p<*0.05, compared with its parallel post-natal day 21 (P21) group. Data are mean±SD values.*

Data analysis

Statistical Package for the Social Sciences (SPSS) 10.0 software package was used to analyze the data. All results are expressed as the means±SD. The statistical significance was assessed by a one factor analysis of variance (ANOVA) or the Kruskal-Wallis non-parametric ANOVA test (when the data were not normally distributed or the variances of the groups differed significantly), followed by the Bivariate test or Pearson correlation test as a *post-hoc* analysis. $p<0.05$ was considered statistically significant.

RESULTS

The hypothyroidism subjects were much decreased in size at weaning even on P50. They showed an infantile appearance with a more rounded head and short tail. The T_4 substitute treatment group had an obvious improvement in appearance but still could not reach the level of the controls of the same age. The P21 hypothyroid group showed decreased weight (43.11±3.08 *vs* 45.92±2.22 g), increased level of TSH level (7.40±1.05 *vs* 3.04±0.62 μIU/ml) and decreased FT_4 level (15.39±1.94 *vs* 19.84±3.42 pmol/l) as compared with the controls ($p<0.05$). The P50 hypothyroid group also showed a decreased weight compared to the controls but no significant difference was found with serum FT_4, FT_3, TSH level between the two groups (Fig. 1, Table 1).

Effects of hypothyroidism on the locomotor activity alteration

The rotating time spent by the P21 hypothyroidism group did not increase significantly compared to the controls (8.25±1.05 *vs* 4.25±1.31 sec) and the treatment groups (8.25±1.0 *vs* 5.50±1.76 sec). In the Wire-hanging test, the P21 hypothyroidism group remained a shorter hang time as compared to the control and the treatment groups (both $p<0.05$). No differences were found among 3 groups on P50. The P50 hypothyroid pups showed a much shorter hang time than the P21 group, but with no significance (all $p>0.05$) (Fig. 2).

Effect of hypothyroidism on emotionality

As shown in the open-field test, both the hypothyroid groups (P21, P50) got the higher score in total

Table 1- *Thyroid hormone analyisis of the pups [free T_3 (FT_3) pmol/l, free T_4 (FT_4) pmol/l, and TSH μU/ml] (means±SD).*

Groups	no.	21d			50d		
		FT_3	FT_4	TSH	FT_3	FT_4	TSH
Normal	12	10.23±2.62	19.84±3.42*	2.12±0.37*	14.14±2.24	28.50±4.83	2.0±0.37
Hypothyroid	12	4.45±1.42	2.97±0.12	7.40±1.05	10.08±2.49	21.25±2.78	3.0±0.57
Treatment	12	7.17±1.36	15.39±1.94*	3.04±0.62*	12.01±2.27	24.48±3.12	2.1±0.29
F value		2.33	14.86	14.58	0.75	0.96	1.71
p value		>0.05	<0.05	<0.05	>0.05	>0.05	>0.05

*: $p<0.05$ compared with the hypothyroid group of the same age.
21d: post-natal day 21; 50d: post-natal day 50.

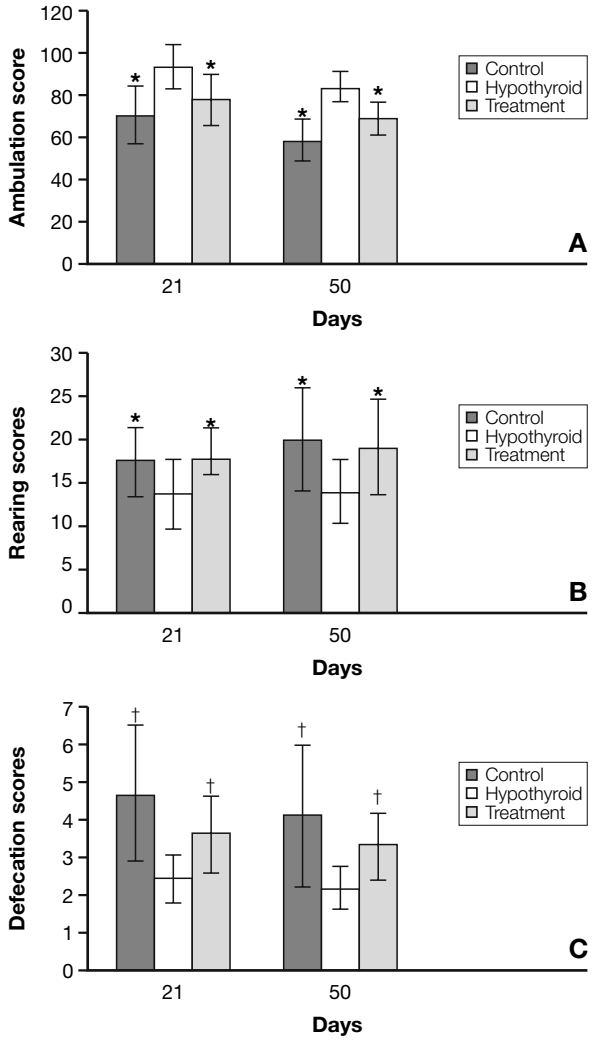

Fig. 3 - *Performance in the open field test. A: scoring in square crossing; B: rearing times; C: the number of fecal boli. *:* p*<0.05, †:* p*<0.01, compared with normal and treatment group of the same age. Data are mean±SD values.*

numbers of squares crossed, but reduced times of rearing and numbers of fecal boli (all $p<0.05$). Compared with the P21 groups, all the P50 groups showed a tendency to lower scores in the total numbers of squares crossed and numbers of fecal boli, increased times of rearing, but with no difference (all $p>0.05$) (Fig. 3).

Effect of thyroidism on learning and memory capability

The passive avoidance task showed that pups with hypothyroidism on P50 were affected by perinatal hypothyroidism. The pups made more mistakes and showed a shortened long-term (24 h) retention interval than the controls and the treatment group ($p<0.001$). There was no difference with memory latency between the control and the treatment group ($p>0.05$) (Fig. 4).

All the P50 pups learned to collect the food pellets during the adaptation period of the Radial maze task. There was no significant difference with RME between the hypothyroid group and the other two groups (both $p>0.05$). Comparisons for WME showed the hypothyroid group had an obvious increase against that of the other two groups (both $p<0.05$), as well as the total errors (both $p<0.05$). No significant difference was found in the total duration spent in the maze (all $p>0.05$). All the data are shown in Figure 5.

Results of TUNEL staining

Brown stained apoptosis neurons can be observed scattered in the CA3 region of the hippocampus tissue in the hypothyroid brain sample under the microscope. Only few denatured and apoptosis neurons can be seen in the brain sample of the P21 control and treatment group. Apoptosis neurons were seldom seen in the P50 control and treatment group. The number increased significantly in both hypothyroid groups (all $p<0.01$). The apoptotic cells in the hippocampus stained with TUNEL assay in the three P21 groups are shown in Figure 6. The numbers of positive cells in the 3 P50 groups fell signif-

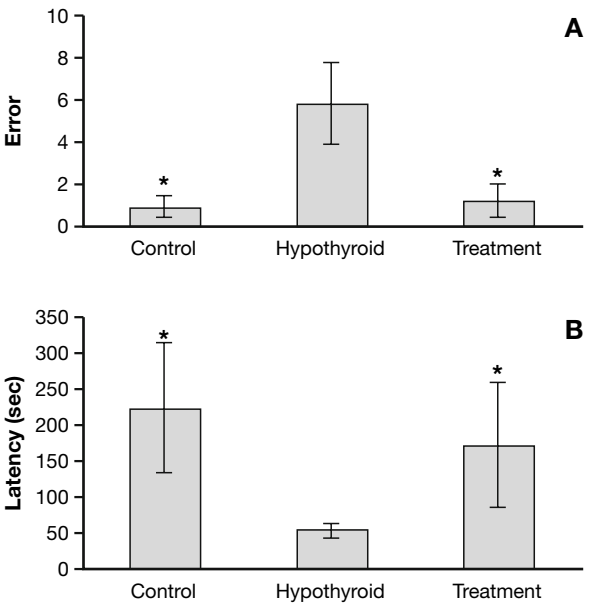

Fig. 4 - *Performance of pups in the passive avoidance test. A: total errors of the groups; B: latency of memory. *:* p*<0.001, compared with the hypothyroid group. Data are means±SD values.*

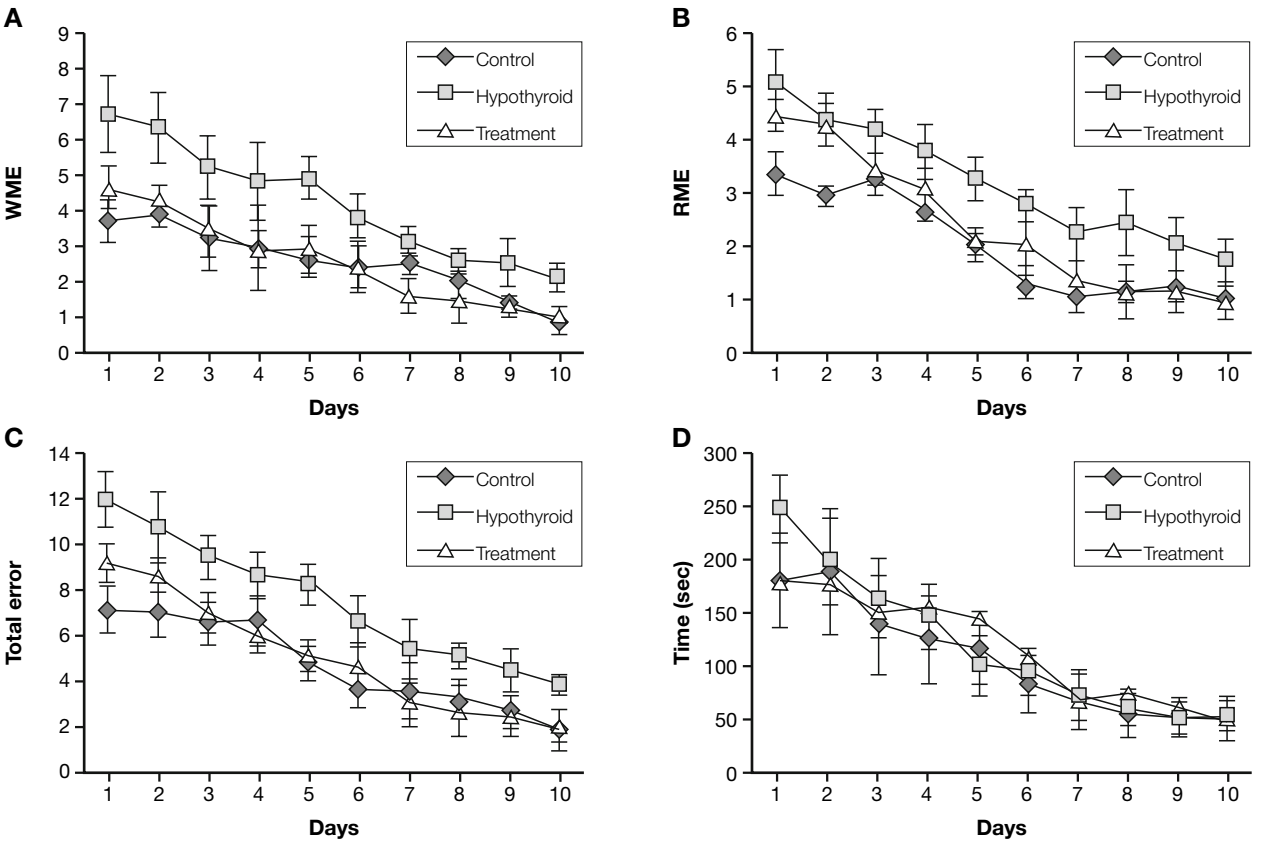

Fig. 5 - *The changes of learning and spacial memory in different groups (A: WME, B: RME, C: total errors, D: total time); WME: working memory errors; RME: reference memory errors. Data are mean±SD values.*

icantly as compared with that of the P21 groups (all $p<0.05$) (Fig. 7). There were no correlations between wire-hanging test and the number of apoptotic cells in the hippocampus (r=0.174, $p>0.05$). The numbers of apoptotic neurons in the CA3 region of P50 (P60) pup's hippocampus were positively correlated with the scores in its open-field test (r=0.726, $p<0.01$), the errors made in passive avoidance test (r=0.805, $p<0.01$) and the number of total errors in the radial maze task (r=0.620, $p<0.05$).

DISCUSSION

Perinatal hypothyroidism is known to markedly retard both maturation and development of the nervous system (9). In the present study, the P21 pups performed normally in the inclined plane test. Performance fell in the wire-hanging test in P21 pups, but was normal in the P50 pups. The result indicated that perinatal hypothyroidism may affect long-term neuromotor activity, and the result may be affected by different tasks. The wire-hanging task is mainly designed to test the muscle strength of the animal. As stated in the previous report, the most striking effects of perinatal hypothyroidism on psychomotor competence are upon muscular tone, but successful turning can already be seen at 5-7 days of age (8, 21). Muscle strength and psychomotor coordination recover quickly (9). Open-field test was considered an important way to measure the emotionality of animals. The ambulation in the test can indicate excitability state; times of rearing indicate the adaptability to the unfamiliar enviroment; number of fecal boli indicate the degree of anxiety of the animal. Open-field task test mainly assesses animals' independent behavior and exploring activities. The activity can reveal whether the central nervous system is in its excitability or depressing state. Animals will have an obvious protective reaction in the unfamiliar field. The open-field test will therefore provide a result of animal's alert, anxiety, and adaptability quantitatively (22). Our results yielded an increase of independent activities, reduction of adaptability and inhibition of

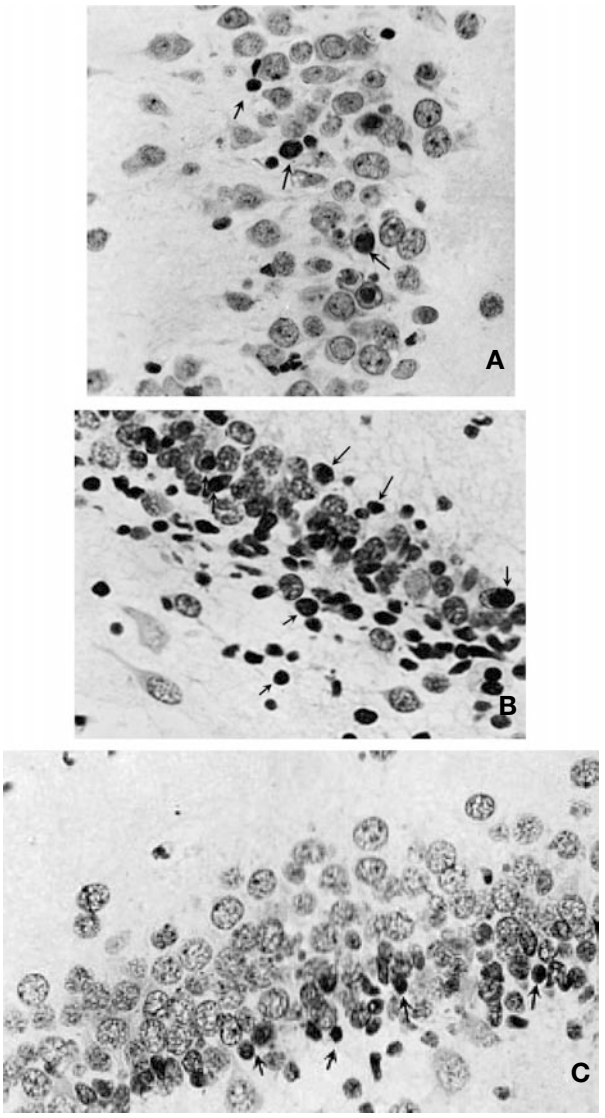

Fig. 6 - *The apoptotic cells in the hippocampus under microscope in different conditions. A: normal; B: hypothyroid; C: after treatment. Arrow: apoptotic cells. Most apoptotic cells exhibit a ring morphology, with densely stained cores (magnification ×400).*

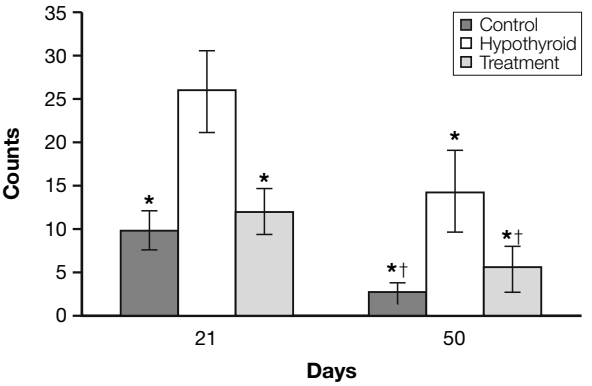

Fig. 7 - *The positive apoptotic neurons counting in the TUNEL staining. *: p<0.01, compared with the hypothyroid group of the same age; †: p<0.05, compared with parallel group of the post-natal day 21 (P21) group. Data are mean±SD values.*

anxiety behavior in the hypothyroid group compared with the control. All the P50 pups had a decrease in activity and an increase in adaptability compared with the P21 pups. This increase in the activities was reported to be the classic behavior change of the hypothyroid pups, and the reaction decreases with age (23).

Previous reports suggested that the inhibiting system of telencephalon began to take an effect in the normal pups at the age of 20 days, so activities decrease in the process to development (8). Vaccari et al. (24) documented that the nigral-striatal dopaminergic pathway is closely intertwined with locomotion and behavior. Hypothyroidism-related decreases in the maximum number of striatal D1- and, reportedly D2-receptors, plus the impairment of D1-coupled second messenger activity, may play a role in the derangement of those neurobehavioral patterns where a dopaminergic regulation is putatively implied. Thus, the activities of the hypothyroid pups still increased on the day of P21 hypothyroid pups. TH also regulates the secretion of other neurotransmitters, such as γ-aminobutyric acid (25), which induce more independent activities, decreasing some anxiety-related activity (26).

Passive avoidance test was one of the tasks involved in learning and memory capability testing for animals. Our results indicated that times into the dark compartment increased in the P50 hypothyroid pups, with a reduction of latency. Perinatal hypothyroidism impaired the recent learning capability. Meanwhile, the electrical stimulation in the test agitates and disturbs the animals, which may influence the experimental results. Other factors such as sensitivity to the electric stimulation and the basic activity of the animals is equally taken into consideration when evaluating the results (27). The present study suggested that the errors in learning increased markedly though hypothyroidism decrease anxiety-like behaviors to some extent. It is not clear whether it is due to less impact on anxiety than on learning ability. Further studies need to be carried out to explain this.

We used the radial maze task to test the spacial memory capability on hypothyroid pups. In this task, animals learned to remember the relative po-

sition between themselves and the baits by referencing fixed subjects. This task can exclude the effects of self-locomotion disorder on learning and memory capability (28). In the present study, WME of the P50 hypothyroid pups increased significantly compared with the normal control and the treatment group. But there was no difference in RME. This spacial memory separation can also appear in the anticholinergic treatment rats doing this task (29). Contrary to this, both the WME and RME increased in the pups with impaired hippocampus function (30). Our result indicated that the impairment of hippocampus functions in the hypothyroid pups is mild, but leads to unstable memory capability. Meanwhile, we found that there was no difference with total time in the maze among the 3 groups. Combined with the results in the open-field test, we hypothesized that it might be due to the increased activity in the hypothyroid pups.

TH involves the regulation of many structures and signaling proteins in the brain, so as to shape the proliferation, migration, differentiation of the neurons as well as the development of the synapse and myelin. Our present study yielded that perinatal hypothyroidism will induce apoptosis of the neurons in the hippocampus. As shown by TUNEL staining, positive apoptotic neurons were observed to be increased in the CA3 region of the hippocampus in the hypothyroid pups. The increased apoptosis has a positive correlation with the errors made both in the passive avoidance test and the radial maze task by the P50 pups. The latency of memory was negatively correlated with the apoptotic neurons. The hormone treatment pups in the present study showed an improvement in locomotion, behavior, learning and memory capability compared with the untreated hypothyroid pups, but still could not reach the normal level. Thus, perinatal hypothyroidism causes partly irreversible brain impairment; alterations of hippocampus structure and ability may be one of the important reasons. The locomotion alterations may also be connected with lack of TH in the regulation of muscle and bone development. In-depth research should be carried out on the extent of brain impairment induced by thyroid hormone deficiency.

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