RESEARCH

Subclinical hypothyroidism in pregnant rats impaired learning and memory of their offspring by promoting the p75^{NTR} signal pathway

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Abstract

Objective: Maternal hypothyroidism during pregnancy can affect the neurodevelopment of their offspring. This study aimed to investigate the effects of maternal subclinical hypothyroidism (SCH) on spatial learning and memory, and its relationship with the apoptotic factors in cerebral cortex of the offspring.

Methods: Female adult Wistar rats were randomly divided into three groups ($n = 15$ per group): control (CON) group, SCH group and overt hypothyroidism (OH) group. Spatial learning and memory in the offspring were evaluated by long-term potentiation (LTP) and Morris water-maze (MWM) test. The protein expression of the p75 neurotrophin receptor (p75^{NTR}), phospho-c-Jun N-terminal kinase (p-JNK), the pro-apoptotic protein p53 and Bax were detected by Western blotting.

Results: The Pups in the SCH and OH groups showed longer escape latencies in the MWM and decreased field-excitatory post synaptic potentials in LTP tests compared with those in the CON group. p75^{NTR}, p-JNK, p53 and Bax expression levels in the cerebral cortex increased in pups in the SCH and OH groups compared with those in the CON group.

Conclusions: Maternal SCH during pregnancy may impair spatial learning and memory in the offspring and may be associated with the increased apoptosis in the cerebral cortex.

Key Words

- subclinical hypothyroidism
- long-term potentiation
- p75 neurotrophin receptor signaling pathway
- cerebral cortex

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Introduction

Thyroid hormones (THs) are vital for fetal neurodevelopment. The prevalence of subclinical hypothyroidism (SCH) in the general population is 4–10% and can reach 5% in pregnant women. Haddow *et al.* investigated the intelligence levels of 62 children aged 7–9 years who were born to mothers with thyroid dysfunction during early pregnancy. They found that even mild and asymptomatic hypothyroidism in pregnant women, including SCH, could affect the development of intelligence in their offspring (1). Several experimental studies also found that maternal TH deficiency during pregnancy could cause irreversible brain damage in the offspring, resulting in varying degrees of mental retardation, cognitive impairment and learning and memory dysfunction (2, 3, 4, 5). We previously found that decreased activation of the cAMP response element-binding protein signaling pathway in pups was related to impaired cognitive function caused by maternal SCH (6). However, the relationship between cognitive impairment due to SCH and the apoptosis in cerebral cortex remains unclear.

The p75 neurotrophin receptor (p75^{NTR}) is an important member of the death receptor family. Although the role of the p75^{NTR}-mediated signal transduction

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pathway in promoting apoptosis is currently unclear, the activation of the JNK signaling pathway is thought to be an important step in this process (7, 8). Previous studies showed that the expression of p75^{NTR} increased in the cerebral cortex of pups from mothers with perinatal hypothyroidism, and the pro-apoptotic protein such as p53 and Bax increased (9, 10). However, whether neuronal apoptosis induced by the p75^{NTR} signaling pathway exists in the offspring of pregnant SCH rats remains unknown.

This study aimed to determine if the p75^{NTR} signaling pathway was activated in the cerebral cortex in the offspring of rats with SCH during pregnancy and to investigate its role in the apoptosis of cerebral cortex.

Materials and methods

Animals

This experiment used 45 female rats (weighing 190–210 g) that had never been pregnant. All female rats were kept in the Experimental Animal Department at China Medical University under specific pathogen-free conditions, in a constant environment at 23–25°C and 55% relative humidity with a 12-h light/darkness cycle, and provided with normal feed and water. Rats were fed normally for 1 week before the experiment. All animal experiments and procedures were approved by the Animal Care and Use Committee at China Medical University, which complies with the National Institutes of Health Guide for the Care and Use of Laboratory Animals (NIH Publications No. 8023, revised 1978). The rats were randomly assigned to three groups: control group (CON, $n=15$), overt hypothyroidism group (OH, $n=15$) and SCH group ($n=15$). Rats in the OH and SCH groups were treated by intraperitoneal (i.p.) injection of 3% pentobarbital sodium (0.1 mL/100 g) and underwent thyroidectomy. CON rats underwent sham thyroid surgery. After surgery, rats were kept at 34 ± 2°C under an electric blanket until they awoke. One month after surgery, rats in the SCH group were injected subcutaneously with L-thyroxine (L-T4, Sigma, USA) 1.0 μg/100 g/day on the back or neck. Rats in the CON and OH groups were injected subcutaneously with physiological saline (50 μL/100 g/day) on the back or neck. Calcium lactate (0.1% w/v) was added to the drinking water for all rats after surgery. After 9 days of injections, all rats were mated with normal male rats (male: female = 1: 2). Then, pregnant rats were placed in solitary confinement until delivery. The female rats were observed daily until they gave birth, and the delivery day was recorded as postnatal day 0 (PND0). All pups were weaned and the mothers stopped receiving subcutaneous injections on PND21. Venous blood (approximately 2 mL) was collected from all pregnant rats via the inner canthus vein on the 9th day after initial subcutaneous injection of L-T4 and on PND0, to detect total thyroxine (TT4) and thyroid-stimulating hormone (TSH) levels. Six to eight pups from each group on PND7 were decapitated on ice and the cerebral cortex was removed for Western blotting. The remaining pups were raised to PND39 and subjected to the Morris water-maze (MWM) test ($n=10$) and long-term potentiation (LTP) experiments ($n=6$), finishing on PND44. The project timeline is described in Fig. 1.

Measurements of TT4 and TSH

Blood samples obtained from the rats were immediately centrifuged at 20,913 ***g*** for 13 min and stored at −80°C for subsequent chemiluminescence immunoassay (Immulite, Diagnostic Products Corporation, Los Angeles, CA, USA) to measure serum TT4 and TSH. The inter- and intra-assay coefficients of variation (CVs) for TT4 was 3.83–7.09% and 1.58–3.68%, respectively. The inter- and intra-assay (CVs) for TSH were 1.87–5.43% and 2.34–3.47%, respectively. Based on >95% specific binding, the limit of detection for TT4 was 1.0 μg/dL. And the results samples below the level of detection were recorded at 1.0 μg/dL for statistical purposes.

MWM test

The MWM test was used to assess the spatial learning abilities of the pups in all groups. The MWM consists of a black circular swimming pool (100 cm diameter, 60 cm

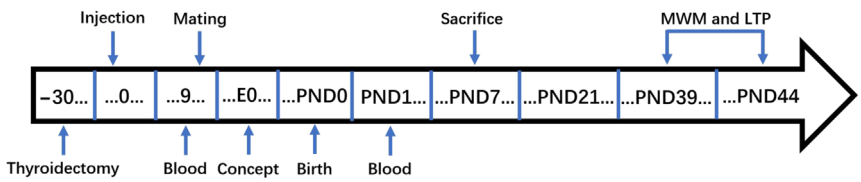

Figure 1
Schematic of experimental timeline. E0, gestational day 0; LTP, long-term potentiation; MWM, Morris Water Maze; PND, postnatal day.

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deep) divided into four equal quarters (I, II, III, IV) by two straight lines. A circular platform (10 cm diameter) is located 1 cm below the surface in the middle of quadrant IV. The rats were allowed to swim freely for 120 s on the first day (PND39) to adapt to the water maze and platform, and training was then started on the following day (PND40). Each pup trained four times a day, and rested for 1 min after each training session. The time required for the pup to climb the platform was defined as the escape latency. If the pup had not found the platform after 120 s, the escape latency was recorded as 120 s. All the pups that completed the experiment were lifted out gently, dried with a towel and then returned to their cages. The procedure was repeated for 4 days (PND40, PND41, PND42, PND43). On the sixth day, the platform was removed and the experiments performed in the previous 4 days were repeated. We recorded the number of times each pup reached the platform area and the time spent in the area for 120 s and assigned this as a probe trial. The escape latency for pups in each group was recorded daily. The time spent in the platform quadrant and the number of times they crossed the platform quadrant were measured in the last day's probe test.

LTP induction *in vivo*

We measured field-excitatory postsynaptic potentials (f-EPSPs) in the hippocampus of the pups in each group using a MED64 planar microelectrode matrix recording system (Alpha Med Science, Osaka, Japan). On PND40, the pups were decapitated on ice after anesthesia with 3% sodium pentobarbital (30 mg/kg, i.p.). Brain tissue was cut into 300-μm thick slices, removed and placed into oxygenated artificial cerebrospinal fluid containing 7.25 g/L NaCl, 0.22 g/L KCl, 2.18 g/L $NaHCO_3$, 0.12 g/L $MgSO_4$, 0.17 g/L KH_2PO_4, 1.8 g/L glucose and 0.22 g/L $CaCl_2$ (pH 7.35–7.45). The temperature of the brain was maintained at 34 ± 1°C and the flow rate of the artificial cerebrospinal fluid was 1–1.5 mL/min. The tissues were kept in a mixture of 95% O_2 and 5% CO_2. Synaptic plasticity in the CA1 area of the hippocampus at 6 weeks can be evaluated by high-frequency stimulation (HFS)-induced increase in f-EPSP after LTP. The baseline level before HFS was measured for 10–15 min and allowed to stabilize. Input/output curves were obtained by increasing the intensity of the stimulus and adjusting it to elicit 70% of the maximal response. The f-EPSP was recorded and the stimulus value corresponding to a 50% amplitude difference was recorded as the HFS. The tissue was stimulated twice with HFS, and LTP was induced 10 s later and recorded for at least 30 min. The percentage f-EPSP (f-EPSP%) increased after HFS was used as an indicator to evaluate LTP.

Western blotting

On PND7, the pups were decapitated on ice. The cerebral cortex was removed immediately and 0.1 g cerebral cortex tissue was added to 200 μL buffer containing protease and phosphatase inhibitors, homogenized by shaking (KeyGenBiotech, Nanjing, China) and centrifuged at 20,913 ***g*** for 10 min at 4°C. Protein concentrations were determined by the bicinchoninic acid method (Beyotime, Shanghai, China), and the samples were then stored at −80°C. Tissue lysates were diluted to the same protein concentrations (5 μg/μL), boiled for 8 min and 10 μL (50 g protein) samples from each group were then electrophoresed in 10% SDS-polyacrylamide gels. The marker was separated at constant voltage of 80 V for 30–60 min, and the proteins were then separated at a constant voltage of 120 V for 1 h. The proteins were then transferred to PVDF membranes (Millipore) at a constant voltage of 70 V for 2 h. Non-specific binding was blocked using a mixture of skimmed milk powder with Tris-buffered saline and Tween 20, with bovine serum albumin for p-JNK. The membranes were then incubated for 4 h in the dark with the following antibodies: rabbit anti-p75^{NTR} (1:1000 dilution; Cell Signaling Technologies); rabbit anti-total JNK (1:1000 dilution; Cell Signaling Technologies); rabbit anti-p-JNK (1:1000 dilution; Cell Signaling Technologies); rabbit anti-p53 (1:1000 dilution; Cell Signaling Technologies) and rabbit anti-Bax (1:1000 dilution; Cell Signaling Technologies). The blots were then incubated for 2 h with horseradish peroxidase-conjugated secondary antibody (1:10,000 dilution; Zhongshan Golden Bridge, Beijing, China) and developed by chemiluminescent Western blotting (ALPHAVIEW, version 1.3; ProteinSimple Inc., San Jose, CA, USA). The optimal time to expose the blot to the membrane was determined by standardization experiments.

Statistical analysis

Data processing and statistics were performed using SPSS 22.0 software (SPSS). The results were expressed as the mean ± standard error of the mean (s.e.m.). Multiple group comparisons were performed using one-way ANOVA test followed by Dunnett's T3 test. Independent sample *t* tests were used for comparisons between two groups. A value of $P<0.05$ was considered to be statistically significant.

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Results

TH levels in pregnant rats

We measured serum TT4 and TSH levels in pregnant rats to confirm successful establishment of the SCH and OH models. TSH levels were significantly higher in the SCH and OH groups compared with those in the CON group before pregnancy ($P<0.05$). TT4 levels were the lowest in the OH group among the three groups ($P<0.05$). And TT4 levels in the SCH group were significantly higher compared with those in the OH group ($P<0.05$). However, there was no significant difference between the SCH group and the CON group ($P=0.755$). On PND1, TSH levels in SCH group were higher than those in CON group, and TT4 levels were similar to those in CON group. These results indicated that the SCH models in rats were established successfully throughout the pregnancy (Fig. 2).

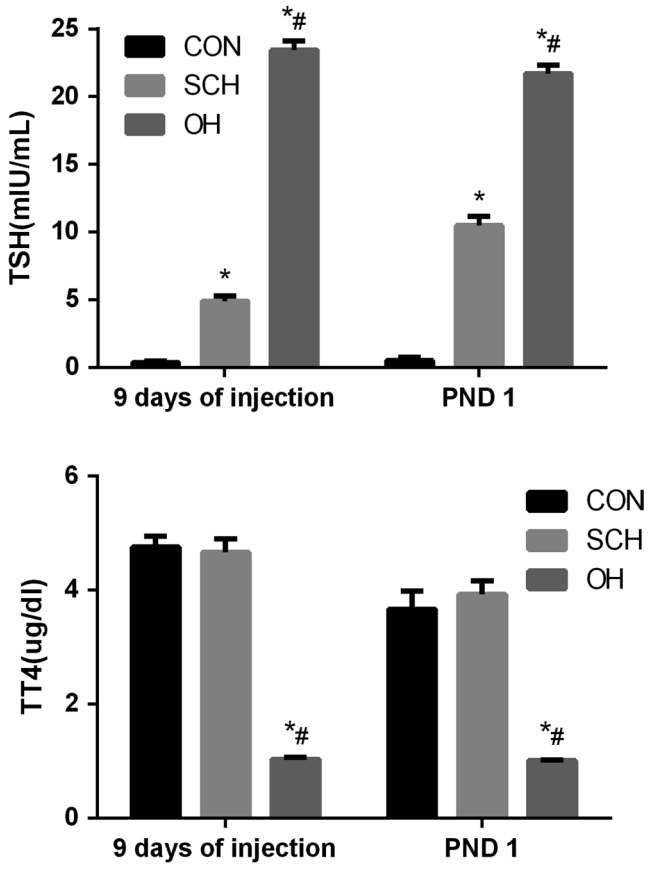

Figure 2
Thyroid-stimulating hormone (TSH) and serum total thyroxine (TT4) levels in pregnant rats (9 days of injection, $n=10$ per group; PND1, $n=6$ per group). *$P<0.05$ vs same day CON group; #$P<0.05$ vs same day SCH group. CON, control; OH, overt hypothyroidism; PND 1, postnatal day 1; SCH, subclinical hypothyroidism; TSH, thyrotropin; TT4, total thyroxine.

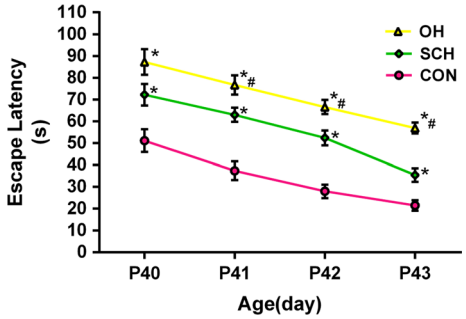

Figure 3
Performance of pups in the MWM test. Data are expressed as the mean±S.E.M. ($n=10$ for each group). Average time to find the hidden platform was longer in the SCH and OH groups compared with the CON group from PND 40–43.

Decline in learning and memory in offspring of rats with SCH during pregnancy

We assessed spatial learning and memory in the offspring of rats with and without SCH using the MWM test. Escape latency decreased with increased training in all groups. Escape latency was significantly longer in the SCH group compared with the CON group, but escape latency was the longest in the OH group ($P<0.05$; Fig. 3). The time spent in the platform quadrant in the probe trial was 29.5±2.4 s in the CON group, 21.4±1.3 s in the SCH group and 17.6±0.5 s in the OH group (all $P<0.05$; Fig. 4). We also calculated the number of times that the pups crossed the platform quadrant, which was 7.98±0.37 times in the CON group, 6.53±0.16 times in the SCH group and 5.30±0.34 times in the OH group (all $P<0.05$; Fig. 5). These results showed that spatial learning and memory reduced in the offspring of mothers with SCH during pregnancy.

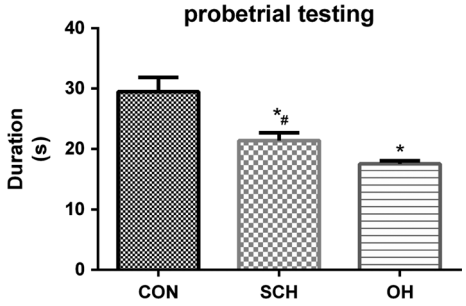

Figure 4
Probe trial test recorded the time spending by each pup in the platform area. Pups in the SCH and OH groups spent less times than pups in the CON group. *$P<0.05$ vs same day CON group; #$P<0.05$ vs same day OH group.

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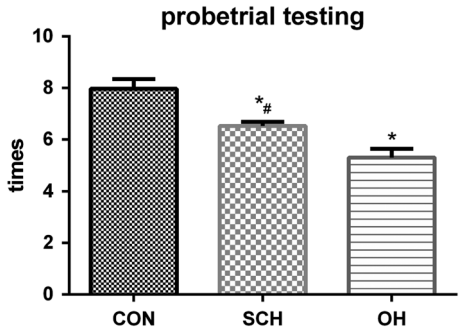

Figure 5
Probe trial test recorded the number of times pups crossed the platform quadrant. Pups in the SCH and OH groups crossed fewer times than pups in the CON group. *P<0.05 vs same day CON group; #P<0.05 vs same day OH group.

LTP in offspring of rats with SCH during pregnancy

The LTP results were evaluated by measuring the percentage of baseline f-EPSP (f-EPSP%) after HFS. There was no significant difference in f-EPSP among the three groups before HFS (P>0.05); however, all three groups showed an increase in f-EPSP after HFS, with significant differences in the f-EPSP% among the three groups. The f-EPSP% reduced in the SCH (175±1%) and OH groups (148±1%) compared with the CON group (209±1%) (n=6, P<0.05). However, the amplitude of the increase in the SCH group was significantly greater than that in the OH group (P<0.05; Fig. 6). These results suggested that SCH during pregnancy could damage LTP in the offspring.

Expression of the transcription factor p75^{NTR} in offspring of rats with SCH during pregnancy

We measured the expression levels of p75^{NTR} in the cerebral cortex of pups from mother rats in the three groups. p75^{NTR} levels were significantly increased in the SCH and OH groups to 1.56-fold and 2.36-fold higher compared with those in the CON group, respectively (n=6, both P<0.05). p75^{NTR} levels in the OH group increased to 1.54-fold higher compared with levels in the SCH group (n=6, P<0.05; Fig. 7).

JNK and p-JNK expression in offspring of rats with SCH during pregnancy

p-JNK expression and activation were determined by Western blotting, and p-JNK and total JNK were identified by rabbit polyclonal antibodies. p-JNK levels increased significantly in the pups of SCH (1.47-fold) and OH group (1.93-fold) compared with those in the CON group (n=6, both P<0.05; Fig. 6). However, there was no significant difference in total JNK among the three groups.

p53 expression in offspring of rats with SCH during pregnancy

Hypothyroidism promotes *N*-methyl-D-aspartate receptor activation, which in turn increases p53 expression, probably by increasing DNA damage and

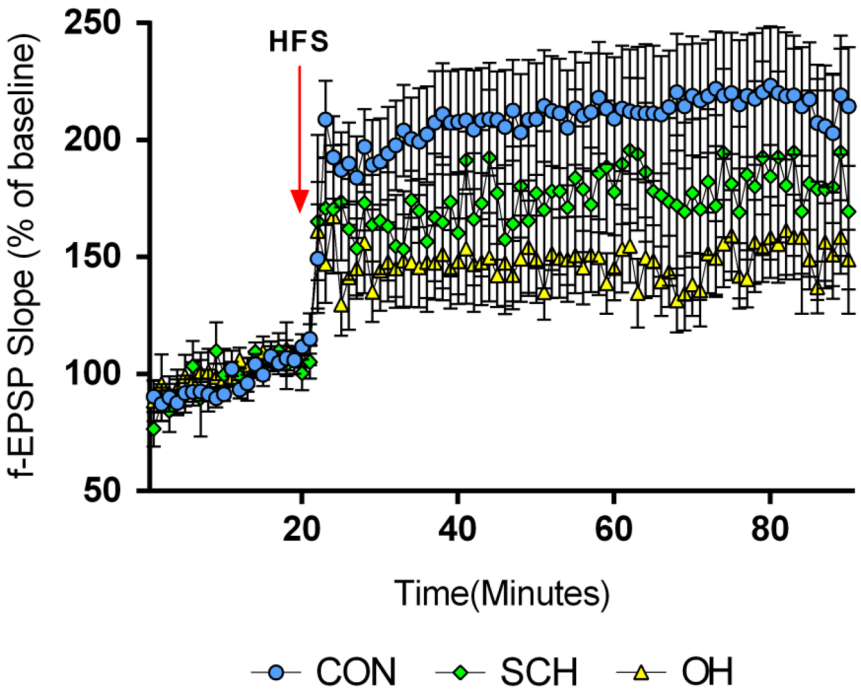

Figure 6
Maternal SCH caused LTP damage in the hippocampal CA1 region in their pups. LTP was induced by HFS and measured as an increase in f-EPSP slope, expressed as percent of the baseline of f-EPSP slope after HFS in different groups. f-EPSP slopes were reduced in the SCH and OH groups compared with the CON group (P<0.05).

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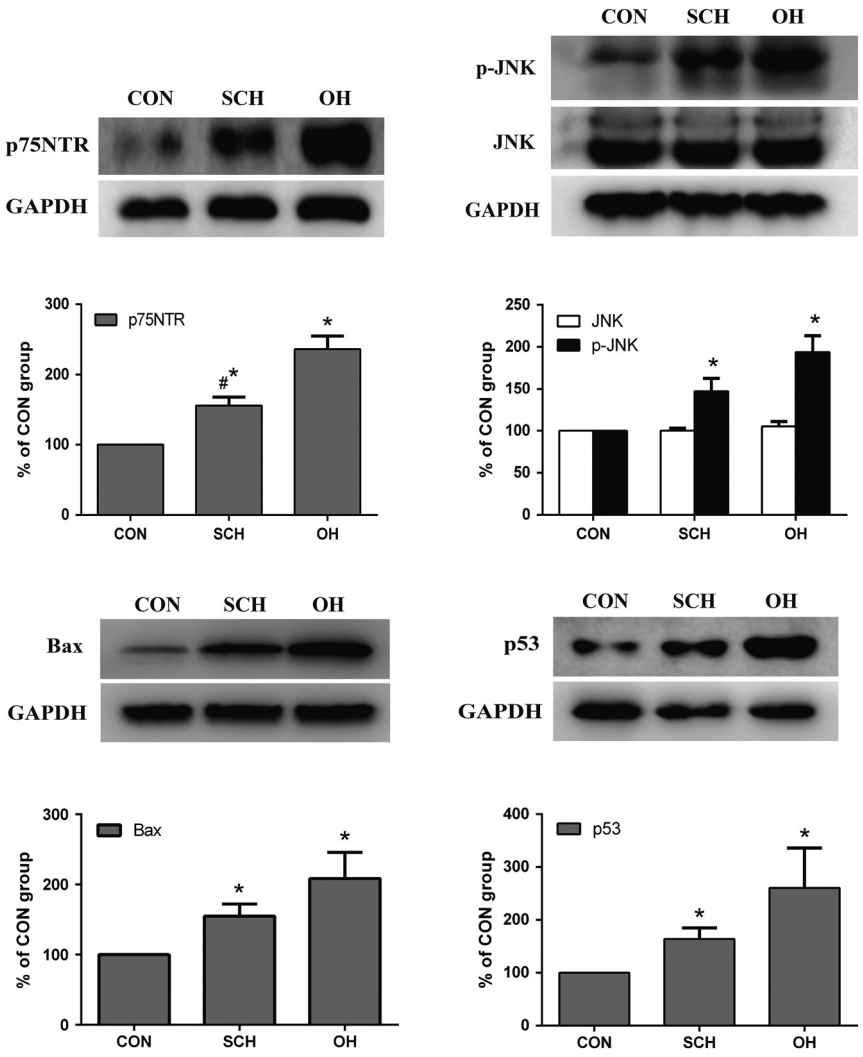

Figure 7
Protein expression levels of p75 neurotrophin receptor ($p75^{NTR}$), phospho-c-Jun N-terminal kinase (p-JNK) and pro-apoptotic proteins p53 and Bax in the cortex of pups ($n=6$ per group). $^{*}P<0.05$ vs CON group, $^{\#}P<0.05$ vs OH group.

triggers apoptosis leading to cell death (11). p53 is an important member of the $p75^{NTR}$ signaling pathway, and it can induce cell apoptosis. We detected p53 and other components of the $p75^{NTR}$ signaling pathway in this study. The expression levels of p53 increased significantly in the cerebral cortex of pups in the SCH (1.64-fold) and OH group (2.16-fold) compared with those in the CON group ($n=6$, both $P<0.05$), as shown by Western blotting (Fig. 7).

Bax expression in offspring of rats with SCH during pregnancy

p53 can induce apoptosis by raising Bax levels, leading to caspase activation (12). Bax expression increased in pups from the SCH (1.55-fold) and OH group (2.08-fold) compared with those in the CON group ($n=6$, $P<0.05$; Fig. 7). These results were consistent with a previous report (9).

Discussion

Epidemiological studies suggest that SCH during pregnancy may affect the offspring's intellectual development (1). Our group has performed a series of experiments to explore the mechanism in it (6). The current study explored the mechanism further from other aspect.

THs play a crucial role in fetal brain development and are involved in the proliferation, differentiation, maturation and migration of neurons in the brain. However, the fetal brain begins to develop before the thyroid, so THs needed for fetal brain development during early pregnancy are therefore derived exclusively from the mother. Moderate or even temporary hypothyroidism in early pregnancy may thus affect the normal migration of cortical neurons and the cellular architecture of the cerebral cortex and hippocampus (13). TH is also involved in apoptosis, which is another important physiological process in cerebral neuron development. Recent research

reported that neonatal hypothyroidism could cause extensive apoptosis of granular cells in the cerebellum (14, 15, 16, 17), while maternal hypothyroidism could induce apoptosis in the fetal cortex (9). We therefore investigated if maternal perinatal SCH could also induce apoptosis in their offspring's cerebral cortex.

Experimental hypothyroidism in neonatal rats was associated with structural defects in the nervous system accompanied by behavioral abnormalities (18). Methimazole and iodine deficiency have traditionally been used to establish models of thyroid deficiency, but these could potentially have other effects on embryogenesis. Surgical removal of the thyroid gland, together with TH supplementation, is thus a preferable method for achieving SCH. We successfully established a surgical model of SCH by this method.

The previous study performed by our group has reported that offspring of rats with either OH or SCH had cognitive impairment (6). Spatial learning and memory, measured by MWM test, were significantly decreased in the SCH group compared with the CON group and were more severely affected in the OH compared with the SCH group. In addition, these differences became more apparent with more training sessions. The probe test also indicated sustained memory impairment in the SCH and OH groups compared with the CON group. These results suggest that the pups born to mothers with either SCH or OH developed irreversible neuronal damage, and the neural network connectivity could not be reconstructed even after prolonged training. This was consistent with previous findings (6, 19, 20).

Hippocampal LTP is considered to be a suitable electrophysiological model for assessing synaptic plasticity in relation to learning and memory. The current results showed that prenatal exposure to OH and SCH led to a smaller increase in amplitude of f-EPSP after HFS, indicating LTP damage, consistent with previous studies (6, 19, 21). This supports the hypothesis that both OH and SCH may cause LTP impairment.

Maternal hypothyroidism can increase neuronal apoptosis during brain development (9), and previous studies showed that hypothyroidism increased apoptosis in the cerebellum (14, 15, 16, 17) and hippocampus (22, 23). Maternal hypothyroidism during pregnancy can also increase apoptosis during cerebral cortex development in the pups, especially in the surface layer of the primary somatosensory cortex (I-III level), which mainly affects neurons (9).

TH modulates levels of nerve growth factor (NGF) in the cerebral cortex, hippocampus and cerebellum in rats (14, 24). Synaptic plasticity, neuronal excitability and cognitive function are all regulated by NGF, which also plays critical roles in regulating hippocampal plasticity and learning in rodents (25, 26, 27, 28). NGF regulates neuronal cell proliferation and apoptosis by binding to two different receptor tyrosine kinase receptors A (TrkA) and p75^{NTR} (29, 30). When NGF levels decreases, the TrkA signal pathway is inhibited and the p75^{NTR} pathway is activated (31), thereby affecting learning- and memory-related protein expression, resulting in neuronal degeneration, apoptosis and mental retardation (32). ProNGF is a precursor protein of NGF and can induce apoptosis in many kinds of cells by combining preferentially with p75^{NTR} (31, 33, 34, 35, 36). p75^{NTR} can also transmit signals independently to promote the apoptosis of a variety of cells, including developing neurons (34, 35). The current results showed that p75^{NTR} expression increased in the cerebral cortex of pups of hypothyroid rats, even in the case of SCH, suggesting that elevated levels of maternal TSH may increase apoptosis in the cerebral cortex in their offspring.

Several models of p75^{NTR}-dependent cell death have demonstrated the involvement JNK activity (29, 35, 37, 38, 39, 40, 41). Increased p75^{NTR} has been reported to activate the JNK pathway, suggesting a link between JNK activation and cell death. Overexpression of the p75^{NTR} adaptor protein NRAGE was shown to induce apoptosis in a JNK-dependent manner (37). JNK activation is also considered to be a molecular switch in stress signal transduction (42). Some stimuli, including cytokines (such as tumor necrosis factor and interleukin-1) and reactive oxygen species, can activate the JNK pathway and play an important role in regulating cell fate, including cell proliferation, gene expression and apoptosis (43). Sherrin *et al.* also reported a role for JNK in the hippocampus in terms of memory and synaptic plasticity (44). JNK expression in the hippocampus and cortex may thus influence learning and memory.

p75^{NTR} can transduce a dichotomous signal with both pro-apoptotic and anti-apoptotic functions. The tumor suppressor p53 is a nuclear phospho-protein that can promote arrest or apoptosis, and it is an essential factor in JNK-mediated neuronal apoptosis. When DNA is damaged, p53 induces the expression of the cyclin-dependent kinase inhibitor p21, leading to cell cycle stagnation (45). Previous studies found that increased JNK activation was consistent with increased expression of the pro-apoptotic proteins p53 and Bax in hypothyroidism (10) (Fig. 8).

Activation of p75^{NTR}, JNK and p53 signaling have also been associated with increased neuronal apoptosis in

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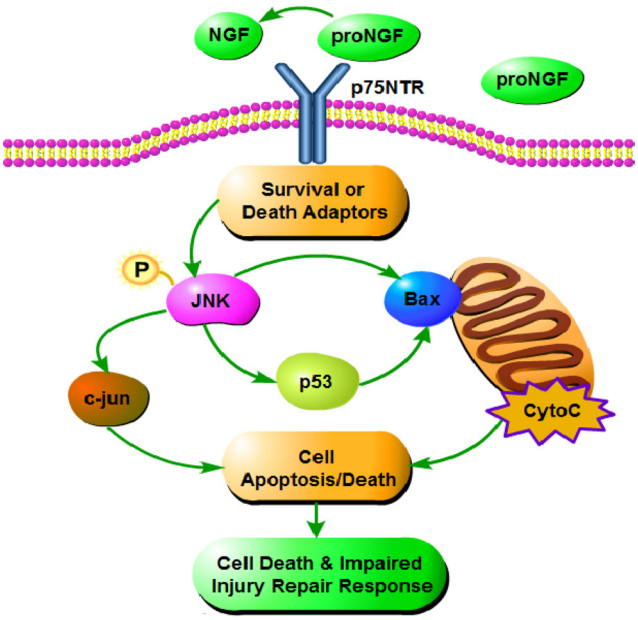

Figure 8
Effects of SCH and OH on p75^{NTR} signaling pathway. ProNGF, as a precursor protein of NGF, can combine with p75^{NTR} preferentially. p75^{NTR} activates the JNK pathway to increase the p53 signaling pathway. p53 and Bax play essential roles in JNK-mediated neuronal apoptosis. In the present study, maternal SCH and OH increased p75^{NTR} expression in the offspring, thus increasing activation of p-JNK, Bax and p53 signaling pathways and thereby increasing neuronal apoptosis.

Alzheimer's disease (46). Our results suggest that similar changes in terms of increased apoptosis occurred in the cerebral cortex of pups exposed to perinatal SCH. This may explain the mechanism involved in the damage to the neurodevelopment of the offspring from SCH mothers partly. However, some other potential mechanisms may exist. Zhang *et al*. found decreased activation of the CREB signaling pathway in pups was related to impairments of cognitive function caused by maternal SCH (6). Lu *et al*. reported maternal SCH affects cytoarchitecture and cell migration in the developing brain of the progeny (47). A recent study of patients with Alzheimer's disease and SCH showed that regional cerebral blood flow was significantly reduced, suggesting that SCH may affect the perfusion of brain regions associated with memory function (48). This may provide a new direction for research into SCH-induced memory deficits.

Limitations

Several limitations exist in our study. We did not perform Western blotting or other molecular biological experiments in the pups following MWM experiments, because we considered that the training may have affected the neurons in the cerebral cortex and thus affected spatial learning and memory. Furthermore, some rats died after thyroid surgery and some fetuses were aborted, which limited the availability of the cortical tissue for PCR, immunofluorescence, immunohistochemistry and other molecular experiments.

Conclusions

In summary, this study showed that perinatal SCH could increase the expression of p-JNK and the pro-apoptotic proteins p53 and Bax and increase neuronal apoptosis in the cerebral cortex of pups exposed to perinatal SCH, by activating the p75^{NTR} signaling pathway. These results help to explain the cellular mechanisms whereby maternal SCH during pregnancy may adversely affect the development of the offspring's cerebral cortex.

Declaration of interest
The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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