

ORIGINAL

# Resveratrol improved the spatial learning and memory in subclinical hypothyroidism rat induced by hemi-thyroid electrocauterization

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**Abstract.** The major purpose of this study was to investigate the effect of resveratrol (RES) on the spatial learning and memory ability in subclinical hypothyroidism (SCH) rat model and the potential mechanism. A SCH rat model was induced by hemi-thyroid electrocauterization and the activity of hypothalamus–pituitary–thyroid (HPT) axis was detected. The spatial learning and memory ability was tested using Morris water maze (MWM) and Y-maze. The protein expressions of synaptotagmin-1 (syt-1) and brain-derived neurotrophic factor (BDNF) in the hippocampus were measured *via* western blot. The results showed that SCH rat model was successfully duplicated. The SCH rats showed impaired learning and memory in the behavioral tests. However, these changes were reversed by the treatment of RES (15mg/kg) and levothyroxine (LT4). Moreover, RES treated rats exhibited reduced plasma TSH level and hypothalamic thyrotropin releasing hormone (TRH) mRNA expression, which suggested that the imbalance of HPT axis in the SCH rats could be reversed by RES treatment. Furthermore, RES treatment up-regulated the protein levels of syt-1 and BDNF in hippocampus. These findings indicated an amelioration effect of RES on the spatial learning and memory in the SCH rats, the mechanism of which might be involved with its ability of modifying the hyperactive HPT axis and up-regulating the hippocampal hypo-expression of syt-1 and BDNF.

**Key words:** Brain-derived neurotrophic factor, Hypothalamus–pituitary–thyroid (HPT) axis, Resveratrol, Subclinical hypothyroidism, Synaptotagmin-1

**SUBCLINICAL HYPOTHYROIDISM** (SCH), defined as an elevated serum thyrotropic-stimulating hormone (TSH) concentration in the presence of serum thyroid-hormone concentrations within normal range [1], is a common thyroid dysfunction that occurs in 4-20 % of the adult population [2, 3]. The risk of SCH for progression to overt hypothyroidism was around 7 % [4]. Although whether SCH patients should be

treated remains to be debated [5, 6] and even no cognitive difference has been found between SCH and euthyroid subjects in some clinical studies [7, 8], more evidence has suggested that SCH is implicated as one of the most risky factors for cognitive decline [9-11]. Apart from the finding that SCH during pregnancy and the early postnatal period could induce structural and functional abnormalities in the developing central nervous system [12, 13], it has been reported that spatial and verbal memories were impaired among SCH patients themselves [14]. Results of functional magnetic resonance imaging (fMRI) also showed that working memory function was impaired in SCH patients [15]. Consistent with the findings in human study, SCH rats showed impaired learning and memory in Morris water maze (MWM) task in our previous study [16]. Although some changes occur to SCH patients could be ameliorated by a levothyrox-

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ine (LT4)-replacement treatment, it has been revealed that neurocognitive impairment, induced by SCH, may not be fully restored by traditional hormone substitution therapies [17]. Moreover, the normal plasma free thyroxine (fT4) concentration in SCH patients and the adverse reaction of LT4 made it difficult to use the precise dose of LT4. Thus, it is necessary to find effective and safe treatments as alternative.

Resveratrol (trans-3,5,4'-trihydroxy-trans-stilbene, RES) is a polyphenol component found mainly in grapes and red wine and possesses diverse beneficial biological and pharmacological activities, including cancer chemopreventive [18] and antioxidant effects [19]. Recently, a growing number of studies have focused on its neuroprotective effect, which demonstrated that RES could improve the impaired learning and memory in healthy elders [20] and some neurodegenerative conditions including Alzheimer's disease (AD) [21], scopolamine exposure [22] or chronic unpredicted mild stress [23]. Moreover, RES treatment could increase the spontaneous locomotor activity, working memory, and spatial memory performance in non-human primates, mouse lemurs [24]. These findings indicated that RES may be a new strategy for therapeutic intervention of diseases associated with memory impairment.

Synaptotagmin-1 (synt-1), an abundant integral membrane protein, directly interacts with synaptosomal-associated protein of 25 kDa (SNAP-25) on the pre-synaptic membrane to facilitate neurotransmitter release [25]. It has been demonstrated that thyroid dysfunction affected synaptic proteins in adult rats [26]. Thyroid hormone deficiency resulted in the down-regulation of synt-1 expression in rat hippocampus [25, 27] and cerebellum [28], and the impairment in hippocampal-dependent tasks of learning and memory [29]. These findings emphasized a close relationship between thyroid dysfunction and synt-1. However, little is known about the effect of synt-1 on the spatial learning and memory impairment induced by SCH, in which the plasma concentration of thyroid hormone remains normal.

Brain-derived neurotrophic factor (BDNF), a protein belonging to the neurotrophic family of growth factors, has significant influence on the crucial process of brain development, including neurogenesis, neuronal differentiation, synaptogenesis and memory formation [30]. Previous studies have documented that thyroid hormones were crucial for BDNF expression [31]. BDNF expression was decreased in the hippocampus

of pups born to rats with SCH [13], while increased in hippocampus of the thyroxine-treated rats [32]. Given the well documented role of BDNF in brain development, adult brain function and plasticity, we propose that alterations in BDNF may underlie some of the persistent neurological impairments associated with SCH.

In the present study, a SCH rat model was induced by hemi-thyroid electrocauterization according to our previous study [33], and the behavioral tasks including MWM and Y-maze were used to explore the effects of RES on the learning and memory of the SCH rats. Moreover, the effect of RES on the activity of hypothalamus-pituitary-thyroid (HPT) axis and the protein expressions of synt-1 and BDNF in hippocampus of the SCH rats were also observed.

## Materials and Methods

### Drugs

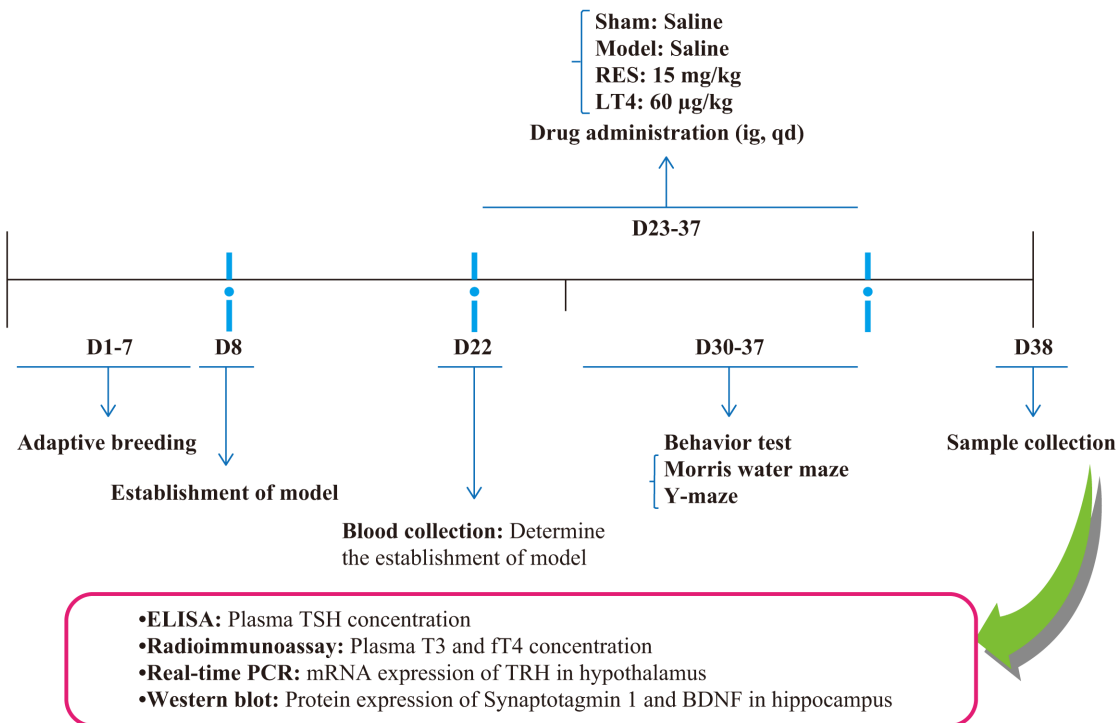
RES was purchased from Sigma Chemical Co. (St. Louis, MO, USA). LT4 was purchased from Berlinchem AG (Berlin, Germany).

### Animals

Forty-one Male Sprague-Dawley rats, aging 2 months, were purchased from Anhui Experimental Animal Center of China. The rats were housed 5 or 6 per cage (43 cm long  $\times$  31 cm wide  $\times$  19 cm high) and maintained under a 12:12 h light/dark cycle (lights on 0700 h). The ambient temperature was maintained at 21-22°C with 50-60% relative humidity. All experimental procedures in this study were approved by Animal Care and Use Committee at Anhui medical university, which complies with the National Institute of Health Guide for the Care and Use of Laboratory Animals (NIH publication No. 85-23, revised 1985).

### Animal model of SCH

Rats were randomly divided into four groups, including a sham group (n=8), a model group (n=11), a RES-treatment group (15 mg/kg/day + model, n=11) and a LT4-treatment group (60  $\mu$ g/kg/day + model, n=11). Thirty-three rats in the model, RES and LT4 group received a hemi-electrocauterization according to the procedures in our previous study [16], and 8 rats taking as sham ones received the same operation but the thyroid tissues were exposed without electrocauterization. In order to prevent the possible hypocalcemia resulted from the destruction of the parathyroid glands by elec-



**Fig. 1** Schedule of the experimental design

trocauterization, rats were provided with 0.1 % (w/v) calcium lactate in the drinking water after surgery.

### Injections

Two weeks after the operation, all the rats received a daily intragastric injection administration of sterile saline solution, RES or LT4 with the corresponding dose. The animals were continuously treated between 0800h and 0840h for 16 days.

### Behavioral tests

Behavioral tests were performed in a soundproof room with neutral environment in the order listed in Fig. 1. Briefly, the MVM test was carried out from day (D) 30 to D35, and the Y-maze from D36 to D37. All of the tests were carried out between 0930h and 1430h, with matching between the groups. The observers were blind to the treatment. The behavioral tests were monitored and recorded by a digital camera interfaced to a computer running the ANY-maze video imaging software (Stoelting Co, Wood Dale, American).

### Morris water maze

MWM was used to test the spatial learning and memory [34]. The maze consisted of black circular pool (diameter 160 cm, height 50 cm, filled with water

containing black pigment at 21-22°C to a height of 30 cm). A black circular platform (9 cm in diameter) was 2.0 cm below the water line in the center of one quadrant, and remained in the same position. Several, constant, large visual cues surrounded the tank at a height of 60 cm to facilitate orientation.

The task consisted of a 4-day acquisition phase with 4 massed trials administered each day and a 1-day memory retention test phase. During the acquisition phase, the rat was placed in the water facing the wall at one random start location of four (north, south, east and west, locating at equal distances from each other on the pool rim). Each rat was allowed to find the submerged platform within 60 s, and rest on it for 30 s. If the rat failed to find the hidden platform within 60 s, it was guided to the platform and allowed to remain there for 30 s. The procedure was repeated for all the four start locations.

On the 5th day (the test phase) following the 4 days acquisition phase, memory retention was determined in a single 60 s probe trial. The underwater platform was removed. The rats were placed into water from the opposite quadrant of the platform, facing the wall, and were permitted to explore the environment for 60 s *ad libitum*. Performance parameters of each rat including

total swim distance, mean swim velocity, and the duration in each quadrant were monitored and recorded.

#### **Y-maze**

Y-maze was made of wood and consisted of three arms with an angle of 120° between each of the two arms. Each arm was 10 cm × 48 cm × 20 cm (length × width × height). The three identical arms were randomly designated: start arm, in which the rat started to explore (always open), novel arm, which was blocked during the 1st trial, but open during the 2nd trial, and other arm (always open). The maze was placed in a separate room with a light. The floor of the maze was wiped by alcohol after each individual trial in order to eliminate olfactory stimuli. The Y-maze test consisted of two trials separated by an inter-trial interval (ITI) to assess spatial recognition memory. The first trial (training) had 10 min duration and allowed the rat to explore only two arms (start arm and other arm) of the maze, with the third arm (novel arm) blocked. 24 h after ITI, the second trial (retention) was conducted. The rat was placed back in the maze in the same starting arm, with free access to all three arms for 5 min, the number of entries and time spent in each arm were analyzed and novelty vs. familiarity was analyzed.

#### **Measurement of plasma concentrations of thyroid hormones**

Two weeks after the operation, blood samples (approximately 1 mL) were collected by angular vein to test whether the SCH rat model was successfully established. Twenty-four hours after the last behavioral test, rats were deeply anesthetized with chloral hydrate, and the blood was taken from the abdominal aorta. Plasma concentrations of TSH were measured with ELISA kits (Cusabio Biotech. Co., LTD, Wuhan, Hubei, China), according to the manufacturer's instructions, and fT4 and triiodothyronine (T3) were measured with radioimmunoassay kit (North institute of Biological Technology, Beijing, China), with the apparatus used in the assay that came from uste Zonkia (AnHui uste ZonKia scientific instruments co., LTD, Anhui China).

#### **RNA isolation and real time PCR**

After blood collection, 8 rats in each group (the rats in the model, RES and LT4 group were SCH rats without or with corresponding treatment) were selected to collect hypothalamus to test the parameters in the present study. Hypothalamus was rapidly dissected and frozen quickly in liquid nitrogen, and

stored at -80°C. Total RNA was extracted using the TRIzol (Invitrogen, Carlsbad, CA) method. cDNA was synthesized using reverse transcriptase (Promega, Wisconsin, USA). Q-PCR was performed using SYBR Green PCR Kit (Applied Biosystems, USA) and an ABI Prism 7000 Sequence Detector system in 25 µL volume for 40 cycles (15 s at 95°C; 60 s at 62°C). The primers used in our study were as follows: rat cyclophilin A 5'-GACTTCACACGCCATAAT-3' and 5'-TCTTCTTGCTGGTCTTGC-3'; Thyrotropin releasing hormone (TRH) 5'-AGCTCAGCATCTTGG AAAGC-3' and 5'-CCAGCAGCAACCAAGTC-3'. The relative amount of target gene was calculated using the  $2^{-\Delta\Delta C_t}$  method.

#### **Western blot assays**

The hippocampi from the same rats in 2.7 were rapidly dissected and collected, frozen in liquid nitrogen, and stored at -80°C. Tissues were homogenized in radioimmunoprecipitation assay (RIPA) buffer (50mM Tris-HCl, pH 7.4, 0.1% SDS, 1% NP-40, 0.25% sodium deoxycholate, 150mM NaCl, 1mM EDTA, 1mM EGTA, and 1mM Na<sub>3</sub>VO<sub>4</sub>). Before homogenisation, protease inhibitor cocktail (Roche, Indianapolis, USA) and the phosphatase inhibitor PhosSTOP (Roche, Indianapolis, USA) were added. Protein quantitation was conducted using a Lowry Protein Assay Kit (Meiji Biotech. Co., LTD., Shanghai, China). The same quantity (approximately 50µg) of protein from each animal was loaded and separated by 15% SDS-PAGE and then transferred onto a polyvinylidene difluoride membrane (Amersham Biosciences, UK). The membrane was blocked with 5% skim milk for 1h, incubated with antibodies targeting syt-1 (1:1000; ImmunoWay, Newark, Delaware, USA), cyclophilin A (1:1000; Cell Signaling Technology, Beverly, MA) or BDNF (1:1000; ImmunoWay, Newark, Delaware, USA) at 4°C over night, and then incubated with a horseradish peroxidase-conjugated secondary antibody (1:10000) at 37°C for 1 h. Blots were developed with the Easy Enhanced Chemiluminescence Western Blot Kit (Pierce Biotechnology, Rockford, IL, USA). Protein bands were scanned and analysed using ImageJ software (NIH).

#### **Statistical analysis**

All the statistical analyses were performed using SPSS (Statistical Package for the Social Sciences) version 12.0.1 (SPSS Inc., Chicago, IL, USA). Data are

**Table 1** Concentrations of plasma total triiodothyronine (T3), free thyroxine (fT4), thyroid-stimulating hormone (TSH) in sham (n=8), model (n=11), RES (n=11) and LT4 (n=11) before and after treatment

Group	n	T3 (nmol/L)		fT4 (pmol/L)		TSH (mIU/L)	
		Before	After	Before	After	Before	After
Sham	8	0.74±0.04	0.70±0.07	8.45±0.23	5.27±0.50	0.45±0.07	0.36±0.05
Model	11	0.77±0.04	0.79±0.08	8.10±0.38	5.72±0.47	2.59±0.33**	1.08±0.21*
RES	11	0.73±0.06	0.56±0.16	8.88±0.03	6.71±0.63	2.49±0.23**	0.65±0.10 <sup>#</sup>
LT4	11	0.74±0.06	0.91±0.15	8.35±0.66	13.97±1.08***	1.43±0.30*	0.14±0.05 <sup>##</sup>

The data were presented as mean ± SEM. \*  $P < 0.05$  and \*\*  $P < 0.01$  compared with sham group. <sup>#</sup>  $P < 0.05$  and <sup>##</sup>  $P < 0.01$  compared with model group.

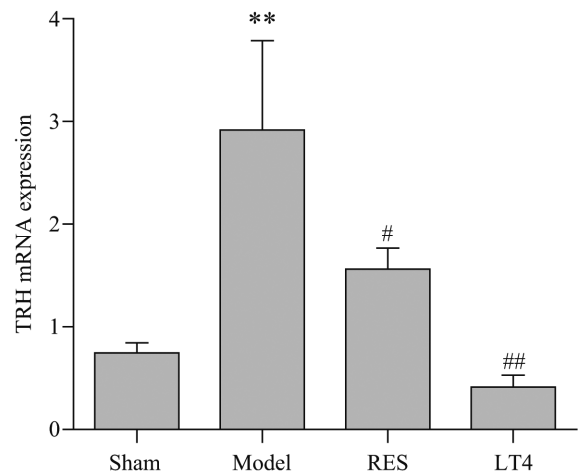
expressed as the mean ± SEM, and  $P < 0.05$  was considered statistically significant. The escape latency declined with each day during the acquisition phase in MWM test was analyzed by repeated measures ANOVA followed by Bonferroni test. Statistical analyses of between-group effects on other behavioural performance, the plasma concentrations of TSH, T3 and fT4, the mRNA expression of TRH, and the protein expression of syt-1 and BDNF were conducted using ANOVA followed by Bonferroni *post hoc* tests. Correlation analysis was performed using a Pearson correlation test.

## Results

### Assessment of SCH model and the effects of RES on the HPT axis

Before the treatment, 33 hemi-thyroid electrocauterized rats in the model, RES and LT4 group showed elevated plasma TSH level ( $F(3,37) = 14.876$ ,  $P < 0.001$ ; Bonferroni: sham vs. model:  $P < 0.001$ , sham vs. RES:  $P < 0.001$ , sham vs. LT4:  $P = 0.034$ ) with normal plasma fT4 ( $F(3,37) = 0.966$ ,  $P = 0.419$ ) and T3 ( $F(3,37) = 0.003$ ,  $P = 1.000$ ) concentrations compared with sham group (Table 1), which defines SCH in humans [36]. If diagnosed SCH according to the measures that both plasma TSH higher than 97.5 percentile of sham group and fT4 among 2.5 and 97.5 percentile of sham group, the success rate of SCH modeling was 75.8% (25/33), with 72.7% (8/11) in the model group, 72.7% (8/11) in the RES group and 81.8% (9/11) in the LT4 group.

After the 16 days' treatment, both RES and LT4 decreased the elevated TSH level (Table 1,  $F(3,37) = 5.914$ ,  $P = 0.002$ ; Bonferroni: sham vs. model:  $P = 0.003$ , RES vs. model:  $P = 0.050$ , LT4 vs. model:  $P < 0.001$ ). Consistent with this result, the hypothalamic

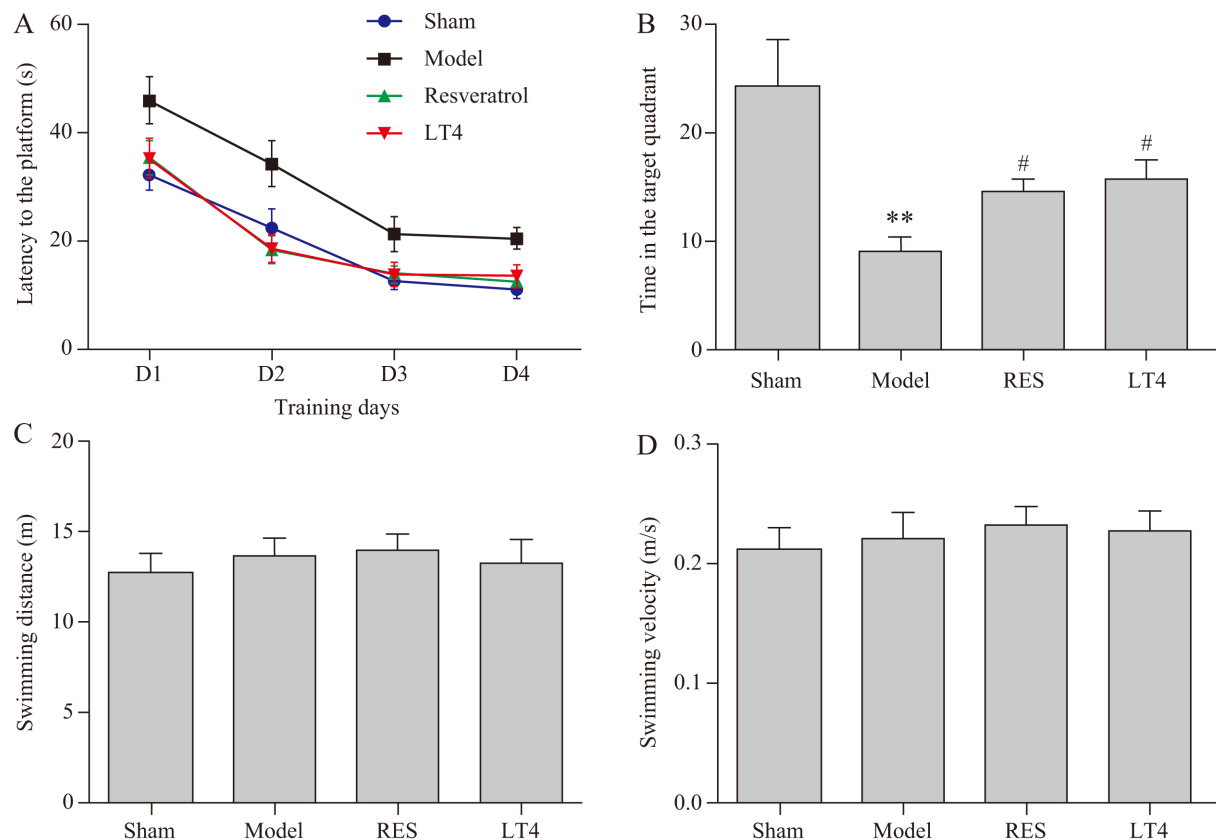
**Fig. 2** The effects of RES on the hypothalamic TRH mRNA expression in the SCH rats.

The mRNA expression of TRH in hypothalamus is illustrated. The data are presented as the mean ± SEM, with n=8 for each group. \*  $P < 0.05$  and \*\*  $P < 0.01$  compared with sham group. <sup>#</sup>  $P < 0.05$  and <sup>##</sup>  $P < 0.01$  compared with model group.

mRNA expression of TRH was inhibited by the treatment of RES or LT4 (Fig. 2,  $F(3, 16) = 10.528$ ,  $P < 0.001$ ; Bonferroni: sham vs. model:  $P = 0.003$ , RES vs. model:  $P = 0.04$ , LT4 vs. model:  $P = 0.001$ ). There was no significant difference in plasma T3 concentrations between groups (Table 1,  $F(3,37) = 1.387$ ,  $P = 0.262$ ). The treatment of LT4, but not RES, increased the plasma fT4 concentrations compared with sham or SCH rats (Table 1, ( $F(3, 37) = 30.909$ ,  $P < 0.001$ ; Bonferroni: LT4 vs. sham:  $P < 0.001$ ; LT4 vs. model:  $P < 0.001$ ).

### Effects of RES on the spatial learning and memory in MWM

For all the rats studied in this experiment, the escape latency declined with each day during the acquisition phase, which was shown in Fig. 3A. Analyzing the



**Fig. 3** The effects of RES on the spatial learning and memory in MWM.

Time in finding the escape platform during the acquisition phase (A), time in the target quadrant during the test phase (B), total swimming distance (C) and velocity (D) were observed. The data are presented as the mean  $\pm$  SEM, with  $n=8$  for sham group and  $n=11$  for model or RES or LT4 group. \* $P < 0.05$  and \*\* $P < 0.01$  compared with sham group. # $P < 0.05$  and ## $P < 0.01$  compared with model group.

latencies using repeated measures ANOVA with experimental treatment as between-subject factor and day as within-subject factor, the results showed that both experimental treatment and training days affected the learning ability (experimental treatment effect:  $F(3, 37) = 10.813$ ,  $P < 0.001$ , days effect:  $F(3, 111) = 58.86$ ,  $P < 0.001$ , and interactive effect:  $F(9, 111) = 0.630$ ,  $P = 0.769$ ). Moreover, when analyzed by Bonferroni test, the SCH rats showed increased escape latency compared with shams during the training days (sham vs. model: D1:  $P = 0.015$ ; D2:  $P = 0.02$ ; D3:  $P = 0.019$ ; D4:  $P = 0.001$ ). RES-treated and LT4-treated rats showed decreased escape latency compared with model ones, which revealed that the spatial learning was ameliorated by RES or LT4 treatment.

In the probe trial, SCH rats showed less time in the target quadrant which previously contained the platform (Fig. 3B), this behavior was reversed in the SCH rats treated with RES or LT4 ( $F(3,37) = 7.943$ ,  $P <$

0.001; Bonferroni: sham vs. model:  $P < 0.001$ , RES vs. model:  $P = 0.05$ , LT4 vs. model:  $P = 0.026$ ). However, there was no swimming distance and speed difference between groups (Fig. 3C and 3D).

#### Effects of RES on the spatial memory in Y-maze

When the arm differences in each group were analyzed, sham, RES-treated and LT4-treated rats spent more time in the novel arm compared with model ones (Fig. 4A) ( $F(3,37) = 6.182$ ,  $P = 0.002$ ; Bonferroni: sham vs. model:  $P < 0.001$ , RES vs. model:  $P = 0.002$ , LT4 vs. model:  $P = 0.026$ ). A negative correlation was found between TSH concentration and time in the novel arm in Y-maze ( $r = -0.370$ ,  $P = 0.017$ ). Similarly, as shown in Fig. 4B, percentage of duration spent in the novel arm of sham, RES-treated or LT4-treated rats was significantly increased compared with model ones ( $F(3,37) = 4.771$ ,  $P = 0.007$ ; Bonferroni: sham vs. model:  $P = 0.002$ , RES vs. model:  $P = 0.004$ , LT4 vs.

model:  $P = 0.04$ ).

The number of the arms that rat visits can be measured as the locomotor activity [35]. As shown in Fig. 4C, decreased number of the arm entries was found in the SCH rats compared with the sham ones, however, neither RES nor LT4 could reverse this change ( $F(3,37) = 6.991$ ,  $P = 0.001$ ; Bonferroni: sham vs. model:  $P < 0.001$ , RES vs. model:  $P = 0.107$ , LT4 vs. model:  $P = 0.111$ ), which indicated that RES treatment had no effect on the locomotor activity of the SCH rats in the Y-maze test.

#### Effects of RES on the protein expression of syt-1 in the hippocampus

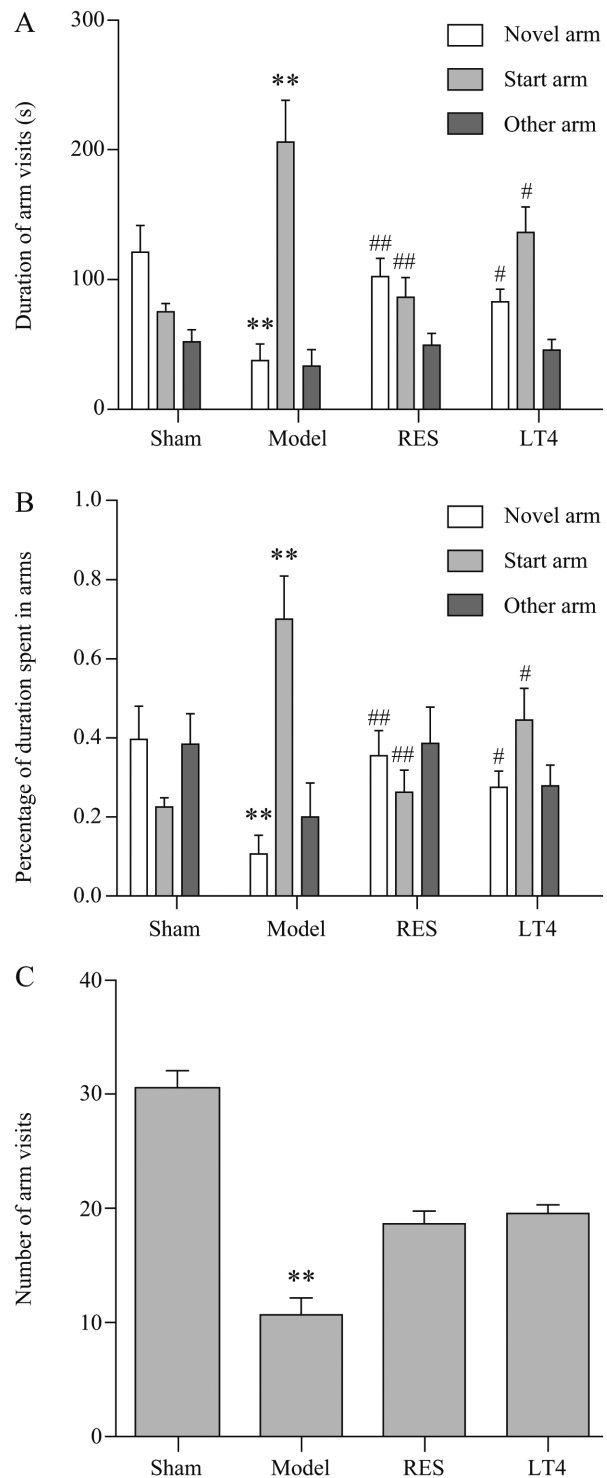
Fig. 5 shows the protein expression level of syt-1 in the rat hippocampus. Compared with that in the sham group, the protein level of syt-1 in hippocampus of the SCH rats was lower, but these changes were reversed by treatment with RES or LT4 ( $F(3,20) = 10.078$ ,  $P < 0.001$ ; Bonferroni: sham vs. model:  $P < 0.001$ , RES vs. model:  $P = 0.007$ , LT4 vs. model:  $P = 0.007$ ).

Interestingly, results of Pearson correlation analysis showed that the syt-1 protein expression was negatively correlated to the plasma TSH concentration ( $r = -0.523$ ,  $P = 0.007$ ) and positively correlated to the time in the target quadrant during the probe trail in MWM ( $r = 0.693$ ,  $P < 0.001$ ), but no correlation was found between the syt-1 protein expression and TRH mRNA expression ( $r = -0.378$ ,  $P = 0.124$ ).

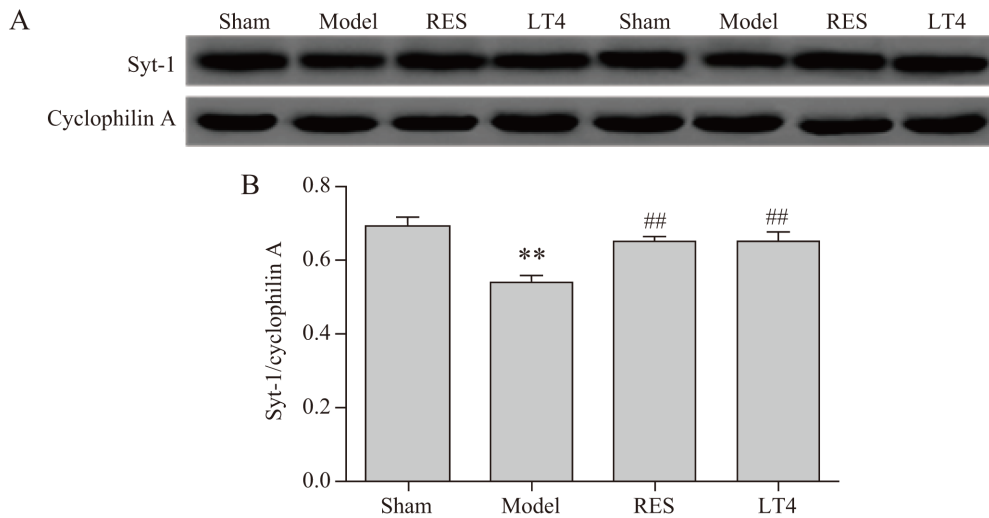
#### Effects of RES on the BDNF protein expression in the hippocampus

As shown in Fig. 6, the protein level of BDNF in hippocampus of the SCH rats was lower than that in the sham ones. However, both RES and LT4 treatment could reverse the change ( $F(3,20) = 17.061$ ,  $P < 0.001$ ; Bonferroni: sham vs. model:  $P < 0.001$ , RES vs. model:  $P < 0.001$ , LT4 vs. model:  $P = 0.003$ ).

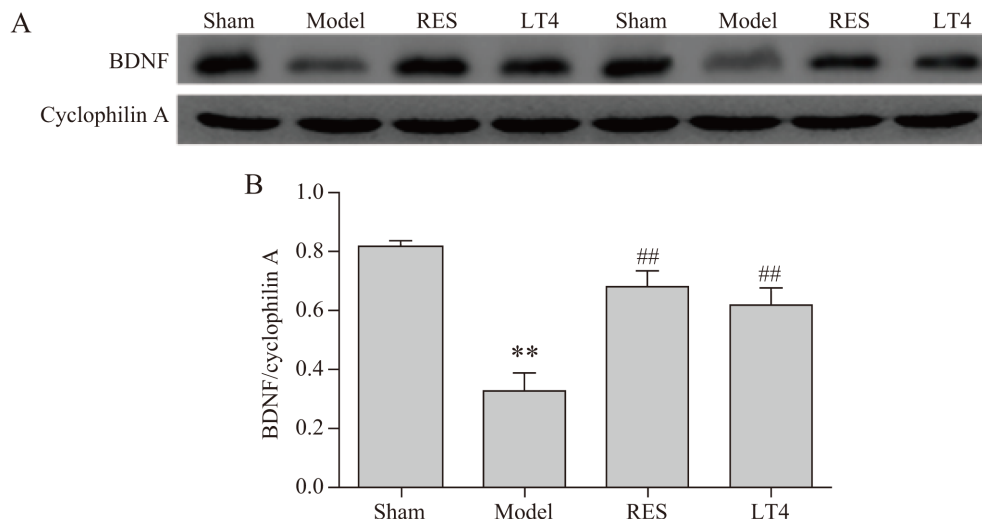
Results of Pearson correlation analysis showed the BDNF protein expression was negatively correlated to the plasma TSH concentration ( $r = -0.756$ ,  $P < 0.001$ ) and the TRH mRNA expression in the hypothalamus ( $r = -0.695$ ,  $P = 0.002$ ). Moreover, a positive correlation was found between the BDNF protein expression and the syt-1 protein expression ( $r = 0.738$ ,  $P < 0.001$ ). The BDNF protein expression was also positively correlated to the time in the target quadrant during the probe trail in the MWM ( $r = 0.523$ ,  $P = 0.009$ ).



**Fig. 4** The effects of RES on the spatial memory in Y-maze. Duration of arm visits (A), percentage of duration spent in arms (B) and total number of arm visits (C) were observed. The data are presented as the mean  $\pm$  SEM, with  $n=8$  for sham group and  $n=11$  for model or RES or LT4 group. \* $P < 0.05$  and \*\* $P < 0.01$  compared with sham group. # $P < 0.05$  and ## $P < 0.01$  compared with model group.



**Fig. 5** The effects of RES on the syt-1 protein expression in hippocampus. Representative blots of syt-1 protein expression in hippocampus (A). Quantitative analysis of syt-1 protein expression (B). The data are presented as the mean  $\pm$  SEM, with  $n=8$  for each group. \* $P < 0.05$  and \*\* $P < 0.01$  compared with sham group. # $P < 0.05$  and ## $P < 0.01$  compared with model group.



**Fig. 6** The effects of RES on the BDNF protein expression in hippocampus. Representative blots of BDNF protein expression in hippocampus (A). Quantitative analysis of BDNF protein expression (B). The data are presented as the mean  $\pm$  SEM, with  $n=8$  for each group. \* $P < 0.05$  and \*\* $P < 0.01$  compared with sham group. # $P < 0.05$  and ## $P < 0.01$  compared with model group.

## Discussion

In the present study, we explored the effect of RES on the spatial learning and memory in the SCH rats induced by hemi-thyroid electrocauterization. The results showed that RES could ameliorate the impaired spatial learning and memory of the SCH rats, as indicated by the decreased escape latency during the training days and increased time in the target quadrant dur-

ing the probe trial in MWM and a longer duration in the novel arm in Y-maze. RES-treated SCH rats exhibited reduced plasma TSH level and hypothalamic TRH mRNA expression. These findings suggested that the imbalance of the HPT axis in the SCH rats was reversed by RES treatment. Moreover, RES treatment up-regulated the protein levels of syt-1 and BDNF in hippocampus of the SCH rats.

Consistent with the result in our previous study [33],

the plasma TSH was increased with normal plasma T<sub>3</sub> and fT<sub>4</sub> concentration by hemi-thyroid electrocauterization in the present study, which indicated that a SCH rat model was duplicated. Based on this, the behavioral tests were conducted.

The MWM test, developed by Morris *et al.* in the early 1980s [12], has become one of the most widely used tasks for testing the spatial learning and memory of rodents [34]. In the present study, SCH rats showed spatial learning and memory impairment in MWM test, which was consistent with the findings in human studies [14, 15]. Similar to the effect of LT<sub>4</sub>, RES treatment could reverse the longer escape latency and shorter duration in target quadrant, which indicated an ameliorative effect of RES on the spatial learning and memory of the SCH rats.

The Y-maze, a simple two-trial recognition test for measuring spatial recognition memory, does not require learning of a rule, and thus is useful for studying memory in rodents [35]. Moreover, the two-trial recognition task is simply and rapidly conducted while minimizing the influences of motivation, emotionality and locomotor activity [36]. Thus, a paradigm using two-trial recognition test in Y-maze task was used in the present study. Consistent with the alleviation of the spatial memory in MWM, SCH rats spent less time in the novel arm compared with sham ones, which could be reversed by the treatment with RES or LT<sub>4</sub>. These results indicated a protective effect of RES on the impairment induced by SCH. It was reported that TSH was positively related to affective and cognitive neurobehavioral deficits in human beings including impaired learning and memory [37, 38]. Consistent with these findings, a negative correlation between the TSH concentration and the duration in the novel arm in Y-maze task was found in the present study.

Syt-1 is crucial for neural development, especially for synaptogenesis [29]. As a multifunction protein, syt-1 is also involved in neurite outgrowth [39]. Reduction in neurite growth can retard subsequent events in central nervous system development, such as synaptogenesis and myelination. Zhu *et al.* [29] reported a down-regulation of syt-1 in hippocampus of adult-onset hypothyroidism rats, which could be restored by the LT<sub>4</sub> treatment. Consistent with this finding, protein levels of syt-1 in hippocampus of the SCH rats were reduced in our study, and these changes could be restored by the treatment with RES and LT<sub>4</sub>. Although the mechanism remains unknown, the reduced expression of syt-1 may

be due to a decreased in the number and/or volume of neurons in hippocampus associated with hypothyroidism, considering it is an abundant constituent of synaptic vesicles [25, 29]. It has been reported recently that higher syt-1 in the limbic system can improve learning and memory [29], and knockout of mouse syt could result in impairments in contextual fear conditioning and associative passive avoidance memory [40]. Thus, the reduction of syt-1 level in hippocampus presented here may contribute to impaired spatial learning and memory ability in the SCH rats, and syt-1 might be a potential candidate for RES in ameliorating the impairment. However, the syt-1 protein expression was negatively correlated to the plasma TSH concentration, not the TRH mRNA expression. We are not exactly sure about the reason. It might be partly ascribed to the different sensitivity of TSH and TRH to the change of syt-1 protein, and the detailed mechanism should be investigated in our future study.

Previous reports have documented that maternal thyroid dysfunction could affect the expression of BDNF and cause neurological defects in neonates [41] and adult rats [30]. In the present study, decreased expression of BDNF was found in the SCH rats, and a positive correlation was found between the BDNF protein expression and the time in the target quadrant during the probe trial in MWM task, which indicated again the role of BDNF in the impaired learning and memory induced by SCH. However, these changes could be reversed by the RES or LT<sub>4</sub> treatment. It has been reported that TSH might exert its function in the brain through BDNF [42]. Clinical studies showed that higher TSH is associated with lower levels and more modest increase of serum BDNF during antidepressant treatment in patients with major depressive disorder [43]. Consistently, in the present study, our results indicated that the hippocampal BDNF protein expression was negatively correlated to the plasma TSH concentration and the TRH mRNA expression in hypothalamus. These findings proved that RES could improve the dysfunction of learning and memory and the decrease of BDNF in SCH rats through its regulating effect on the imbalanced HPT axis.

Due to the current lack of evidence regarding the optimal treatment strategy in individuals with SCH, LT<sub>4</sub> is the most widely acceptable form of replacement therapy. However, risks of treatment have been mainly associated with overtreatment, the rate of which ranged from 14% to 21% [44]. Consistently, with the dose

parallel to the routine dose used clinically, LT4-treated SCH rats showed significantly higher plasma FT4 concentration than the sham rats in the present study. Moreover, LT4 treatment could result in some possible adverse effects including atrial fibrillation, angina pectoris, congestive heart failure, and symptoms associated with excess thyroid hormone such as nervousness and palpitations [45]. Thus, better drugs are needed to improve the efficacy and safety of SCH treatment. A growing interest has emerged regarding the value of RES for its efficacy in metabolic regulation and neuroprotection with high safety margins [46, 47]. Results of animal studies showed that with the dose of 10 or 20 mg/kg, RES could significantly improve the cognitive impairment induced by intracerebroventricular streptozotocin [48] and chronic cerebral hypoperfusion [49]. In terms of human beings, chronic treatment of RES with the dose ranging from 200 mg [20] to 1000 mg [50] daily was used in clinical trials and demonstrated well tolerated [51]. Consistent with our previous study which demonstrated the anti-depressant effect of RES [52], the dose of 15 mg/kg was selected in the present study, and our results showed its ameliorative effect on the spatial learning and memory ability in a SCH rat

model. These findings might provide further evidence to its neuroprotective effect. However, with the efficacy of RES, its working mechanism, dose-relationship, and long-term side effects should be determined using different doses in our future study.

In conclusion, our results demonstrated that RES improved the spatial learning and memory in the SCH rats. This effect may be due, at least in part, to the regulation of HPT axis and the expression of syt-1 and BDNF in hippocampus.

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## Disclosure

All authors declare that they have no conflicts of interest.

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