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The role of the Wnt/BDNF pathway in maternal SCH-induced autism-like phenotypes in offspring rats: behavioral and molecular mechanisms

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Maternal subclinical hypothyroidism (SCH) has been associated with neurodevelopmental disorders, but the molecular mechanisms underlying its impact on offspring behavior remain poorly understood. This study investigates the role of the Wnt/BDNF signaling pathway in the development of autism-like behaviors in male offspring rats born to SCH mothers. Our findings demonstrate that maternal SCH induces significant behavioral abnormalities in the offspring, including increased grooming behavior and deficits in social interaction, which are hallmarks of autism spectrum disorder (ASD). These behaviors correlate with alterations in hippocampal protein expression, particularly a decrease in Brain-Derived Neurotrophic Factor (BDNF) and key signaling molecules involved in neuronal survival, such as cAMP response element-binding protein (CREB) and B-cell lymphoma 2 (Bcl-2). Additionally, we observe a marked upregulation of mTOR gene expression and a downregulation of Wnt signaling in the hippocampus of SCH-exposed offspring. These molecular changes are consistent with disrupted synaptic plasticity and neurogenesis, which are critical processes for cognitive and social development. Our study further reveals that impaired Wnt/BDNF signaling may play a pivotal role in the pathogenesis of autism-like behaviors in these offspring. Moreover, sex-specific differences were observed in the behavioral manifestations, with male offspring showing more pronounced deficits, suggesting a gender-dependent sensitivity to maternal SCH. This research provides novel insights into the molecular pathways by which maternal thyroid dysfunction contributes to neurodevelopmental disorders, offering potential targets for therapeutic intervention.

INTRODUCTION

Autism spectrum disorder (ASD) is a complex neurodevelopmental disorder characterized by core features such as impaired social interactions, repetitive behaviors, and restricted interests [1]. In recent years, the prevalence of ASD has significantly increased. This trend is not only linked to changes in diagnostic criteria and increased public health awareness but may also be profoundly influenced by environmental factors [2]. According to data from the Centers for Disease Control and Prevention (CDC), the prevalence of ASD in children has risen from approximately 1 in 44 in 2018 to 1 in 36 in 2023. Although the rapid rise in prevalence is concerning, the underlying mechanisms remain poorly understood [3, 4].

Recent studies have increasingly focused on maternal thyroid dysfunction during pregnancy, particularly thyroid hormone deficiency, as a potential key factor influencing offspring neurodevelopment [5, 6]. Maternal thyroid dysfunction during pregnancy is associated not only with an increased risk of psychiatric disorders such as attention deficit hyperactivity disorder (ADHD) and schizophrenia but also considered one of the important risk factors for ASD [7, 8]. A meta-analysis has shown that offspring of mothers with isolated hypothyroxinemia

(IH) have a 1.8-fold increased risk of developing ASD [9]. Furthermore, a study in the Netherlands found that severe thyroid hormone deficiency in early pregnancy can nearly quadruple the risk of ASD in offspring by the age of six [10].

Recent large-scale meta-analyses and cohort studies have further substantiated the link between maternal subclinical hypothyroidism (SCH) and adverse neurodevelopmental outcomes in offspring. Evidence indicates that SCH during early gestation—characterized by elevated maternal TSH with normal free thyroxine—correlates with significantly lower scores in cognitive domains, particularly receptive and expressive language abilities, as early as 12 months of age [11]. Furthermore, children born to mothers with SCH show increased risks of gross motor delays and reduced developmental quotient scores, even when thyroid autoantibodies are absent. Longitudinal assessments extending into early childhood have also demonstrated persistent deficits in adaptive behavior and neurocognitive function [12, 13]. These findings suggest that even mild maternal thyroid hormone insufficiency, if unrecognized or untreated, may disrupt critical neurodevelopmental windows, exerting long-term effects on brain maturation and functional integration.

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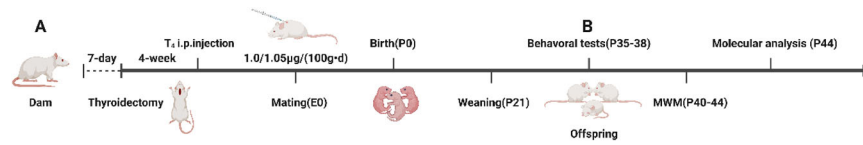
Fig. 1 Schematic diagram of the timing of assessing the behavior of offspring from maternally pregnant SCH rats. A Experimental design (thyroidectomy, T_4 injection, mating, birth, and weaning). **B** Timeline of offspring behavioral tests and molecular analysis. T_4 thyroxine, E embryonic day, P postnatal day, MWM Morris water maze.

Table 1. Serum TT4 and TSH levels in each group (Dams).

Group ($n = 12$)	E7		E13		P1	
	TT_4 (ug/dl)	TSH (mIU/L)	TT_4 (ug/dl)	TSH (mIU/L)	TT_4 (ug/dl)	TSH (mIU/L)
Control	3.67 ± 0.13	0.18 ± 0.33	4.05 ± 0.18	0.95 ± 0.29	3.98 ± 0.15	0.45 ± 0.25
SCH	3.45 ± 0.28	$1.98 \pm 0.57^*$	3.87 ± 0.08	$2.05 \pm 0.52^*$	4.07 ± 0.21	$2.55 \pm 0.35^*$

Data are presented as group mean ± SEM.

E7 embryonic day 7, *E13* embryonic day 13, *P1* postnatal day 1, *SCH* subclinical hypothyroidism, *TSH* thyrotropin, *TT4* total thyroxine.

*$p < 0.05$, versus same-day control.

Thyroid hormones play a crucial role in fetal brain development, especially during early pregnancy when the fetal hypothalamus-pituitary-thyroid (HPT) axis is not fully matured [13, 14]. Thyroid hormones not only regulate neuronal differentiation, migration, and synaptogenesis but also play a key role in the functional maturation of the central nervous system [15]. Therefore, maternal thyroid dysfunction during pregnancy may have profound effects on fetal neurodevelopment through complex molecular and cellular mechanisms [16]. These findings suggest that investigating the relationship between maternal thyroid dysfunction and ASD is of great importance for uncovering the mechanisms underlying the rising prevalence of ASD and for developing intervention strategies.

Although previous studies have indicated an association between maternal thyroid dysfunction during pregnancy and an increased risk of ASD, the exact underlying mechanisms remain unclear. In this study, we used a maternal subclinical hypothyroidism (SCH) model to systematically investigate the impact of SCH on offspring's autism-like behaviors and its potential molecular mechanisms. The results show that maternal SCH during pregnancy not only leads to noticeable autism-like behaviors in offspring but also causes a significant reduction in neuronal density in the hippocampal region. Further analysis revealed that key molecules related to neurogenesis and synaptic plasticity, including brain-derived neurotrophic factor (BDNF), cAMP response element-binding protein (CREB), and the anti-apoptotic protein Bcl-2, were significantly downregulated. Meanwhile, excessive activation of the mTOR signaling pathway and significant inhibition of the Wnt/β-catenin signaling pathway, along with the imbalance between these two signaling pathways, play an important role in the behavioral changes induced by maternal SCH.

Building on established evidence and our preliminary data, we hypothesize that maternal subclinical hypothyroidism (SCH) disrupts offspring neurodevelopment via suppression of Wnt/β-catenin signaling, leading to impaired CREB-dependent transcription and subsequent downregulation of BDNF. This cascade is predicted to drive synaptic destabilization and neuronal apoptosis, ultimately manifesting as autism-like behavioral phenotypes. Critically, we identify Bcl-2 and mTOR as pivotal regulatory hubs within this pathway, modulating both cell survival and synaptic integrity. Our findings delineate a novel mechanistic link between maternal endocrine dysfunction and neurodevelopmental pathology, while nominating actionable targets for early biomarker development and precision therapeutics in ASD.

MATERIALS AND METHODS
Animals
Twenty-four female and fifteen male Wistar rats (aged 6–8 weeks, weighing 180–220 g) were used, all housed in a specific-pathogen-free (SPF) barrier environment at the Experimental Animal Center. The housing conditions were maintained at a controlled room temperature of $22 \pm 2 \, ^{\circ}C$, 50% relative humidity, and a 12 h light/dark cycle. Prior to experimentation, all animals underwent a 7-day acclimatization period, during which they were provided with ad libitum access to tap water and standard laboratory chow. The animal facility was regularly cleaned, with bedding and cages replaced to ensure optimal hygiene and animal welfare. All animal experiments were conducted in strict accordance with the "Guide for the Care and Use of Laboratory Animals" and approved by the Animal Care and Use Committee of China Medical University (Approval No. KT20242238). Efforts were made to minimize animal usage and alleviate pain or discomfort. A detailed experimental timeline is shown in Fig. 1.

Establishment of the SCH maternal rat model
The 24 female rats were randomly divided into a control group and a subclinical hypothyroidism (SCH) group, with 12 rats in each group. Following our previous study [17], all surgeries were performed under general anesthesia with intraperitoneal injection of 3% sodium pentobarbital (0.1 ml/100 g). After anesthesia, the rats were fixed on the surgical table, and a midline incision was made on the neck. Subcutaneous tissue and the sternocleidomastoid muscle were dissected to expose the thyroid glands on both sides of the trachea. A midline incision was made at the thyroid isthmus, and the thyroid was carefully separated from the trachea using forceps, avoiding damage to the recurrent laryngeal nerve. After the surgery, the incision was sutured, and penicillin was administered to prevent infection. To prevent hypocalcemia caused by parathyroid dysfunction due to total thyroidectomy, a 0.1% (w/v) calcium lactate solution was added to the drinking water postoperatively, and the rats were fed a standard rat chow.

After thyroidectomy, rats in the experimental group were subcutaneously injected daily with 1.0–1.05 μg/100 g of L-T_4 (Sigma, USA) for supplementation. Successful establishment of the SCH model was indicated by a significant increase in serum TSH levels and normal TT_4 levels in the experimental group. One week later, the female rats were housed with normal male Wistar rats (male: female = 1:2). On the following morning, the presence of a vaginal plug was observed in the females. If a plug was found, the female was designated as embryonic day 0 (E0). During pregnancy, female rats were housed individually until delivery, which was marked as postnatal day 0 (P0). Drug interventions were discontinued after delivery, and normal drinking water was resumed. Blood samples of approximately 2 ml were collected from each female rat on embryonic days 7 (E7), 13 (E13), and postnatal day 1 (P1) to measure serum total T_4 (TT_4) and TSH levels (Table 1).

Serum hormone level assessment

Blood samples collected from the rats were immediately centrifuged at 13,000 rpm for 15 min and stored at −80 °C. Total thyroxine (TT$_4$) was measured using the electrochemiluminescence method (Roche Diagnostic Products, Los Angeles, California, USA). Thyroid-stimulating hormone (TSH) levels were determined using an enzyme-linked immunosorbent assay (ELISA) kit (Cloud-Clone Corp., Houston, Texas, USA). The detection range and intra-assay coefficient of variation (CV) for TT4 were 3.38–4.26% and 1.37–1.79%, respectively. For TSH, the detection range and intra-assay CV were <10 and <12%, respectively. All samples were measured in duplicate.

Behavioral testing

Unless otherwise specified, all behavioral tests were conducted on offspring rats aged 5–8 weeks, with 6–8 animals per group. The animals were habituated to the testing room for at least 1 h prior to each behavioral experiment. A minimum of 1 day of rest was allowed between each test. All behavioral experiments were independently repeated 3 times, and data were recorded by two blinded observers.

Self-grooming behavior

Behavioral experiments were conducted following previously described methods [18]. Each rat was placed individually in a clean, standard open space (40 cm × 40 cm), with a total experimental duration of 20 min, consisting of a 10 min habituation phase and a 10 min testing phase. During the habituation phase, rats were allowed to freely explore the environment to become familiar with the setup. The testing phase involved measuring the total time spent and the number of grooming actions performed by the rat within the 10 min period. A timer and video equipment were used to record the experiment from a distance of 1.5 meters from the cage for subsequent analysis. Self-grooming behavior was defined as actions such as licking the paws, licking the legs, nose washing, face washing, or scratching the head, body, fur, or tail with the paws. The recorded videos were used to analyze the frequency and duration of grooming behavior, ensuring accuracy and comprehensiveness in the analysis.

Open field test (OFT)

The open field test was used to assess the locomotor activity of the rats, following previously described methods [18]. The open field apparatus was made of gray plywood, measuring 40 cm × 40 cm, with walls 30 cm high. Each rat was placed individually in the center of the open field, and its locomotor behavior was continuously recorded by a camera fixed above the apparatus. The test duration was set to either 10 min or 30 min. Movement data were analyzed using Ethovision 11.0 (Noldus) software. Between tests, the apparatus was thoroughly cleaned with 70% ethanol and dried with clean paper towels to avoid odor interference. The center area of the open field, defined as 25% of the total area (approximately a 25 cm × 25 cm square), was used for further analysis of the rats' activity in the new environment and the time spent in the center area.

Three-chamber social test

The three-chamber test was used to assess the social behavior and social novelty preference of the offspring rats. The test consisted of three phases, each lasting 10 min with a 3–4 h interval between phases: the acclimation phase, social phase, and social novelty preference phase. Prior to each phase, the test rat was placed in the center chamber, with the two side doors closed.

Phase 1 (Acclimation phase). The test rat was allowed to freely explore the three-chamber arena for 10 min to familiarize itself with the experimental environment.

Phase 2 (Social phase). In one side chamber, an unfamiliar Wistar rat of the same age as the test rat (Stranger 1) was placed in a metal cage; in the other side chamber, an identical empty metal cage was placed. The test rat was free to explore all three chambers, and the time spent sniffing Stranger 1 was recorded to assess social interaction.

Phase 3 (Social novelty preference phase). The empty metal cage was replaced with a cage containing a novel Wistar rat (Stranger 2). The test rat was allowed to freely move between the chambers containing the familiar rat (Stranger 1) and the novel rat (Stranger 2). The time spent in each chamber and the time spent sniffing each stranger were recorded to assess the rat's preference for social novelty.

All parameters, including the time spent by the test rat in each chamber and the time spent sniffing Stranger 1 and Stranger 2, were recorded and analyzed using the automated Noldus Observer software (Ethovision 11.0). Data analysis was performed by researchers unaware of the experimental treatment to ensure objectivity. After each test, the apparatus and related items were thoroughly cleaned to avoid odor interference. To minimize the potential effects of target cage odors on the results, multiple unfamiliar rats from different home cages were used in the experiment.

Morris water maze (MWM)

The Morris Water Maze (MWM) test was performed on male offspring rats for behavioral assessment, following the previously described methodology [17]. Before the experiment, the rats were allowed to swim freely in the pool without a platform for 60 s to acclimatize to the environment.

Spatial learning phase. In subsequent trials, the rats were required to find a hidden platform submerged underwater within 60 s. If the rat failed to find the platform within the allotted time, the experimenter manually guided it to the platform, where the rat remained for 30 s. Training was conducted three times a day for four consecutive days.

Spatial memory test. On the fifth day, the platform was removed, and rats were tested for spatial memory. The following parameters were recorded and analyzed using Noldus Observer software (Ethovision 11.0): escape latency, platform crossing frequency, time spent in the platform quadrant, and average swimming speed. These metrics provided a comprehensive evaluation of the rats' learning and memory abilities.

Tissue processing and histological staining

Hippocampal tissue morphology was observed via Hematoxylin and Eosin (H&E) staining and Nissl staining at postnatal day 44 (P44). Rats were anesthetized using 10% chloral hydrate and then subjected to cardiac perfusion with cold physiological saline followed by 4% paraformaldehyde (PFA) in 0.01 M phosphate-buffered saline (PBS, pH 7.4) for 30 min. Following perfusion, the brain was removed and stored overnight in 4% PFA PBS at 4 °C, followed by cryoprotection in 30% sucrose at 4 °C.

Coronal sections were paraffin-embedded and sliced into continuous 5 μm thick coronal sections of the prefrontal cortex, which were then stained using H&E and Nissl staining (blue hematoxylin). Nissl body total integrated optical density (IOD) in the hippocampal CA1 region was measured under 40× and 400× magnifications using a Cast-grid microscope (MetaMorph/DP10/Bx41, UIC/OLYMPUS, US/JP) and image analysis software (MetaMorph Offline 4.65).

Western blot

Hippocampal samples from rats were collected and processed according to our previous methods. Protein was extracted using a BCA kit (Pierce™, Rockford, IL, USA) and mixed with 0.2% bromophenol blue and 7.5% β-mercaptoethanol. Protein samples were separated by 10% SDS-PAGE and transferred to a nitrocellulose membrane. The membrane was blocked in TBS-T (50 mM Tris-HCl, pH 7.4, 100 mM NaCl, 0.2% Tween-20) with 5% non-fat milk while gently shaking for 30 min.

The membrane was then incubated overnight at 4 °C with primary antibodies, including BDNF (1:1000; Abcam), CREB (1:1000; Cell Signaling Technology), Bcl-2 (1:1000; Cell Signaling Technology), mTOR (1:1000; Cell Signaling Technology), Wnt1 (1:1,000; Proteintech), Gapdh (1:1000; Cell Signaling Technology) and β-actin (1:1000; Cell Signaling Technology). After incubation, the membrane was washed twice with distilled water and then washed with TBS-T, followed by incubation with horseradish peroxidase-conjugated anti-mouse/anti-rabbit IgG (1:10,000–15,000) for 1 h at room temperature.

The membrane was further washed with TBS-T, and the procedure was repeated five times. The optical density values of the target bands were analyzed using a gel image processing system (Bio-Rad).

Quantitative real-time PCR

Total RNA was extracted from the hippocampal tissue of offspring rats using TRIzol reagent (Thermo Fisher Scientific, USA) according to the manufacturer's instructions. RNA concentration was determined using a microplate reader (Molecular Devices, USA). Next, total RNA was reverse transcribed into cDNA using the Revert Aid First Strand cDNA Synthesis Kit (Fermentas, USA) following the manufacturer's protocol.
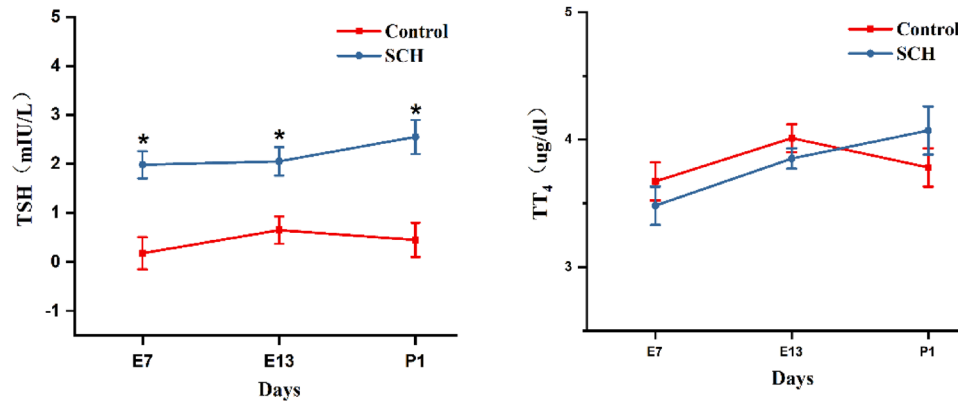
Fig. 2 Serum levels of thyroid-stimulating hormone (TSH) and total thyroxine (TT₄) were measured across all groups of dams on embryonic day 7 (E7), embryonic day 13 (E13), and postnatal day 1 (P1) ($n = 12$ per group). Compared with the control group, significant differences were observed in the SCH group. Statistical significance was indicated as *$P < 0.05$. E7 embryonic day 7, E13 embryonic day 13, P1 postnatal day 1, SCH subclinical hypothyroidism, TT₄ total thyroxine.

Quantitative PCR (q-PCR) was performed using the Quantitect SYBR Green PCR Kit (Thermo Fisher Scientific, USA) on the ABI PRISM 7500 real-time system (Applied Biosystems, USA). The amplification parameters were as follows: 95 °C for 30 s, 95 °C for 5 s, 60 °C for 30 s, for 40 cycles, with signal detection at 60 °C.

The data were quantified using the $2^{-\Delta\Delta Ct}$ method and normalized to β-actin expression levels. The primer sequences (Sangon Biotech) used were: mTOR: 5′-ATCGTGCTGTTGGGTGAGAG-3′ and 5′-TGGATCTC-CAGCTCTCCGAA-3′ Wnt1: 5′-AACAGTAGTGGCCGATGGTG-3′ and 5′-GGGTTCTGTCGGGATCAGTCG-3′ β-actin: 5′-GGCTGTATTCCCCTCCATCG-3′ and 5′-CCAGTTGGTAACAATGCCATGT-3′.

Statistical analysis
All data are expressed as mean ± standard error of the mean (SEM). Statistical analyses were performed using SPSS software. Group comparisons were conducted using one-way analysis of variance (ANOVA) followed by Tukey's post-hoc test. For the escape latency data from the Morris water maze (MWM), one-way repeated measures ANOVA was used. All graphs and analyses were conducted using GraphPad Prism 8 (GraphPad Software Inc.). The level of statistical significance was set as follows: *$P < 0.05$, **$P < 0.01$, NS indicates no significant difference.

RESULTS
Assessment of thyroid hormone levels in maternal rats
To verify the successful establishment of the maternal SCH model, we measured serum TT₄ and TSH levels on gestational days 7, 13, and at parturition. The results showed that the TSH levels in the SCH group were significantly higher than those in the control group at all time points ($P < 0.05$), while the TT₄ levels showed no significant differences compared to the control group. These findings are consistent with the diagnostic criteria for SCH, confirming the successful establishment of the maternal SCH rat model. Furthermore, on postnatal day 40, the average body weight of the offspring rats was as follows: 122.11 ± 3.66 g for the control group and 117.00 ± 4.75 g for the SCH group, with no significant difference between the two groups (Table 1, Fig. 2).

Stereotypic behaviors in SCH offspring rats
Stereotypic behaviors were assessed by measuring the frequency and duration of behaviors such as paw licking, face and nose washing, and body scratching during free exploration (Fig. 3A). The results showed that male offspring in the SCH group exhibited significantly higher frequencies and durations of self-grooming behaviors compared to the control group ($P < 0.01$), while there were no significant differences in total distance traveled. In female offspring, the SCH group also showed a significantly higher frequency of self-grooming behaviors compared to the control group ($P < 0.05$), although no significant differences were found in

the duration of activity or total distance traveled. These findings suggest that maternal SCH induces increased stereotypic behaviors, particularly in male offspring, and that these differences are not due to changes in motor activity. This is a hallmark feature of stereotypic behavior, potentially indicating autistic-like traits (Fig. 3B–D).

Open field test (OFT)
To exclude potential confounding factors of motor dysfunction affecting social behavior, we assessed the motor performance of the rats using the Open Field Test (Fig. 4A). The results showed that male rats in the SCH group spent significantly less time in the center of the open field compared to the control group ($P < 0.05$), indicating pronounced anxiety-like behavior in the SCH male offspring (Fig. 4B). In contrast, there were no significant differences in the time spent in the center of the open field between the SCH and control female rats. Furthermore, no significant differences were found in the total distance traveled between either the male or female groups (Fig. 4C).

Social interaction deficits in SCH offspring rats
Social behavior of the two groups of offspring rats was assessed using the three-chamber social test (Fig. 5A, B). The results showed that male rats in the control group spent significantly more time sniffing stranger 1 (Stranger 1) than the empty cage, indicating normal social behavior ($P < 0.01$) and suggesting no social deficits in the control group male rats. In contrast, male rats in the SCH group did not show a significant difference in the time spent sniffing Stranger 1 and the empty cage, indicating notable social interaction deficits. Additionally, in the social novelty preference and motivation test, control group male rats spent significantly more time sniffing Stranger 2 (novel mouse) than Stranger 1 ($P < 0.01$), displaying normal social novelty preference behavior. However, male rats in the SCH group did not show a significant difference in the time spent sniffing Stranger 1 and Stranger 2, suggesting autism-like behavior (ASD-like behavior), including a lack of novelty preference and reduced social behavior (Fig. 5C, D).

In female rats, control group females spent significantly more time sniffing Stranger 1 (Stranger 1) compared to the empty cage, demonstrating normal social behavior ($P < 0.01$) with no social behavior deficits. Similarly, SCH group females did not show a significant difference in the time spent sniffing Stranger 1 and the empty cage, indicating clear social behavior deficits. In the social novelty preference test, only the control group females exhibited normal social novelty preference behavior, spending significantly more time sniffing Stranger 2 than Stranger 1 ($P < 0.01$). In contrast, SCH group females showed no significant difference in
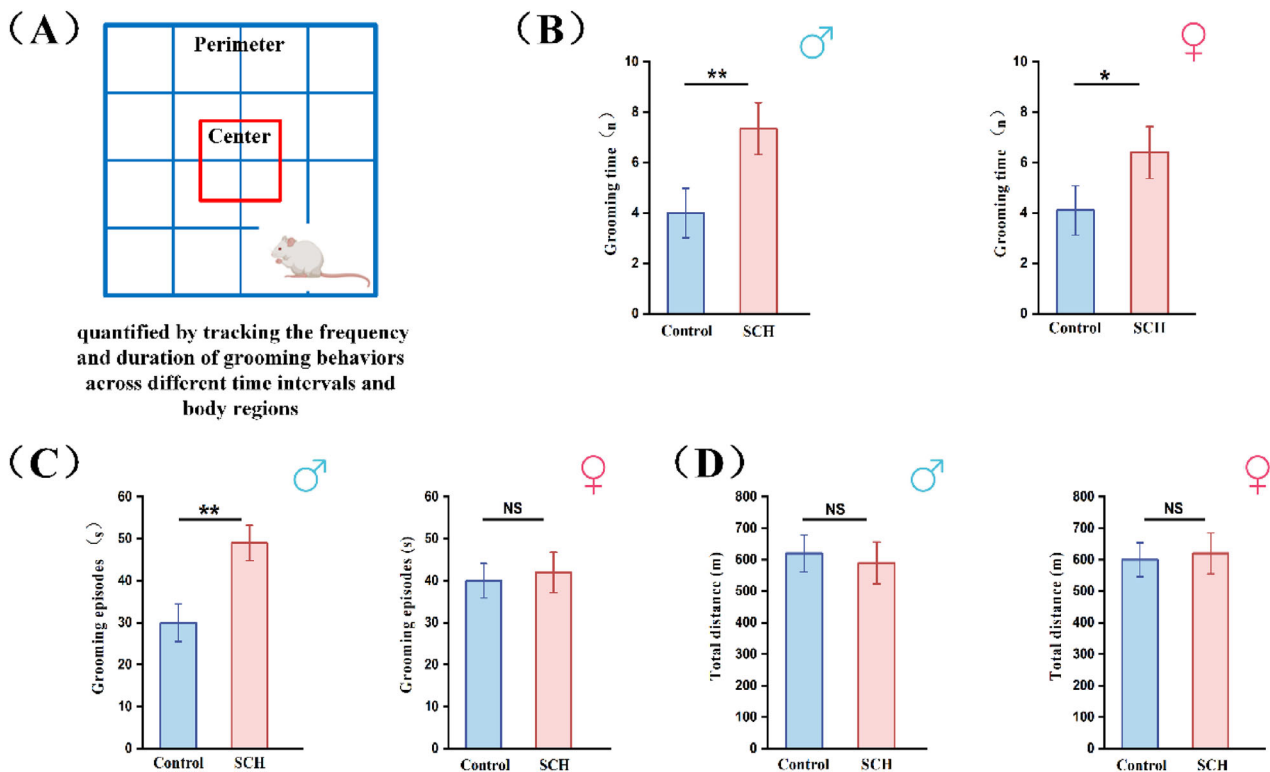
Fig. 3 Self-grooming test was performed on two groups of offspring rats at postnatal day 35 (P35). Parameters measured included the number of self-grooming sessions, duration of grooming, and total distance traveled. Data are expressed as mean ± SEM. **A** shows the schematic diagram of the experiment. **B** shows the number of self-grooming sessions recorded during the test, and **C** shows the duration of the grooming behavior. **D** shows the total distance traveled by the offspring rats ($n = 8$ for each group). Non-significant differences in some parameters compared to controls are indicated by NS. Statistical significance is indicated by *$P < 0.05$ and **$P < 0.01$. P35 postnatal day 35, SCH subclinical hypothyroidism.
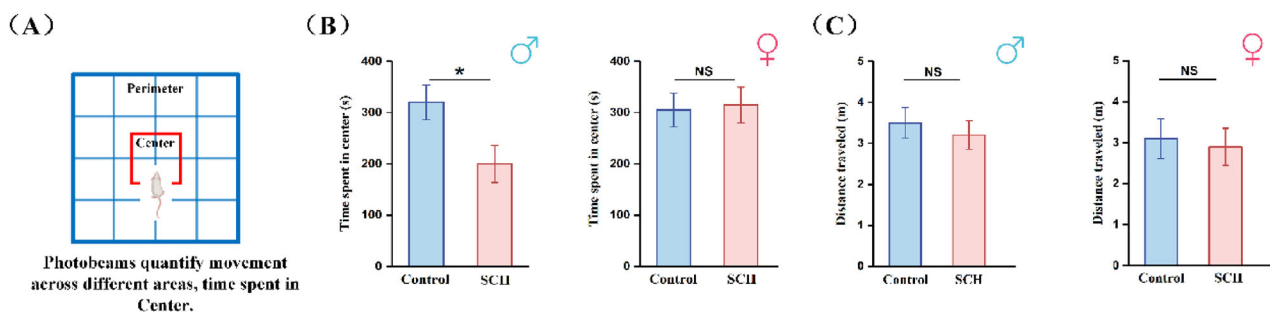

Fig. 4 Open field tests were conducted on offspring rats from two groups at postnatal day 36 (P36). The parameters measured included time spent in the center square and total distance traveled in the open field. Data are expressed as mean ± SEM. **A** Schematic representation of the experiment; **B** Time spent in the center square; **C** Total distance traveled. For male rats: Control group ($n = 6$) and SCH group ($n = 8$); for female rats: $n = 8$ per group. NS indicates no significant differences. P36 postnatal day 36, SCH subclinical hypothyroidism. Statistical significance: *$P < 0.05$.

the time spent sniffing Stranger 1 and Stranger 2, suggesting a significant loss of social novelty preference (Fig. 5C, D).

Spatial learning and memory impairment in SCH offspring rats

Autism is often associated with intellectual disabilities and memory impairments, so we conducted the Morris Water Maze (MWM) test to assess these aspects. The experiment was performed only on male offspring rats. On the first day of spatial learning, rats were in the adaptation phase, and there were no significant differences between groups in the escape latency (time to reach the hidden platform). Starting from the second day, with increasing training sessions, the escape latency gradually decreased for each group of rats. However, from the second to the fifth day, the SCH group

exhibited a slower reduction in escape latency, and the time spent finding the hidden platform was significantly longer than that of the control group. ANOVA analysis revealed that this difference was statistically significant ($P < 0.01$, Fig. 6B).

In the final probe trial phase, the SCH group spent significantly less time in the target quadrant compared to the control group, and the difference was statistically significant ($P < 0.01$, Fig. 6C). Additionally, the frequency of crossing the platform area was significantly lower in the SCH group than in the control group, with this difference also being statistically significant ($P < 0.05$, Fig. 6D). However, there was no significant difference between the SCH and control groups in average swimming speed, indicating that the SCH group exhibited impairment in spatial learning and memory (Fig. 6E).
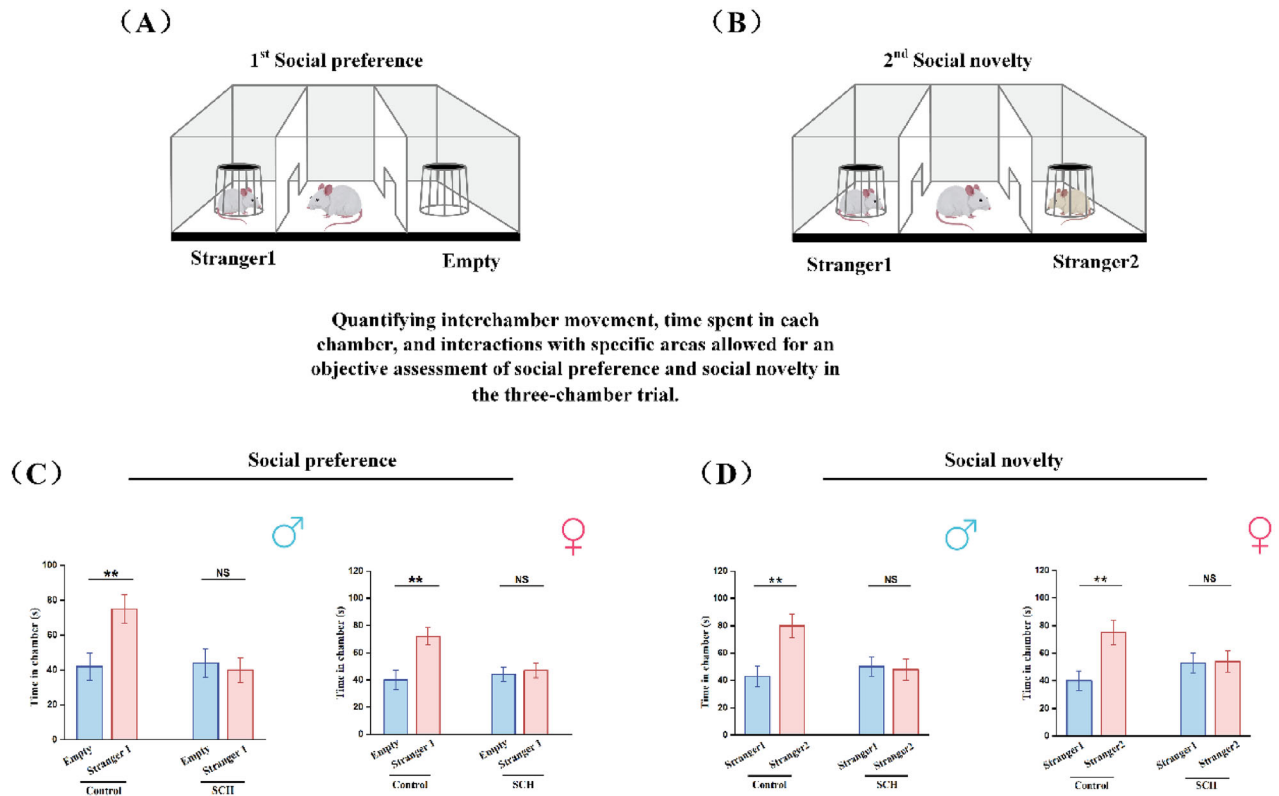
Fig. 5 Three-chamber sociality tests were performed on offspring rats from each group on postnatal days 37–38 (P37–38). The tests included **A** a social preference test (Stranger 1 vs. Empty) and **B** a social novelty and motivation test (Stranger 2 vs. Stranger 1). Data are expressed as mean ± standard error (mean ± SEM). **A, B** Diagrams representing the experimental setup; **C** Results of the social preference test; **D** Results of the social novelty test (*n* = 8 per group). NS indicates no statistically significant differences compared with the control group. P37–38 postnatal days 37–38, SCH subclinical hypothyroidism. Statistical significance: *P < 0.05, **P < 0.01.
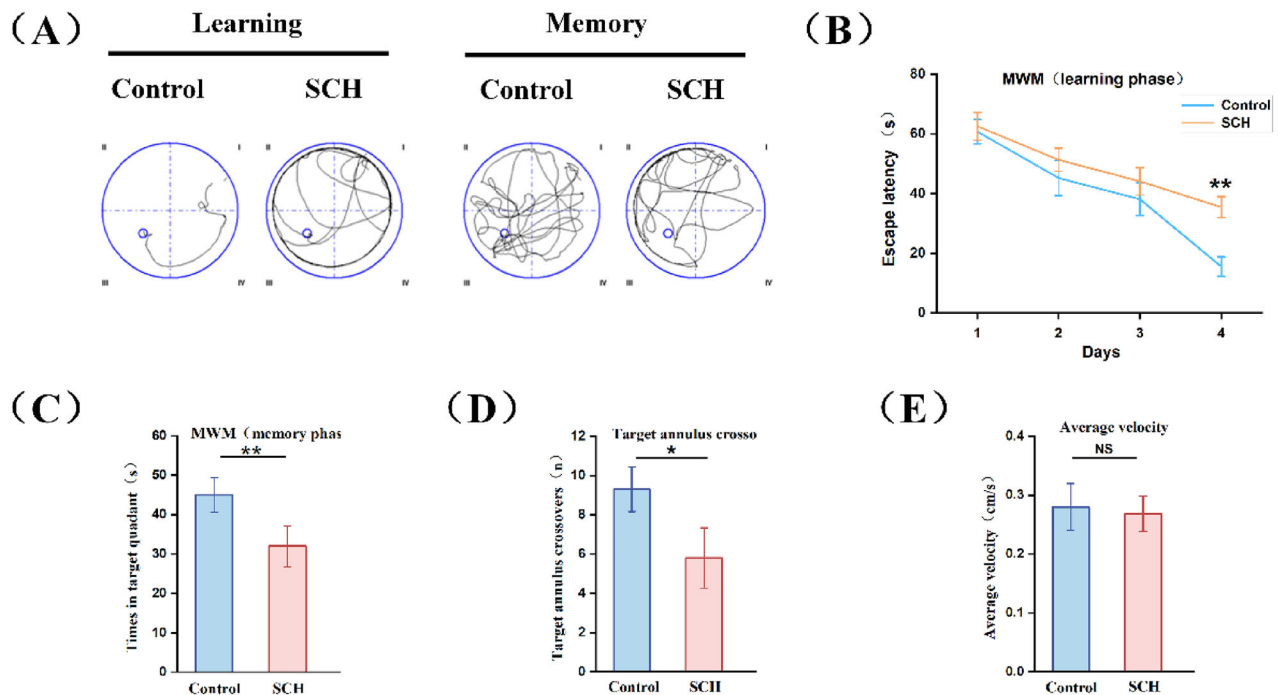

Fig. 6 Behavioral performance in the Morris water maze test (P40–44). Control and SCH offspring male rats were compared in terms of latency to find the hidden platform **B**, time in the target quadrant **C**, frequency of crossing the platform **D**, average swimming speed **E**, and swimming traces on day 5 **A**. Data are presented as mean ± SEM (*n* = 8 per group). NS no significant difference compared with control. P40–44 postnatal day 40–44, SCH subclinical hypothyroidism. *P < 0.05, **P < 0.01.
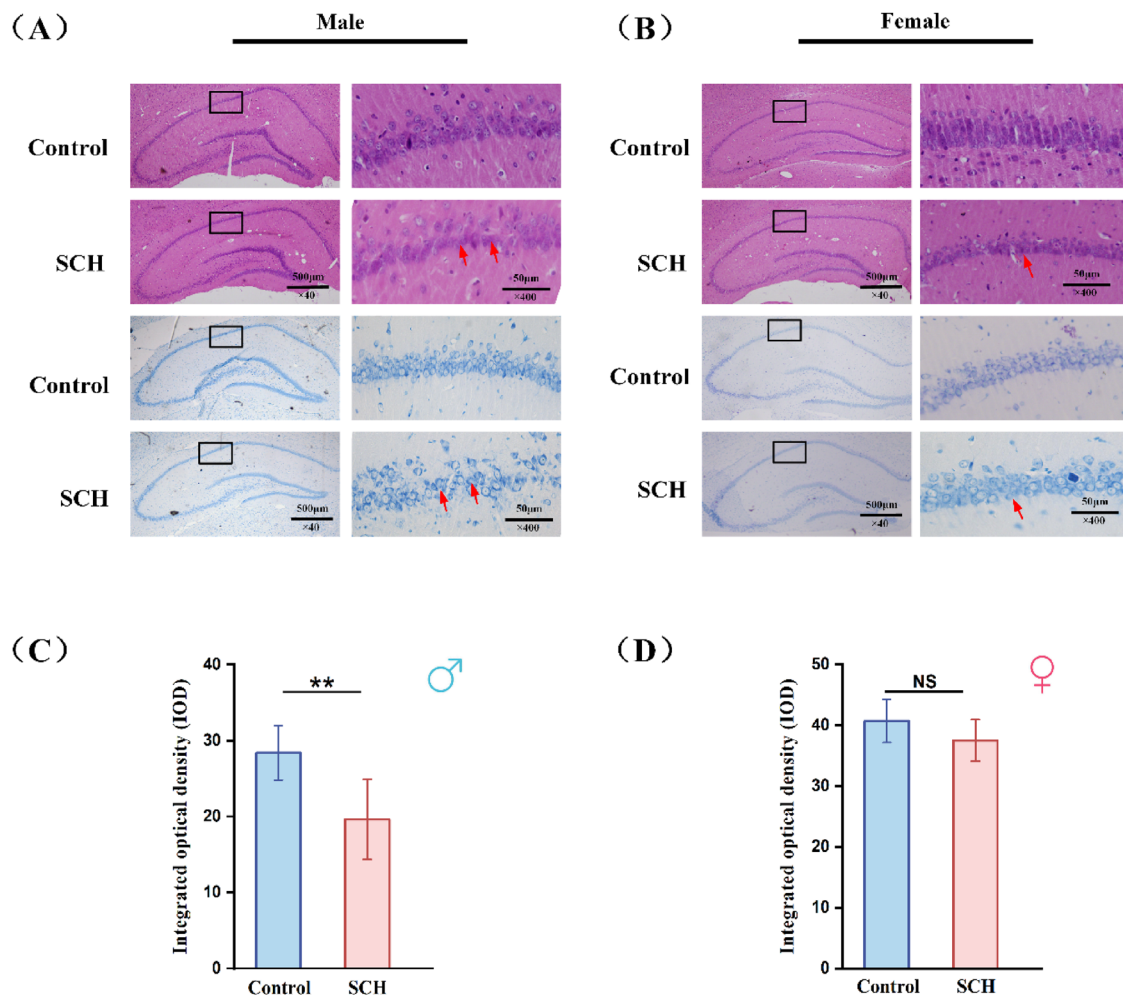
Fig. 7   Effect of maternal SCH during pregnancy on Nissl bodies in the CA1 region of the hippocampus of male and female rat offspring (P44, HE staining ×40 and 400, Nissl staining ×40 and 400). Data are presented as mean ± SEM. **A, B** Representative images of hippocampal CA1 region in different groups of rat offspring. Integrated optical density (IOD) results of Nissl bodies in CA1 area. (×40 and ×400; scale bars = 50 and 500 μm). **C**: male rats; **D**: female rats ($n = 6$ per group). NS no significant difference compared with control. P44 postnatal day 44, SCH subclinical hypothyroidism. $**P < 0.01$.

Hippocampal CA1 neuron damage in SCH offspring rats

In the Nissl staining of the hippocampal CA1 region, red arrows highlight representative Nissl bodies. The results revealed that the integrated optical density (IOD) of Nissl bodies in the hippocampal CA1 region of male offspring in the SCH group was significantly lower than that of the control group ($P < 0.01$). Further observations indicated that the Nissl bodies in the SCH group exhibited fragmentation or complete loss, with irregular neuronal alignment and a disrupted layered structure. These alterations suggest that maternal SCH leads to the dissolution and loss of Nissl bodies in the hippocampal neurons of male offspring, potentially compromising neuronal integrity and function (Fig. 7A, B).

In contrast, while the IOD of Nissl bodies in the hippocampal CA1 region of female offspring in the SCH group was slightly lower than in the control group, the difference was not statistically significant (Fig. 7C, D). These findings imply that maternal SCH-induced damage to hippocampal CA1 neurons may exhibit sex-specific effects, with a more pronounced impact on male offspring.

Effect of SCH on hippocampal CREB, BDNF, and Bcl-2 levels in offspring rats

The results showed that maternal subclinical hypothyroidism (SCH) significantly affected the expression of hippocampal CREB

and its downstream molecule BDNF in offspring rats. In male offspring, the hippocampal CREB levels in the SCH group were significantly lower than those in the control group ($P < 0.01$), and the expression of the downstream molecule BDNF was also significantly reduced ($P < 0.01$). Additionally, the expression of the anti-apoptotic protein Bcl-2 was significantly lower in the hippocampus of male offspring in the SCH group compared to the control group ($P < 0.05$) (Fig. 8B, C).

In female offspring, the expression of hippocampal CREB and BDNF in the SCH group was also significantly lower than that in the control group ($P < 0.01$), showing a similar trend to that in males. However, unlike the male offspring, the expression of Bcl-2 in the hippocampus of female offspring in the SCH group was slightly decreased but did not reach statistical significance (Fig. 8D). These results suggest that maternal SCH affects hippocampal function in offspring by modulating the CREB-BDNF signaling pathway, and this effect exhibits some gender-specific differences.

Effect of SCH on mTOR and Wnt gene and protein expression in offspring rats

Maternal subclinical hypothyroidism (SCH) significantly affected the gene and protein expression of mTOR and Wnt in offspring rats. In male offspring, the mTOR gene expression in the SCH group was significantly higher than in the control group ($P < 0.01$),
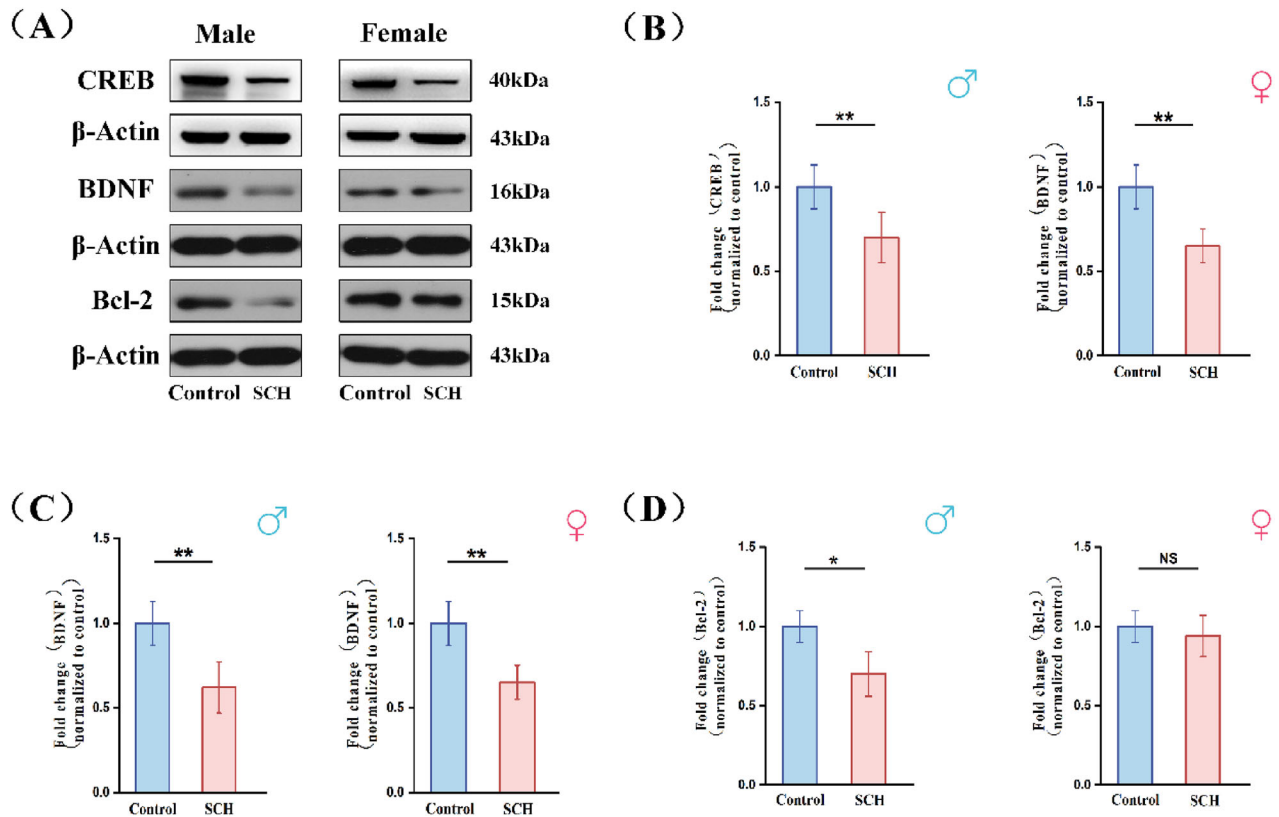
Fig. 8 **Effect of maternal SCH on expression levels of thyroid hormone-related proteins in the hippocampus of male and female rat offspring (P44).** Data are presented as mean ± SEM. **A** Representative images of hippocampal tissue from mice of both groups on the left side. **B**–**D** Histograms show quantification of CREB, BDNF and Bcl-2 expression, left: male rats; right: female rats ($n = 6$ per group). NS no significant difference compared to control. P44 postnatal day 44, BDNF brain-derived neurotrophic factor, CREB cAMP response element binding protein, SCH subclinical hypothyroidism. $*P < 0.05$, $**P < 0.01$.

and the mTOR protein level was also significantly increased, consistent with the changes in mTOR gene expression ($P < 0.01$, Fig. 9A, E). In contrast, the Wnt gene expression in the SCH group of male offspring was significantly lower than that in the control group ($P < 0.01$), and Wnt1 protein levels were also significantly downregulated ($P < 0.01$, Fig. 9C, F). These results suggest that SCH exerts a notable regulatory effect on the mTOR and Wnt signaling pathways in male offspring.

In female offspring, the effects of SCH on mTOR and Wnt expression were weaker. Specifically, the mTOR gene expression in the SCH group was slightly elevated, but the difference did not reach statistical significance (Fig. 9B, E). However, the expression of both Wnt gene and protein was significantly decreased in the SCH group ($P < 0.05$, $P < 0.01$, Fig. 9D, F). These gender differences indicate that the impact of maternal SCH on brain signaling pathways in offspring may be gender-specific.

DISCUSSION

In this study, male offspring rats exhibited significant social deficits and increased self-grooming behavior, which may be closely related to the induction of anxiety. Anxiety is considered a common comorbid symptom of ASD-like behaviors [19]. Specifically, anxiety may affect the social motivation and behavior of rats, thereby exacerbating social deficits. It is known that anxiety symptoms often lead to avoidance of unfamiliar environments or social interactions. This avoidance behavior may manifest as social deficits in the experiment, such as reduced sniffing time towards unfamiliar rats and a lack of preference for social novelty [20]. In addition, anxiety may increase self-stimulatory behaviors, such as self-grooming, which to some extent reflects the animals' adaptive

response to environmental stressors [21]. In our study, the male offspring exhibited higher levels of anxiety, which may have interfered with their social behavior and emotional regulation, thereby affecting the development of ASD-like behaviors. Therefore, anxiety, as a confounding factor, may interact with social deficits, and this mechanism warrants further investigation.

Maternal SCH significantly increased repetitive behaviors and exacerbated social deficits in male offspring, core symptoms of ASD, which were closely associated with a marked reduction in the density of hippocampal CA1 neurons. The hippocampus, a core brain region involved in learning, memory, and emotional regulation, has been implicated in the pathophysiology of ASD [22–24]. Nissl staining analysis suggests that maternal SCH may disrupt hippocampal structure by downregulating the CREB-BDNF signaling pathway, thus inhibiting neuronal survival and synaptic plasticity. Meanwhile, the excessive activation of mTOR signaling and the significant inhibition of Wnt signaling likely act synergistically in this process, providing a molecular explanation for the onset of ASD-like behaviors [25].

Molecular mechanism studies indicate that maternal subclinical hypothyroidism (SCH) significantly downregulates the expression of CREB and BDNF in the offspring hippocampus, leading to the disruption of neural network functions. CREB is a core transcription factor involved in regulating synaptic plasticity [26, 27], and its reduced activity directly suppresses BDNF expression [28, 29]. BDNF is closely associated with neuronal survival, differentiation, cognitive function, learning and memory, as well as social behavior [30–32]. Therefore, dysfunction of the CREB-BDNF signaling pathway may destabilize neural networks, contributing to the development of ASD-like behaviors [33, 34]. These molecular and cellular changes collectively form the essential mechanisms underlying the onset of
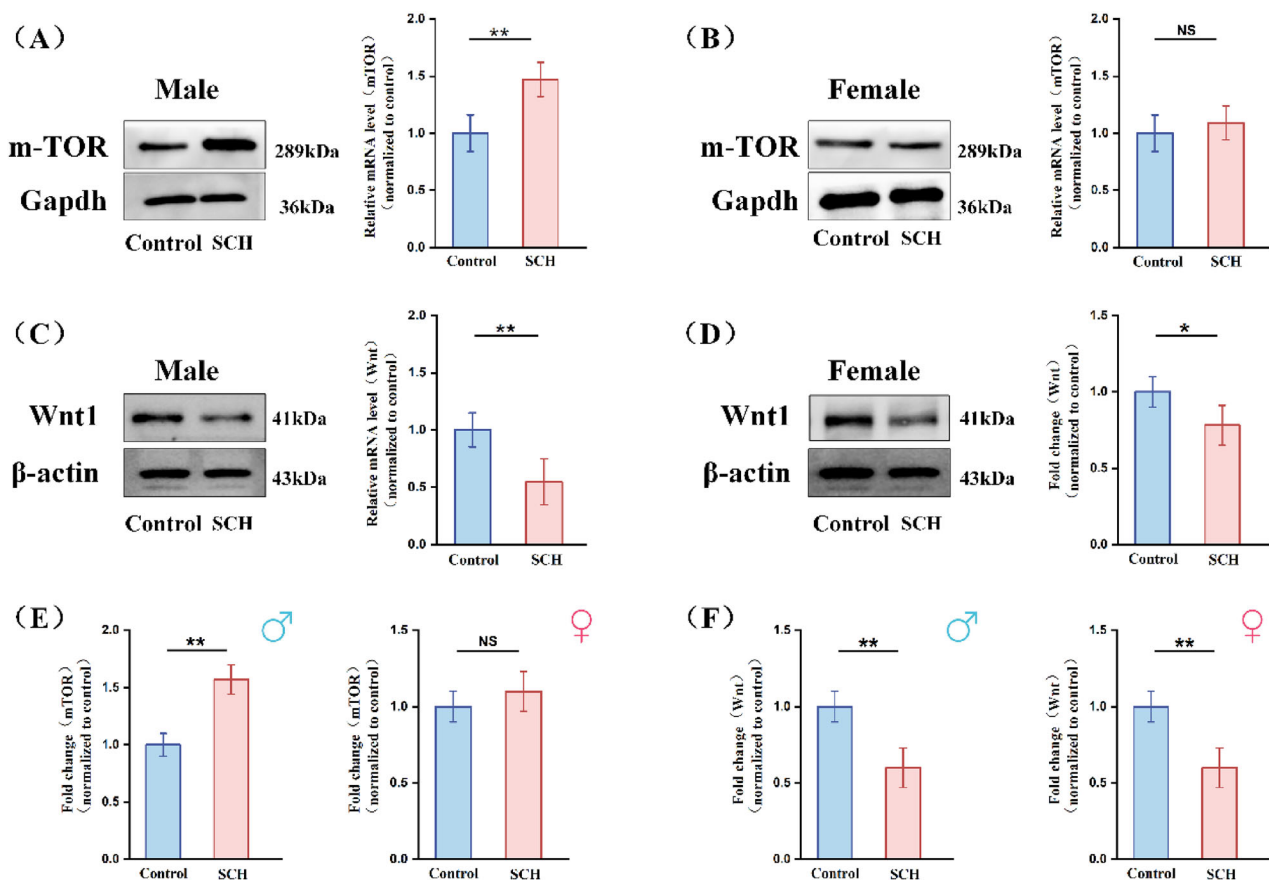
Fig. 9 Effects of maternal SCH on hippocampal m-TOR and Wnt protein and gene expression in male and female offspring at postnatal day 44 (P44). Data are presented as mean ± SEM. **A–D** Protein expression in offspring from both groups; **E, F** mRNA expression in offspring from both groups ($n = 6$ per group). NS indicates no significant differences compared with the control group. P44 postnatal day 44, SCH subclinical hypothyroidism. Statistical significance: *$P < 0.05$, **$P < 0.01$.

ASD-like behaviors. This aligns with emerging views from large-scale integrative analyses, which highlight dysregulation of Wnt/β-catenin, BDNF, and CREB pathways as central to ASD pathophysiology and as potential therapeutic targets across genetic and environmental models of autism [35–37]. Notably, changes in BDNF expression not only directly affect synaptogenesis but may also indirectly influence neuronal survival and network integration through the regulation of key anti-apoptotic factors like Bcl-2 [38, 39]. The disruption of the CREB-BDNF signaling pathway caused by maternal SCH likely triggers cascading effects at multiple levels of neural networks, including reduced synaptic density, decreased neuronal survival, and weakened network integration. These molecular and cellular changes collectively form the essential mechanisms underlying the onset of ASD-like behaviors.

Our findings align with and extend previous epidemiological studies reporting an increased risk of ASD in offspring born to mothers with thyroid dysfunction during pregnancy, including both overt and subclinical hypothyroidism. Several large-scale cohort studies and meta-analyses have shown that maternal thyroid hormone deficiency is associated with neurodevelopmental abnormalities, but the underlying mechanisms remain largely unclear [6, 9, 10].

Unlike most prior studies that primarily focused on behavioral assessments or circulating hormone levels, our work integrates both phenotypic and molecular data, providing insight into the hippocampal CREB–BDNF–Wnt/mTOR–Bcl-2 signaling cascade as a potential mechanistic axis linking maternal SCH to ASD-like behaviors. Furthermore, while sex differences in ASD prevalence have been widely reported [40, 41], few experimental studies have examined how maternal SCH differentially affects male and female offspring at both behavioral and molecular levels. By including both sexes in our design, we contribute new evidence of sex-specific vulnerability, with male offspring showing more pronounced deficits and molecular alterations. Together, these comparisons situate our study within the existing body of maternal–fetal neurodevelopmental research and underscore its added value in elucidating how subclinical, rather than overt, maternal thyroid dysfunction may initiate ASD-related trajectories through defined neurobiological pathways.

Maternal subclinical hypothyroidism (SCH) inhibits offspring hippocampal Wnt signaling and abnormally upregulates mTOR signaling, both of which contribute to the formation of ASD-like behaviors, reflecting a complex molecular regulatory network. Wnt signaling, a critical pathway in neurodevelopment [42, 43], plays a pivotal role in neuronal migration and differentiation, while also maintaining neural network stability by promoting synaptogenesis and neural network connectivity [44, 45]. The significant downregulation of Wnt signaling due to maternal SCH may lead to a reduction in synaptic connections and disruption of the neural network structure, providing a structural basis for the manifestation of core ASD behaviors. On the other hand, the aberrant upregulation of mTOR signaling plays an important role in neurodevelopmental disorders [46, 47]. As a key pathway regulating cell metabolism, protein synthesis, and synaptic plasticity [48, 49], mTOR hyperactivation may result in inadequate synaptic pruning or abnormal protein accumulation, leading to overloading and dysfunction in neuronal connections [50]. This process may further exacerbate the neural network abnormalities

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caused by the downregulation of Wnt signaling, offering functional support for the occurrence of ASD-like behaviors.

It is noteworthy that the downregulation of Wnt signaling and the upregulation of mTOR signaling may exhibit a synergistic effect, forming a positive feedback loop through mutual promotion [50]. For example, the weakening of Wnt signaling could reduce its negative regulation on the mTOR pathway, thereby further enhancing mTOR signaling [51]; conversely, the overactivation of mTOR signaling may hinder the synthesis and expression of proteins involved in the Wnt pathway, further exacerbating the suppression of Wnt signaling [52, 53]. This bidirectional regulatory pattern reveals the complex molecular network underlying maternal SCH-induced ASD-like behaviors, not only suggesting a potential interaction mechanism between Wnt and mTOR signaling, but also providing an important theoretical foundation for future targeted interventions.

This study is the first to find that maternal SCH significantly reduces the expression levels of Bcl-2 in the hippocampus of offspring. The anti-apoptotic function of Bcl-2 is crucial for neural development [54], as it maintains neuronal survival and function by regulating the mitochondrial apoptotic pathway [55]. Thyroid hormones not only play an essential role in regulating the expression of brain-derived neurotrophic factor (BDNF) [56], but have also been shown to directly activate Bcl-2 family members to prevent neuronal apoptosis [57]. Disruptions in maternal thyroid function, such as thyroid hormone deficiency, have been reported to be associated with a significant increase in neuronal apoptosis [58]. In a mouse neural progenitor cell model, deletion of the Bcl-2 gene not only induced neuronal apoptosis but was also significantly associated with anxiety-like behaviors [59]. Notably, the downregulation of Bcl-2 may occur predominantly in brain regions associated with memory and social behavior, such as the hippocampus and prefrontal cortex, providing a direction for further research. Additionally, the potential interaction between Bcl-2 and the CREB-BDNF signaling pathway may jointly contribute to the development of ASD-like behaviors by regulating neuronal survival and synaptic formation [37, 60].

Sex-specificity is one of the key findings of this study. The effects of maternal SCH on male offspring are significantly stronger than on female offspring, which may be due to the greater sensitivity of male rats to maternal thyroid hormone deficiency, possibly related to the neuroprotective effects of estrogen. The subtle changes observed in female offspring may not manifest in significant ASD-like behaviors due to the presence of intrinsic protective mechanisms [61, 62]. Male offspring exhibit more pronounced behavioral abnormalities (such as increased repetitive behaviors and social deficits) and molecular disruptions, which aligns with the higher incidence and severity of ASD in males [63]. X-chromosome-linked genes may confer a higher susceptibility to neurodevelopmental disorders in males, while androgens may play a critical role in the specific regulation of brain development [64]. The molecular mechanisms underlying these sex differences warrant further exploration in future studies.

To further elucidate the mechanistic implications of our findings, we adopted a rigorous, pathway-oriented perspective to interpret the observed alterations in neuronal morphology and neurotrophic signaling. The reduced Nissl body density in the hippocampus suggests compromised neuronal integrity or impaired ribosomal activity, likely reflecting functional disturbances at the cellular level. Concurrently, the downregulation of BDNF, CREB, and Bcl-2 represents a coherent molecular signature closely associated with ASD-like behavioral phenotypes. These molecules are well-established regulators of neuronal survival, synaptic plasticity, and affective behavior, underscoring their central role in neurodevelopmental processes. Importantly, their dysregulation may arise from convergent upstream signals, including glucocorticoid exposure, estrogen imbalance, or neuroimmune activation, suggesting that maternal SCH disrupts neurodevelopment through multifaceted molecular pathways.

Importantly, these findings lay a mechanistic foundation that warrants deeper exploration. To this end, we have initiated a series of ongoing investigations, including: (1) assessment of apoptosis-related markers (e.g., cleaved caspase-3, Bax/Bcl-2 ratio, TUNEL) to evaluate neuronal viability; (2) locus-specific epigenetic modulation of CREB, BDNF, and Wnt1 using CRISPR/dCas9 platforms to determine their functional relevance; and (3) longitudinal profiling across key developmental windows to capture the temporal dynamics of molecular and behavioral alterations. These efforts aim to move beyond correlative evidence and establish causal links between maternal SCH and neurodevelopmental disruption.

Although our current data strongly associate maternal SCH with ASD-like phenotypes in offspring, functional validation of Wnt/BDNF pathway involvement remains ongoing. Future studies will incorporate pathway-specific pharmacological interventions, such as gestational T4 supplementation and Wnt pathway agonists, in conjunction with the aforementioned genetic approaches. Developmental trajectory analyses from early post-natal stages through adolescence will further define the timing and progression of these signaling disturbances. Collectively, these strategies are expected to clarify the mechanistic under-pinnings of maternal thyroid dysfunction and enhance the translational value of our findings.

While our findings offer valuable mechanistic insights into how maternal SCH may shape offspring neurodevelopment across molecular, cellular, and behavioral levels, several questions remain to be addressed. For instance, the precise role of Bcl-2 in mediating behavioral phenotypes via apoptotic pathways requires further experimental validation. Moreover, the cross-sectional design used here captures only a static snapshot of a dynamic developmental process. Future longitudinal studies will be essential to delineate the temporal trajectory of molecular disruptions induced by maternal SCH. Finally, a deeper dissection of the interactions among Wnt, mTOR, and CREB–BDNF signaling may help clarify the broader regulatory network underlying ASD-like phenotypes. Collectively, these directions represent promising avenues for expanding upon the foundational findings presented in this study.

CONCLUSION

This study highlights the regulatory role of maternal SCH on the CREB-BDNF signaling pathway, Wnt/mTOR axis, and Bcl-2 anti-apoptotic mechanisms, establishing their critical involvement in the induction of ASD-like behaviors. Our findings provide molecular evidence for the influence of maternal health on fetal brain development and offer potential targets for early prediction and intervention of ASD. Future research should combine long-itudinal experiments and multi-level molecular validations to further elucidate the mechanisms by which maternal SCH affects offspring brain development, ultimately contributing to new strategies for mitigating the potential impact of maternal health on neurodevelopmental disorders in offspring.

DATA AVAILABILITY
The data supporting the findings of this study are available from the corresponding author upon reasonable request.

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AUTHOR CONTRIBUTIONS

Dijie Liu and Kai Tao conceived and supervised the study. Dijie Liu was responsible for designing the experiments, performing data analysis, and drafting the manuscript. Ying Sun conducted the molecular and behavioral experiments, while Jialin Hao performed the histological and imaging analyses. Shiyong Wang contributed to the preparation of figures. All authors participated in the discussion of the results, revised the manuscript, and approved the final version for submission.

COMPETING INTERESTS

The authors declare no competing interests.

ETHICS APPROVAL

All experimental procedures involving animals were performed in accordance with the relevant guidelines and regulations for the care and use of laboratory animals. The study protocol was reviewed and approved by the Animal Care and Use Committee of China Medical University, Approval No. KT20242238). No human participants were involved in this study.

ADDITIONAL INFORMATION

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