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ORIGINAL RESEARCH

The Effects of Maternal Atrazine Exposure and Swimming Training on Spatial Learning Memory and Hippocampal Morphology in Offspring Male Rats via PSD95/NR2B Signaling Pathway

Dandan Wang[1] · Bai Li[1] · Yanping Wu[1] · Baixiang Li[1]

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Abstract

Atrazine (ATR), a widely used herbicide, has been previously shown to damage spatial memory capability and the hippocampus of male rats during the development. It has also been indicated that physical exercise can improve learning and memory in both humans and animals, as a neuroprotective method. Our aim here was to investigate the effect of maternal ATR exposure during gestation and lactation on spatial learning and memory function and hippocampal morphology in offspring and to further evaluate the neuroprotective effect of swimming training and identify possible related learning and memory signaling pathways. Using Sprague-Dawley rats, we examined behavioral and molecular biology effects associated with maternal ATR exposure, as well as the effects of 8 or 28 days swimming training. Maternal exposure to ATR was found to impair spatial learning and memory by behavioral test, damage the hippocampal morphology, and reduce related genes and proteins expression of learning and memory in the hippocampus. The extended, 28 days, period of swimming training produced a greater amelioration of the adverse effects of ATR exposure than the shorter, 8 days, training period. Our results suggest that maternal ATR exposure may damage the spatial learning and memory of offspring male rats via PSD95/NR2B signaling pathway. The negative effect of ATR could be at least partially reversed by swimming training, pointing to a potential neuroprotective role of physical exercise in nervous system diseases accompanying by learning and memory deficit.

Keywords Atrazine · Swimming training · Spatial learning memory · Hippocampus · PSD95 · NR2B

Abbreviations

ATR	Atrazine
IEG	Immediate-early gene
JNK	c-Jun N-terminal kinase
NMDA	N-methyl-D-aspartic acid
NR2B	NMDA receptor subunit 2B
PI3 K	Phosphatidylinositol 3-kinase
p-JNK	Phosphorylated c-Jun N-terminal kinase
PSD95	Postsynaptic density protein-95

Dandan Wang and Bai Li are Co-first authors.

✉ Baixiang Li
libaix@ems.hrbmu.edu.cn

Dandan Wang
2521118720@qq.com

Bai Li
1198233993@qq.com

Yanping Wu
279154533@qq.com

[1] Department of Toxicology, School of Public Health, Harbin Medical University, Harbin 150086, China

Introduction

Atrazine (2-chloro-4-ethylamino-6-isopropylamino-1,3,5-triazine, ATR) is a low-cost and highly effective broad spectrum herbicide that has been widely used to control grasses and weeds in agriculture for decades (Eldridge et al. 1994). Although it was banned in the European Union, ATR is still frequently detected in aqueous environments in Europe (Dong et al. 2017; Rimayi et al. 2018) because of its high persistence. Since ATR is still used extensively in China, the USA, South Africa, and other countries, exposure to ATR remains a potential public health risk and needs to be assessed and further reduced.

As ATR has high water solubility and mobility in the soil, it easily washes into deep water, which means that long-term, high-dose application can affect the quality of groundwater. Since ATR persists in the environment for

long periods, it is easily enriched in organisms, resulting in chronic or sub-chronic poisoning of humans, animals, and plants. Increasing evidence indicates that ATR is an environmental endocrine disruptor that has selective effects on multiple organs and tissues (Zaya et al. 2011; Xing et al. 2013; Sai et al. 2016) and that it plays important roles in the endocrine, reproductive, immune, and nervous systems (Weber et al. 2013; Wirbisky et al. 2016; Liu et al. 2017).

In terms of nervous systems, animal experiments and epidemiological studies both have shown that exposure to certain herbicides may lead to deficits in spatial learning and memory, and can increase the risk of developing neurodegenerative diseases, such as Parkinson's disease (PD) and Alzheimer's disease (AD) (Chen et al. 2012; Van et al. 2012; Mostafalou and Abdollahi 2017). Although it is known that animals and humans are particularly susceptible to environmental factors during the gestational and lactational periods, the possible effects of ATR exposure during these periods have received only limited attention. Our previous studies initially indicated that gestational and lactational exposure to paraquat and maneb or developmental exposure to ATR may result in behavioral, learning, and memory impairment in rats (Li et al. 2016; Li et al. 2018), but the exact biological mechanism linking ATR with decreased learning and memory function, especially in the gestational and lactational periods, requires further in-depth study.

Epidemiological studies have shown that moderate physical exercise helps to improve learning and memory function and to reduce the risk of AD and similar neurodegenerative diseases (Prakash et al. 2015; Northey et al. 2017). Studies in both humans and animals also have shown that physical activity, especially aerobic exercise, can have positive effects on multiple aspects of brain function including learning and memory (Hillman et al. 2008), and can help to decrease psychopathological symptoms and improve learning and memory performance in psychotic patients (Oertel-Knöchel et al. 2014). The swimming training used in the present study is a type of aerobic exercise. A series of recent studies have shown that aerobic exercise can improve synaptic plasticity in the hippocampus and can mediate neurogenesis by increasing levels of neurotrophic and growth factors (Liu and Nusslock 2018; Rendeiro and Rhodes 2018) and thereby improve spatial learning and memory (Erickson et al. 2011). We questioned, therefore, whether physical exercise can alleviate ATR-induced learning and memory impairment.

The postsynaptic density 95 (PSD95), an essential scaffolding protein during synaptogenesis and neurodevelopment, specifically binds to the C-terminus of the N-methyl-D-aspartic acid (NMDA) receptor subunit 2B (NR2B) through its PDZ domains (Kornau et al. 1995). The combination of PSD95 and NR2B has been shown to play a key role in learning and memory deficit, depression, epilepsy, and AD (Feyissa et al. 2009; Ying et al. 2010; Coley and Gao 2017). Thus, some studies indicated that the combination of PSD95 and NR2B implicates in the synaptic plasticity and the learning and memory processes (Delint-Ramírez et al. 2008; Bustos et al. 2014). The downstream neuronal survival pathway associated with NMDA receptor in synapses is mediated mainly through phosphatidylinositol 3-kinase (PI3K)/protein kinase B (Akt) signaling pathways. The γ isoform of PI3K (PI3Kγ, 110 kDa) acts a crucial part in NMDA receptor-mediated synaptic plasticity and hippocampus-dependent learning and memory function (Jae-Ick et al. 2011; Choi et al. 2014). In downstream targets, since the immediate-early gene (IEG) c-fos is an indirect marker of neuronal activity and is related to learning and memory (Tanabe et al. 2010; Gallo et al. 2018), it is also regarded as an objective sign of learning and memory function. In the upstream path of the combination of PSD95 and NR2B, there was a report showing that Wnt5a regulates postsynaptic structure and directs the gathering of PSD95 in hippocampal neurons through the noncanonical Wnt5a/JNK pathway by phosphorylation of JNK (p-JNK) (Farías et al. 2009). Simultaneously, it has been shown a strong relationship between deregulation of the Wnt signaling and learning and memory deficit in AD (De Ferrari et al. 2014; Riise et al. 2015; Folke et al. 2018). In short, we would like to initially explore whether Wnt5a/JNK mediates the binding of PSD95 and NR2B to regulate the expressions of PI3 K and c-fos (Fig. 1), and whether this signaling pathway will participate in the learning and memory regulation of this study.

The experiments were designed to examine the following three aspects. Firstly, molecular biology experiments were used to confirm that maternal ATR exposure can cause learning memory damage to offspring rats. Secondly, the ability of swimming training to help improve or repair the learning memory impairment of offspring of dams exposed to ATR was assessed. Thirdly, relevant physiological mechanisms that may be involved in the regulation of learning and memory were explored. Overall, we intend to explore the impacts of maternal ATR exposure and exercise training on learning and memory ability of offspring from a molecular point of view and provide a theoretical basis for further research work in related fields.

Materials and Methods

Ethics Statement

The animal experiments in the present study were approved by the Medical Ethics Committee of Harbin Medical University (Harbin, China, HMUPHIRB2016006, 4 March 2016),

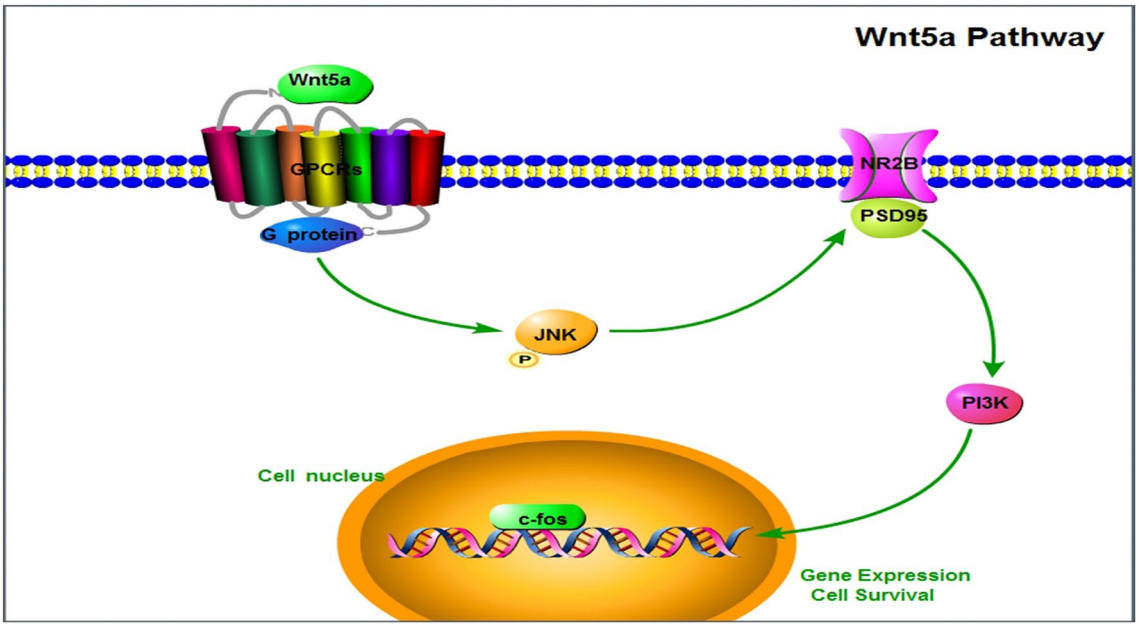

Fig. 1 The mechanism of Wnt5a/JNK mediating PSD95/NR2B signaling pathway

and the procedures were according to the National Institutes of Health Guidelines for the use of laboratory animals.

Animals, Exposure, and Groups

Twenty male and forty female, 8 weeks, Sprague-Dawley (SD) rats were purchased from Charles River Laboratory (Beijing, China) and kept under a 12:12-h light–dark cycle at the constant temperature of 20 ± 2 °C and humidity of 50 ± 10%, with free access to food and water. After acclimatization for 1 week, female SD rats were mated and, till detection of the vaginal plugs, were kept separately. Thirty-two pregnant dams were selected and were further randomly divided into two groups on gestation day (GD) 1: control group ($n = 8$, 1% methylcellulose; Sigma, St. Louis, MO, USA) and treatment group ($n = 24$, 100 mg/kg ATR; CAS:1912-24-9). The ATR dosage was selected based on a previous report that the oral maximum tolerated dose (MTD) of ATR is estimated to be 100 mg/kg in rats [40]. The dams were weighed and administered the ATR intragastrically twice each week from GD 5 to postnatal day (PND) 21 (on Monday and Thursday), by which time all the pups were weaned. Three healthy male pups were randomly selected from each litter and continued feeding without exposure to ATR until 1.5 months of age. Pups from dams in the treatment group were then randomly divided into three groups: ATR group (no swimming training), ATR + 8DST group (8 days of swimming training), and ATR + 28DST group (28 days of swimming training). At 3 months of age, eight pups were randomly selected from each group (pups from control, ATR, ATR + 8DST, and ATR + 28DST group dams) for behavioral testing. The remaining pups were anesthetized, and then the hippocampi were separated on ice immediately for further experiments and analysis. The experimental design is summarized in Fig. 2.

Swimming Training

From 1.5 months of age, the ATR + 8DST group and the ATR + 28DST group carried out 8 days and 28 days of swimming training, respectively. Swimming training was carried out between 13:00 and 18:00 pm, with each rat receiving 20-min training in each session. The swimming pool was the same as the equipment used in the Morris water maze (MWM) test.

Behavioral Tests

Morris Water Maze

At 3 months of age, rats were submitted to spatial orientation, learning and memory evaluation in the MWM. The device consisted mainly of a circular pool 180 cm in diameter and 58 cm deep, divided into four equal quadrants and a circular escape platform 10 cm in diameter and 40 cm high, in one of the quadrants. The device was surrounded with four different visual landmarks to aid spatial navigation. Simultaneously, the pool was filled with water to a level 1.5–2 cm higher than the escape platform and the temperature of the water was maintained at 20–22 °C throughout

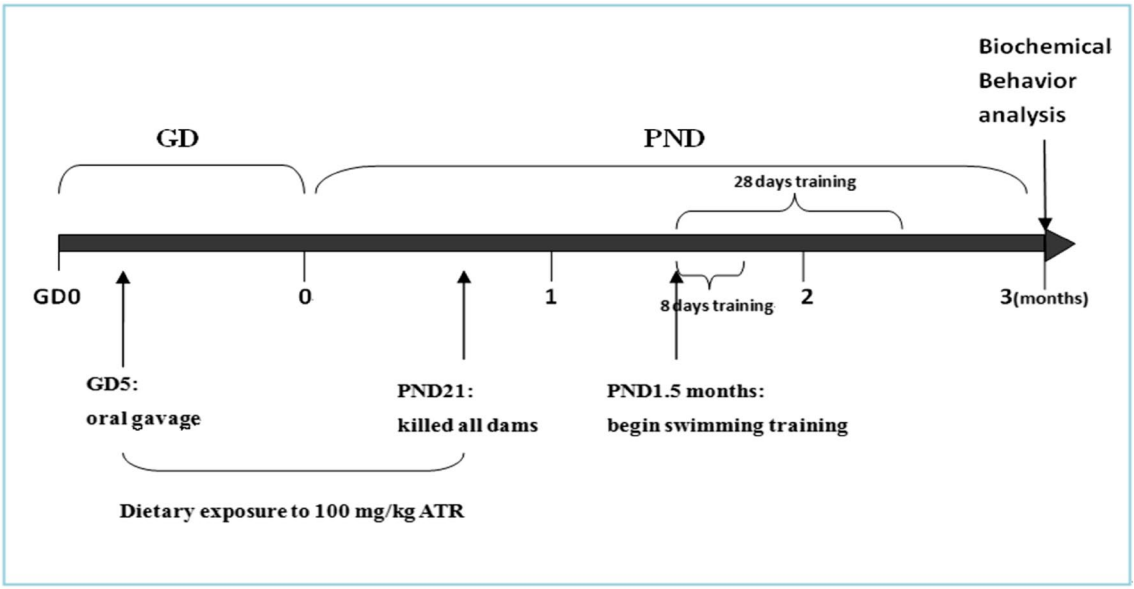

Fig. 2 Timeline of experimental procedures

the entire session. The experiment consisted of two stages: training and testing. During the training stage, the rat was placed into the water facing the pool wall in one of the four quadrants, in a random order each day. For each trial, the rat was allowed to reach the escape platform at the maximum of 90 s. The rat failed to reach the escape platform within 90 s, and was then kept on the platform for 10 s to familiarize itself with its surroundings. Latency and average speed of reaching the platform were measured each day. During the spatial probe test stage, on the sixth day, the escape platform was removed from the pool. The rat was allowed a single 90-s trial period to reach the platform position from a random starting location. The number of times the platform was crossed and the percentage of time in the target quadrant over a period of 90 s was recorded and analyzed.

Grip Strength

A YLS-13A grip strength meter (Yiyan Technology Co., Ltd, Jinan, China) was used to evaluate the limb strength of the rats and also to identify degree of nerve damage and recovery. The grip strength meter was placed horizontally and the two front feet under the instrument were adjusted to stabilize the horizontal bubble on the instrument at the center of the black circle. The power was switched on and the "Settings" button was used to set the time and number of tests. The "Run" button was pressed and the running and reading indicators then showed that the instrument was in "Run" mode. The "Measure" button was pressed and the animal was gently placed on the grip plate. The rat's tail was grasped and the animal was gently pulled back in the horizontal direction, being careful not to yank. After the animal had grasped the grip plate, it was pulled evenly backwards until it loosened its claws. At this point, the instrument automatically recorded the maximum grip of the animal and emitted an acoustic prompt. The instrument was equipped with a foot pedal, which was equivalent to the "Measure" button. If one-handed operation was inconvenient, the foot pedal was used instead of the "Measure" button. Grip force measurements were automatically saved in the memory of the instrument and values copied to an Excel spreadsheet, using the "YLS Data Communication Program" software.

Histomorphology

Hematoxylin and Eosin (H&E) Staining

To observe the form and arrangement of hippocampal tissues, the animals were fixed by cardiac perfusion with chilled 0.01 M phosphate-buffered saline (PBS, pH 7.2), followed by 4% paraformaldehyde in PBS. The hippocampi were separated and then sliced before H&E staining and observation using a BX51 optical microscope (Olympus Corporation, Tokyo, Japan).

Scanning Transmission Electron Microscopy

After animals were anesthetized with pentobarbital sodium, the hippocampi were quickly removed to cut into small pieces and then fixed in chilled 2.5% glutaraldehyde in 0.01 M PBS (pH 7.2) for 24 h. Finally fixed in the 1% osmium tetroxide for 2 h, the samples were then observed

by a JEM-2100 scanning transmission electron microscope (JEOL Ltd., Tokyo, Japan).

Double-Labeled Immunofluorescence

The animals were perfused through the hearts with PBS (pH 7.2), followed by chilled 4% paraformaldehyde in 0.01 M PBS. The entire brains were removed and post-fixed in 4% paraformaldehyde for 24 h. These brains were then transferred to series sucrose gradient solution until they sank and were then cut into 8-μm coronal sections. The sections were blocked with 5% goat serum (Solarbio, Beijing, China) for 1 h, followed by incubation with mouse anti-rat PSD95 (1:200 dilution, Cell Signaling, Danvers, MA, USA) and rabbit anti-rat NR2B (1:200 dilution, Abcam, Cambridge, MA, USA). On the second day, the sections were incubated with Alexa Fluor®488 goat anti-mouse IgG (1:500 dilution, Cell Signaling) and Alexa Fluor®594 goat anti-rabbit IgG (1:500 dilution, Cell Signaling), followed by nuclear staining with DAPI (Beyotime, Shanghai, China) for 5 min. Sections were mounted on coverslips with Antifade Mounting Medium (Beyotime) and viewed using an Axio Observer A1 inverted fluorescence microscope (Zeiss, Jena, Germany).

RNA Collection and Quantitative Real-Time PCR

To examine gene expression levels of Wnt5a, JNK, PSD95, NR2B, PI3K, and c-fos mRNA in the hippocampus, and Nurr1 mRNA in the midbrain, total RNA was isolated with TRizol reagent and then used to synthesize into cDNA with a SYBR Premix Ex Taq II Reagent Kit and gDNA Eraser reverse transcriptase, according to the manufacturer's protocol (Takara, Tokyo, Japan). The RNA concentration was measured at 260 nm and 280 nm using a Nanodrop One spectrophotometer (Thermo, Waltham, MA, USA). The cDNA was amplified using an ABI 7500 real-time PCR instrument (Applied Biosystems™; Life technologies, Gaithersburg, MD, USA), which can automatically calculate the Ct value of each group and β-actin. For the result analysis, the target gene was normalized to β-actin expression by calculating $\triangle\triangle$Ct. The $2^{-\triangle\triangle Ct}$ method was used to calculate the relative levels of target gene. The following primer sequences (Generay, Shanghai, China) were used: β-actin: (forward: GAGAGGGAAATCGTGCGT, reverse: GGAGGAAGAGGATGCGG); Wnt5a: (forward: AGGTCAACAGCCGCTTCAACTC, reverse: TAAACTGGTCATAGCCACGCCC); JNK: (forward: ATGAAAGGGAGCACACAATAGAGGA, reverse: AGACGGCGAAGACGACGGAT); PSD95: (forward: AAGCGGGAATATGAGATAGACG, reverse: ATAGAGGTGGCTGTTGTACTGG); NR2B: (forward: AGTAGGTGGTGACGATGGAAA, reverse: ATAATGGCGGATAAGGATGAG); PI3 K: (forward: CTTGAGCCAGTGATTCAGGAC, reverse: GGTGACAAAATAGGGATGATAGAG); c-fos: (forward: CACTCTGGTCTCCTCCGT, reverse: ATTCTCCGTTTCTCTTCCTC).

Western Blot

To assess ATR-induced and post-exercise training protein expression levels of Wnt5a, JNK, p-JNK, PSD95, NR2B, PI3K, and c-fos in the hippocampus, and Nurr1 in the midbrain, total protein was extracted in lysis buffer containing a phosphatase inhibitor and phenylmethanesulfonyl fluoride (Beyotime), followed by centrifugation. The protein concentrations were measured using the BCA protein assay kit (Beyotime), with equal amounts of each sample (50 μg). Total protein was separated by 10% SDS-PAGE and then transferred to PVDF membranes. The membranes were blocked with 1% BSA in TBST for 45 min at room temperature, followed by incubation at 4 °C overnight with primary Wnt5a antibody (1:1000 dilution, R&D, Minneapolis, MN, USA), JNK antibody (1:1000 dilution, ImmunoWay), p-JNK antibody (1:1000 dilution; ImmunoWay), PSD95 antibody (1:1000 dilution, Cell Signaling), NR2B antibody (1:1000 dilution, Abcam), PI3 K antibody (1:500 dilution, ImmunoWay), c-fos antibody (1:1000 dilution, ImmunoWay), or with β-actin antibody (1:1000 dilution, ImmunoWay) as the control. After incubation with HRP-conjugated goat anti-rabbit IgG (1:5000 dilution, ZSGB-Bio, Beijing, China) or HRP-conjugated goat anti-mouse IgG (1:5000 dilution, ZSGB-Bio) for 1 h, the protein complexes were visualized using the enhanced chemiluminescence reagents BeyoECL Plus (Beyotime). Immunoreactive signals of the membranes were then imaged using a Tanon-5200 chemiluminescence imaging system (Tanon Science & Technology Co., Shanghai, China). Band intensities of targeted proteins were determined using Image J v1.50 software (NIH., Bethesda, MD, USA).

Statistical Analysis

One-way ANOVA test was applied to compare differences among all groups. The differences of escape latency measured in the MWM test were analyzed using repeated-measures two-way ANOVA test. Bonferroni's multiple comparison test was used for pairwise comparisons. A value of $P < 0.05$ was considered to be statistically significant. The mean ± standard error (SE) was calculated and presented graphically using SPSS 19.0 software.

Results

Body Weight and General Appearance

There was no difference in body weight or general appearance between ATR-treated dams and control dams during pregnancy and lactation. There was also no difference among the four groups of offspring.

Effects of ATR Exposure and Swimming Training on Spatial Learning Memory

Morris Water Maze Test

Latency to reach the platform of all groups decreased during the training phase. However, there was no obvious difference in latency time for arrival the platform among all groups. But the ATR+28DST group reached the platform a little shorter than the ATR group on the 5th day (Fig. 3a). Besides, there was also no significant change in the average speed of pups among the groups (Fig. 3b). In the spatial probe test stage, there was a significant reduction in the percentage of time in the target quadrant (Fig. 3c) and the number of crossing platforms (Fig. 3d) in the ATR group ($P < 0.05$). The performance of animals that had received 28 days of swimming training was noticeably improved compared with the ATR group in the number of crossing platforms (Fig. 3d). And in the percentage of time in the target quadrant, there was an increasing trend in swimming training groups compared with the ATR group, but not statistically significant.

Grip Strength Test

The results of the grip strength test in the ATR group and the swimming training groups are shown in Fig. 3e. The maximum grip of the ATR group was significantly decreased ($P < 0.05$) compared with that of the control group. The grip strength of rats in the ATR+28DST group that had received swimming training was greater than that of rats in the ATR group ($P < 0.05$). Differences in grip strength between the ATR+8DST group and the ATR group were not statistically significant.

Effects of ATR Exposure and Swimming Exercise on Hippocampal Histomorphology

Hematoxylin and Eosin (H&E) Staining

Histomorphological analysis revealed some slight alterations in the hippocampal CA1 area of the ATR group and the swimming training groups (Fig. 4A). In the control group, the hippocampal pyramidal neurons presented a regular, orderly, compact alignment, containing a large, rounded, and distinct nucleus in each neuron (Fig. 4A1–2). However, in the ATR group, the hippocampal pyramidal neurons showed nuclear pyknosis and an obvious change in cell arrangement and even the presence of microglia

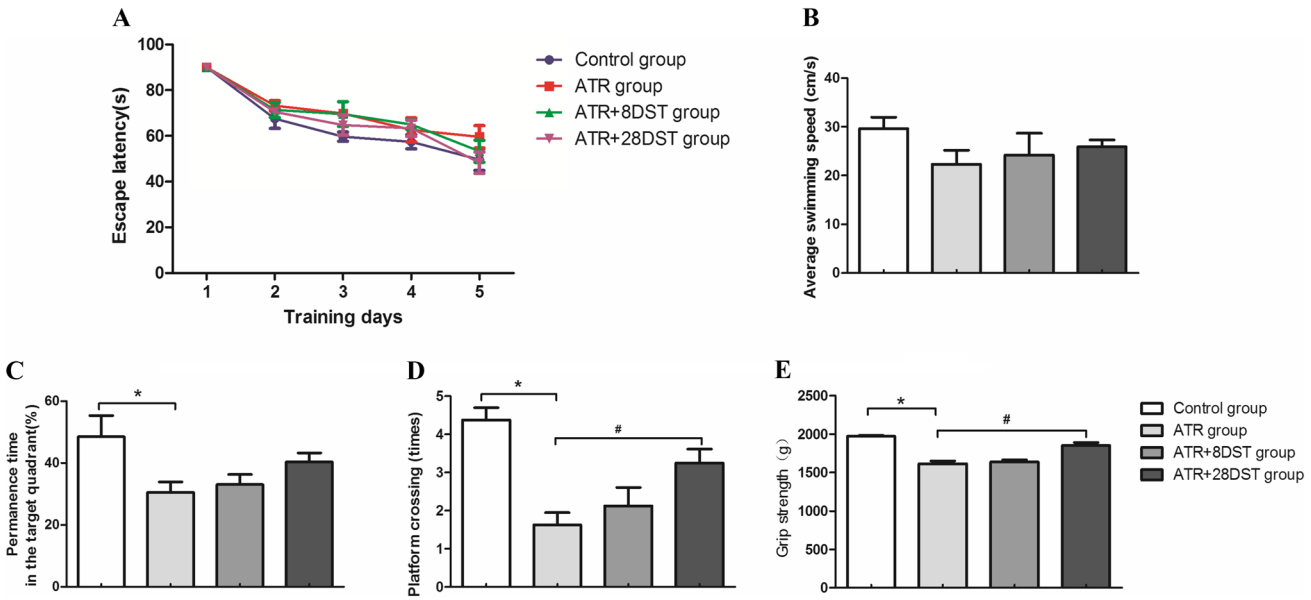

Fig. 3 **a** Escape latency of rats reaching the platform in the MWM during training trial; **b** Average swimming speed of rats in the MWM; **c** Percentage of time that the rat spent in the target quadrant; **d** Number of times rats passed the platform site in the MWM; **e** Grip strength of rats in different groups (*$P < 0.05$ vs. control group; #$P < 0.05$ vs. ATR group; $n = 8$)

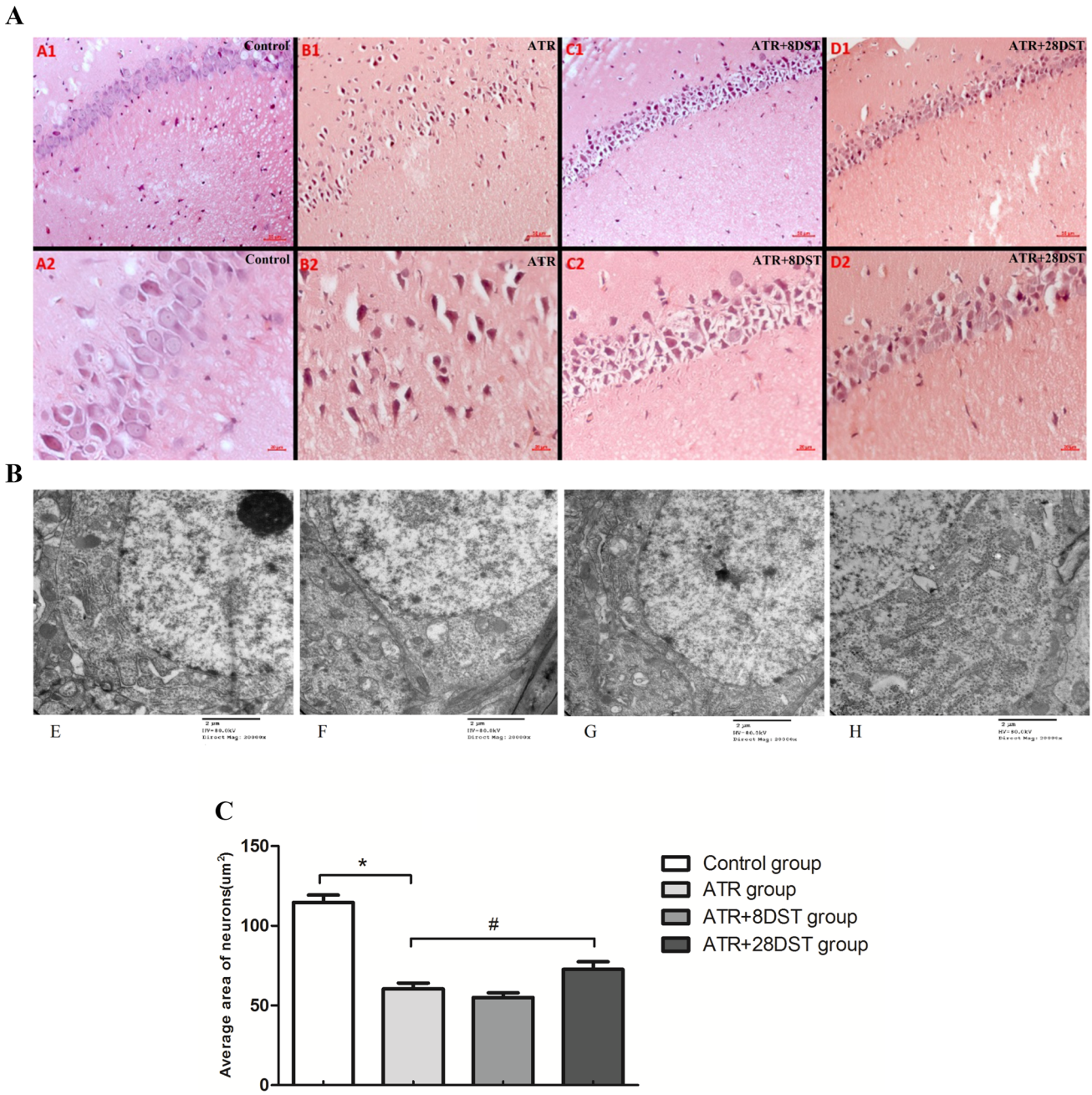

Fig. 4 **a** Optical microscopy images showing morphology of hippocampus in ATR group and groups that received swimming training: A1, B1, C1, D1; scale bar = 50 μm; A2, B2, C2, D2; scale bar = 20 μm. **b** Electron microscopy images showing effects of ATR and swimming training on microstructure of hippocampal cells: E, F, G, H; scale bar = 2 μm. Obvious changes, including swollen mitochondria, fuzzy nuclear membranes, or enlarged endoplasmic reticulum, are marked with red arrows. **c** The hippocampal neuronal soma size in 400× magnification was measured using Image-Pro Plus (*$P < 0.05$ *vs.* control group; #$P < 0.05$ *vs.* ATR group; $n \geq 5$)

infiltration (Fig. 4B1–2). After swimming training, the arrangement of pyramidal neurons in the hippocampus was obviously improved, especially in the ATR+28DST group (Fig. 4D1–2). Statistical analysis showed an obvious decrease in neuronal soma size after maternal exposure to ATR, compared with that in the control group ($P < 0.05$, Fig. 4C). There was no statistical difference between ATR+8DST and ATR group or ATR + 28DST and ATR group.

Scanning Transmission Electron Microscopy

The electron microscope observation showed subtle differences in ultrastructure of the hippocampal CA1 area

among the ATR group, the swimming training groups and control group (Fig. 4B). In the control group, hippocampal neurons had distinct nuclei and smooth double-layer karyolemma with chromatin hypodispersion. And, there were mitochondria with obvious cristae, clear endoplasmic reticulum, and Golgi apparatus, well distributed in the cytoplasm (Fig. 4E). In the ATR group, an obvious change in hippocampal neurons was that karyolemma were blurred and shriveled, and chromatin particles were condensed in the nuclei. The mitochondria were swollen and flawless, the Golgi and endoplasmic reticulum became flat (Fig. 4F). After swimming training, the mitochondria and endoplasmic reticulum in hippocampus neurons were noticeably improved, compared with the ATR group. The effect increased with increased training time, and was most marked in the ATR+28DST group (Fig. 4H).

Immunofluorescence Double Staining of PSD95 and NR2B

Double-labeled immunofluorescence analysis was used to assess position and quantity of PSD95 (green) and NR2B (red) proteins in the hippocampal CA1 area (Fig. 5). The hippocampal nuclei (blue) arranged closely and orderly were used to localize target proteins (Fig. 5a). Statistical analysis showed an obvious decrease in PSD95 and NR2B protein expression after maternal exposure to ATR, compared with that in the control group ($P < 0.05$, Fig. 5b). After swimming training, PSD95/NR2B double positive neurons were significantly increased in the ATR+28DST group compared with the ATR group ($P < 0.05$). There was no statistical difference between ATR+8DST and ATR group.

Effects of ATR Exposure and Swimming Training on Gene Expression

Real-time PCR analysis showed that gene levels of Wnt5a, JNK, PSD95, NR2B, PI3K, and c-fos mRNA in the hippocampus were significantly reduced in the ATR group compared with the control group ($P < 0.05$; Fig. 6). After swimming training, all gene levels in the ATR+28DST group showed an obvious increase compared with the ATR group ($P < 0.05$). However, except for NR2B mRNA, there were no significant changes in Wnt5a, JNK, PSD95, PI3K, and c-fos mRNA between the ATR + 8DST group and the ATR group.

Effects of ATR Exposure and Swimming Training on Protein Expression

In comparison with the control group, ATR significantly decreased protein expression levels of Wnt5a, JNK, p-JNK, PSD95, NR2B, PI3 K, and c-fos in the hippocampus ($P < 0.05$; Fig. 7), as determined by western blot analysis. However, the expression levels of all proteins in the ATR+28DST group and JNK and p-JNK in ATR+8DST group were significantly increased by swimming training compared with the ATR group ($P < 0.05$). Differences in levels of Wnt5a, PSD95, NR2B, PI3 K, and c-fos in the hippocampus between the ATR+8DST group and the ATR group were not statistically significant.

Discussion

ATR, a herbicide with widely acknowledged neurotoxicity, may be a important risk factor for the neurodegenerative diseases, such as AD and PD (Shaw 2011; Sun et al. 2014). The present study firstly demonstrated our previous speculation that gestational and lactational exposure to ATR leads to dysfunction in learning and memory in offspring male rats. Learning and memory deficit is a very important symptom of central nervous system damage, which are the main characteristics of AD and also symptoms of PD (Millan et al. 2012; Coley and Gao 2017). Currently, although there is no reliable medical treatment to treat this type of neurodegenerative disease with learning and memory deficit, the neuroprotective effects of exercise training have been confirmed in AD, PD, etc. (Patki et al. 2014; Cyna et al. 2016). Some studies have suggested that exercise training maintains synaptic plasticity and protects learning and memory impairment in AD rat models (An et al. 2013; Dao et al. 2015). Therefore, we hypothesized and further confirmed that appropriate exercise training might also improve ATR-induced learning and memory impairment.

Rats are capable of spontaneous swimming and do not generally show strong resistance behaviors when they receive swimming training. Therefore, swimming training can reduce the reaction to other adverse stimuli (Kramer et al. 1993). In the MWM test, there were no obvious changes in escape latency and average swimming speed of the offspring among four groups. However, it could still be found that there is a slight decrease in the ATR group compared with the other groups. Following maternal ATR exposure, percentage of time in the target quadrant and number of crossing platforms both decreased significantly compared with the control group, indicating that ATR exposure during gestation and lactation can lead to a decrease in spatial learning and

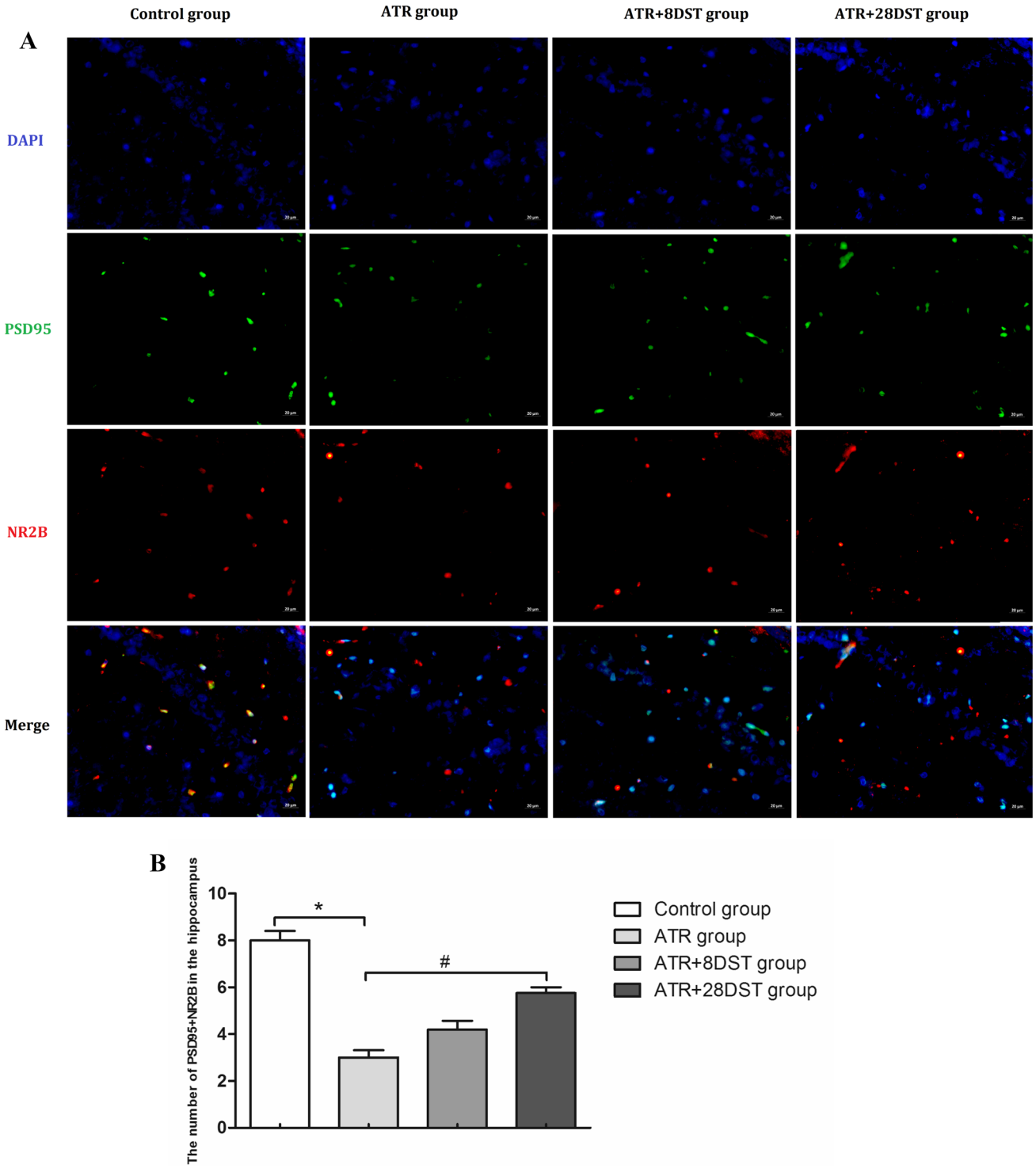

Fig. 5 **a** Double-labeled immunofluorescence analysis of PSD95 (green) and NR2B (red) proteins in the hippocampal CA1 area. Scale bar = 20 μm. **b** Counts of $PSD95^{+}$+$NR2B^{+}$ cells were measured using Image-Pro Plus (*$P < 0.05$ *vs.* control group; $^{\#}P < 0.05$ *vs.* ATR group; $n \geq 5$)

memory in offspring rats. The above results were mostly consistent with our previous MWM tests in ATR or paraquat and maneb (PQ-MB) developmental exposures (Li et al. 2016; Li et al. 2018). However, the result was different from a previous scholar's report that ATR exposure revealed no significant differences on rats in the MWM

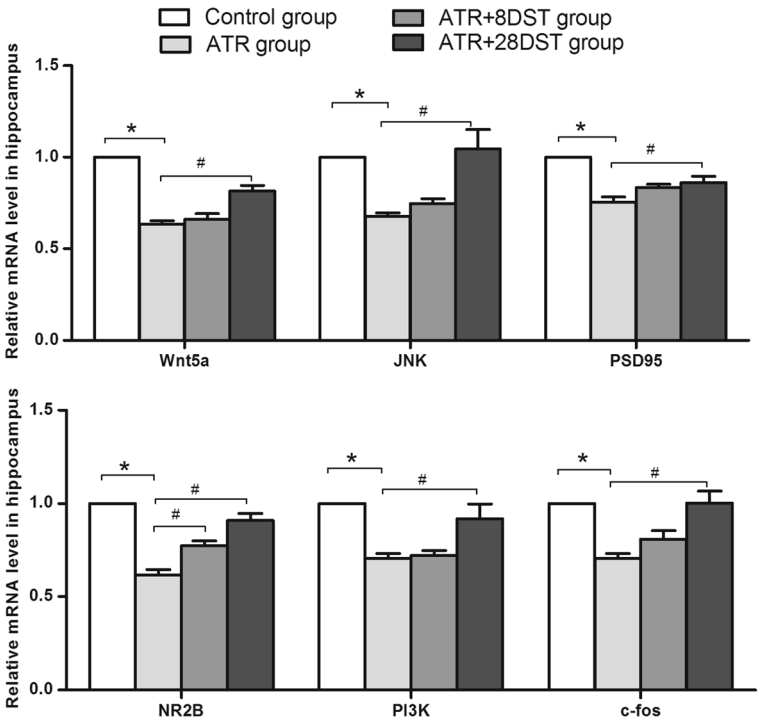

Fig. 6 Real-time PCR analysis of Wnt5a, JNK, PSD95, NR2B, PI3K, and c-fos mRNA expression in hippocampus (*$P < 0.05$ *vs.* control group; #$P < 0.05$ *vs.* ATR group; $n \geq 10$)

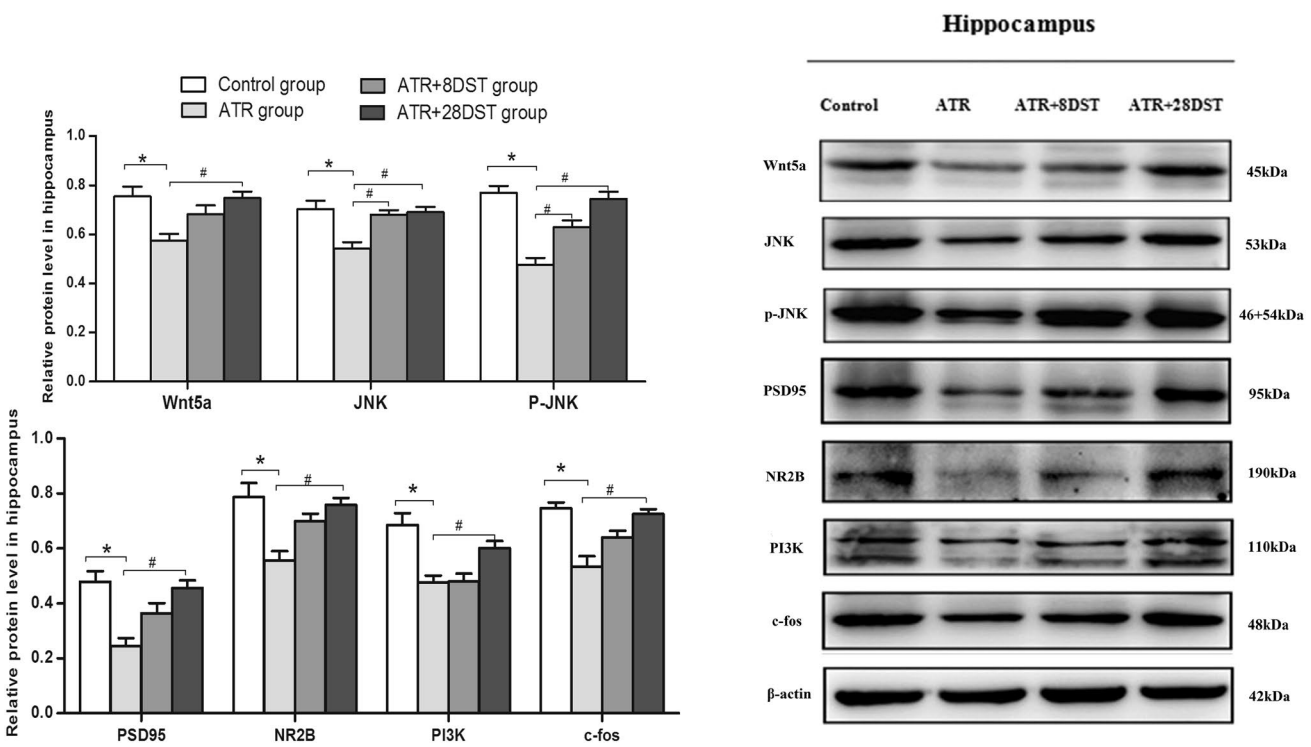

Fig. 7 Western blot analysis showing protein expression levels of Wnt5a, JNK, p-JNK, PSD95, NR2B, PI3K, and c-fos in hippocampus (*$P < 0.05$ *vs.* control group; #$P < 0.05$ *vs.* ATR group; $n \geq 10$). Representative western blots of proteins, together with pixel densities

test (Walters et al. 2015). We speculated that there may be no toxic effects on rats due to too low exposure dose of ATR. Next, after 8 days and 28 days of swimming training, there was a noticeably improved in the number of crossing platforms and a slight increase in percentage of time in the target quadrant especially in ATR+28DST group, compared to the ATR group. In order to avoid the situation that long-trained rats are accustomed to swimming in the water, we have increased the rat grip strength test, unrelated with water. The result was consistent with the number of crossing platforms in the MWM. Therefore, we preliminarily concluded that long-term swimming training is likely to improve learning and memory deficit in offspring caused by maternal ATR exposure during gestation and lactation.

It is worth noting that the changes in hippocampal CA1 area have a good correlation with learning and memory impairment in human brain (Fouquet et al. 2012; Counts et al. 2014). Many alterations in hippocampal gene expression underlie the learning and memory improvement caused by lifelong exercise (Stranahan et al. 2010). Histomorphological examination of the hippocampal CA1 area in offspring rats showed a disordered arrangement of pyramidal neurons, with atrophic nuclear membranes and expanded organelles after maternal ATR exposure. These changes, which were obviously present in the ATR group, were probably a direct result of ATR exposure, since it was also found in our previous histomorphometric analysis (Li et al. 2016; Li et al. 2018). Significantly, the improvements were clearly seen in the morphology of hippocampal tissue after 28 days of swimming training, again illustrating the ability of swimming training to reverse the negative effects of maternal ATR exposure on learning and memory function of the offspring from morphological aspect. These important findings above all were consistent with brain histomorphological and functional impacts of motor training in rats in the current series of studies, which training can improve motor function and enhance dendritic growth and plasticity, promote nerve myelinization, regenerate, and repair of injured brain tissue (Li et al. 2000; Biernaskie and Corbett 2001; Rustemeyer et al. 2010).

To determine the potential mechanism of learning and memory function, we preliminary analyzed the immunofluorescence signal intensity of PSD95 and NR2B in the hippocampal CA1 area of offspring rats. Immunofluorescence double staining showed that PSD95 colocalized with the NR2B receptor in hippocampal neurons significantly decreased following maternal ATR exposure, while the decrease in expression levels in offspring was obviously prevented by swimming training, which is in agreement with previous reports. Water maze training can rapidly induce the recruitment of PSD95 and NR2B to the synaptic lipid raft (Delint-Ramírez et al. 2008), while perturbing the interaction of PSD95 with NR2B receptor weakens spinal nociceptive plasticity (D'Mello et al. 2011). Furthermore, we again demonstrated that the gene and protein levels of PSD95 and NR2B were significantly downregulated in the hippocampus of ATR-treated rats without swimming compared with the control rats, accompanying a spatial learning and memory deficit in the MWM task. Moreover, there was an obvious up-regulation in the gene and protein levels of PSD95 and NR2B in ATR-treated rats with 28 days of swimming training compared with ATR-treated rats without swimming, accompanying a slightly improved spatial learning and memory in the MWM task. The above results further reveal that the down-regulation or up-regulation of PSD95 and NR2B is closely related to spatial learning and memory function in the study. Consistent with the present study, previous report has also indicated that the decreased expressions of PSD95 and NR2B in hippocampus in long-term spontaneous recurrent seizure might lead to behavioral defect, including spatial learning and memory deficit (Sun et al. 2009). Subsequently, we measured the hippocampal levels of Wnt5a, JNK, p-JNK, PI3 K, and c-fos mRNA and proteins in 3-month-old rats of all groups. Consistent with the expressions of PSD95 and NR2B, all gene and protein expression levels were significantly decreased after ATR exposure, and their expression levels increased significantly after 28 days of swimming training compared with the ATR exposure. Consistent with the expressions of Wnt5a, JNK, and p-JNK in the present study, it has been found that the Wnt-5a/JNK pathway promotes accumulation of the postsynaptic membrane scaffold protein PSD95 in hippocampal neurons (Farías et al. 2009). The current results are also supported by a research showing that aerobic exercise can increase levels of Wnt and Wnt receptor, and improve the function of the nervous system (Fang et al. 2017). This suggests that the Wnt5a/JNK pathway can participate in PSD95/NR2B-mediated learning memory function in offspring rats. Further, we found that the expression of NR2B, PI3K, and c-fos in consistency is reduced in ATR-exposed rats and up-regulated in 28 days of swimming-trained rats. The similar expression was also observed in the following studies. The deletion or inhibition of PI3 Kγ disrupted NMDA receptor-dependent Long-Term Depression (LTD) and reversal learning in the MWM task (Jae-Ick et al. 2011). Lower concentrations of acute fluoxetine treatment reduced c-fos expression via PI3K/Akt signaling pathway (Li et al. 2017). The level of c-fos was significantly up-regulated in the hippocampus of NR2B overexpression-induced memory-enhanced mice (Li et al. 2011). All the above show that NR2B might affect the IEG c-fos expression via PI3K/Akt pathway, along with the regulation of learning and memory.

Collectively, our current studies firstly indicated that gestational and lactational exposure to ATR may have adverse effects on offspring, resulting in changes in spatial learning

and memory ability. We have also shown, for the first time, that swimming training can reduce the adverse learning and memory effect caused by maternal ATR exposure. It was found that Wnt5a/JNK mediates the binding of PSD95 and NR2B to regulate the expressions of PI3K and c-fos, which can participate in the learning and memory regulation of this study. Long-term swimming training was also found to significantly improve learning and memory compared with short-term training. Taken together, this study provides a scientific basis for the neurotoxicity of ATR and the effects of gestational and lactational exposure to ATR on spatial learning and memory in offspring, and lays a theoretical foundation for the ability of exercise training to repair learning and memory function.

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Compliance with Ethical Standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical Approval All applicable international, national, and/or institutional guidelines for the care and use of animals were followed. This article does not contain any studies with human participants performed by any of the authors.

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