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Involvement of NMDAR/PSD-95/nNOS–NO–cGMP pathway in embryonic exposure to BPA induced learning and memory dysfunction of rats

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ABSTRACT

Bisphenol A (BPA), can lead to learning and memory impairment, but the underlying mechanism is poorly understood. Researchers have indicated that the N-methyl-D-aspartate receptor (NMDAR)/post-synaptic density protein 95 (PSD-95)/neuronal nitric oxide synthase (nNOS)-nitric oxide (NO)-cyclic guanosine monophosphate (cGMP) pathway greatly contributes to learning and memory process. Pregnant rats were exposed to 0, 0.05, 0.5, 5 and 50 mg/kg/day BPA via oral gavage from gestational day (GD) 5 to GD 19. Morris water maze, transmission electron microscope, western blot, real time PCR, biochemical analysis and ELISA were used to analyze the changes in behavior, synaptic ultrastructure, protein and gene expression of NMDAR, PSD-95, nNOS, together with nNOS activity, NO (Nitrate reductase method) and cGMP levels of the rat pups at different growth stages. Results of this research displayed that exposure to 0.5 mg/kg/day BPA could damage the spatial learning ability of rats at postnatal day (PND) 56. However, spatial memory ability could be affected by exposure to BPA at doses up to 5 mg/kg/day. Moreover, the thickness of the postsynaptic density decreased after exposure to BPA at doses of 5 and 50 mg/kg/day. Levels of NR1, NR2A, PSD-95 protein and mRNA were downregulated to some extent after exposure to BPA, whereas the expression of NR2B increased at GD 20 but decreased at PND 21 and 56. Contrarily, the nNOS expression along with the enzyme activity were promoted after exposure to BPA. Meanwhile, the NO and cGMP levels were suppressed at GD 20 but promoted at PND 21 and 56. In conclusion, these results demonstrated that NMDAR/PSD-95/nNOS–NO–cGMP pathway could be affected by embryonic exposure to BPA, which may involve in the spatial learning and memory dysfunction of rats in later life.

1. Introduction

As a component of epoxy resin and polycarbonate plastic, bisphenol A (BPA) has been widely employed in the production of consumer products, such as food packaging, thermal receipts, medical equipment, children's toys and consumer electronics (Healy et al., 2015; Negev et al., 2018). Due to the ubiquitous use in daily necessary products, the existence of BPA in human fluids has been proved (Careghini et al., 2015; Rubin, 2011; Vandenberg et al., 2007). Previous researchers have revealed that exposure to BPA is correlated with the risk of human diseases involving multiple organ systems, including reproductive, cardiovascular, endocrine and nervous systems (Rochester, 2013; Silva et al., 2019). The negative influence of exposure to BPA on the central nervous system, especially on the dysfunction of learning and memory ability is increasing and is of concern (Bowman et al., 2019; Fang et al., 2017; Hu et al., 2017; Zhou et al., 2017). However, the underlying molecular mechanism remains unclear.

Learning and memory ability is associated with several brain regions, and the hippocampus is supposed to be principal in this process (Ekstrom and Ranganath, 2018; El-Falougy and Benuska, 2006; Mhaouty-Kodja et al., 2018). As a fact that synaptic plasticity greatly contributes to learning and memory process, the alterations in synaptic structure and function at several aspects,

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including presynaptic neurotransmitter transmission, postsynaptic protein expression and dendrite growth, are referred to be related with learning and memory dysfunction (Lynch, 2004). Long-term potentiation (LTP) is one of the molecular mechanisms involved in the learning and memory process, and changes in N-methyl-D-aspartate receptor (NMDAR) expression or function are regarded as symbolic signs of abnormal LTP induction in many learning and memory disorders (Bailey et al., 2015; Morris, 2013). Briefly, the postsynaptic cellular Ca^{2+} influx induced by NMDAR stimulation can trigger the activation of multiple cell signaling pathways, which play important roles in LTP induction and maintenance of glutamatergic synapses.

The NMDAR are receptors composed of 4 subunits (NR1, NR2 and NR3 subunits), while NR1, NR2A and NR2B subunits are ubiquitously expressed in hippocampus, of which NR1 acts as functional subunit, and NR2A or NR2B functions as accessory subunit (Baez et al., 2018; Wang et al., 2019; Yashiro and Philpot, 2008). Previous studies have suggested that NR2A and NR2B could be linked with neuronal nitric oxide synthase (nNOS) via postsynaptic density protein 95 (PSD-95) forming NMDAR/PSD-95/nNOS complex, and the main biological effect of this complex is to catalyze the synthesis of nitric oxide (NO) (Christopherson et al., 1999; Li et al., 2017; Yan et al., 2004). NO has been a well-documented retrograde messenger in LTP, which can cross the synaptic gap and then bind to the guanylyl cyclase-coupled receptor of presynaptic neuron, enhancing cyclic guanosine monophosphate (cGMP) release (Hopper and Garthwaite, 2006; Prast and Philippu, 2001). The cGMP can promote protein phosphorylation by protein kinases and can result in presynaptic vesicle exocytosis and transmitter release, which play pivotal roles during the induction and maintenance of LTP (Arancio et al., 1996).

NMDAR/PSD-95/nNOS complex acts as a role in the specificity of nNOS to synthesize NO and cGMP. This cell-signaling pathway along with its downstream effectors is important in the LTP process. Previous researchers have explored the effects of exposure to BPA on the expression of NMDAR subunits, but the available results are inconclusive (Hu et al., 2017; Jardim et al., 2017; Kumar and Thakur, 2014; Xu et al., 2010). Besides, whether NMDAR/PSD-95/nNOS–NO–cGMP pathway participate in BPA-induced learning and memory dysfunction remains unclarified. With the special metabolic state during pregnancy, pregnant women are more susceptible to BPA, and exposure to BPA at this stage may have a profound impact on the offspring. In addition, free BPA can cross the placental barrier and blood brain barrier of the rat pups (Hu et al., 2017). Therefore, we carried out this study to explore the effects of embryonic exposure to BPA on learning and memory ability of rats by focusing on the alterations in NMDAR/PSD-95/nNOS–NO–cGMP pathway.

2. Materials and methods

2.1. Animal care and use statement

The present study was designed to minimize the animal suffering and use amount according to the National Institutes of Health Guidelines in China, which were also permitted by the Animal Ethics Committee of Shenyang Medical College.

2.2. Animals

Ten-week-old Sprague Dawley (SD) rats were used in this study purchased from Liaoning Changsheng biotechnology (Certificate number: SCXK2015-0003, Liaoning, China). Rats were cared with food (SPF-grade, soy-free rodent diet) and water ad libitum in sterilized polypropylene cages with the presence of wood chips at 25 ± 2 °C and 55 ± 5% relative humidity, light cycle (half day light and dark). As the existence of BPA in the commonly used plastic water bottles might be the main oral exposure source of rats, BPA-free glass bottles were used during the study to limit the orally exogenous exposure.

2.3. Experimental procedures

After adapting for one week, female rats were mated with male rats randomly. Vaginal smears were used to confirm the pregnancy of rats. The day was defined as gestational day (GD) 0 when the positive sperm was observed under the microscope. Sixty pregnant rats were randomly divided into five groups, twelve in each group. They were fed separately and given corn oil contained 0, 0.05, 0.5, 5 and 50 mg/kg/day BPA from GD 5 to GD 19 (from implantation stage to the former day of parturition) through oral gavage, BPA is dissolved in corn oil. Half of pregnant rats and their fetal pups in each group were anaesthetized and sacrificed at GD 20. Other pregnant rats were kept to spontaneous labor. The day of the pups' birth was recorded as postnatal day (PND) 1, and six pups of each litter were preserved to keep litter and sex balance. At PND 21, mother rats were euthanized and the pups were weaned and feed until PND 56. At GD 20, PND 21, and PND 56, the pups were decapitated under deep ether anesthesia, then the hippocampal tissues were immediately frozen in liquid nitrogen after separated, and kept in −80 °C freezer before western blot, real-time PCR, nNOS activity, NO levels and ELISA analyses. The detailed experimental flow-chart is shown in the Supplementary Fig. 1. As it is still controversial about whether there is gender difference in BPA induced learning and memory impairment, the pups used in each experiment were half-male and half-female.

2.4. Reagents and laboratory wares

Antibodies against NR1 (#sc-1467), NR2A (#ARG52360.100), PSD-95 (#ab2723), nNOS (#ab1376), NR2B (#21920-1-AP), and β-actin (#66009-1-Ig) were purchased from Santa Cruz Biotechnology (California, USA), Arigo (Taiwan, China), Abcam (Cambridge, UK), and Proteintech (Wuhan, China), respectively. The cGMP assay, nNOS activity and NO level detecting kits were purchased from R&D Systems (Minneapolis, USA) and Nanjing Jiancheng Bioengineering Co. Ltd (Nanjing, China), respectively. Trizol and SYBR Premix Ex Taq II reagent were purchased from Takara (Tokyo, Japan).

2.5. Morris water maze (MWM)

MWM place trials and probe trials were performed to test spatial learning and memory ability of rat pups at PND 56 (six in each group), respectively. Escape latency (place trial) and time spent in the target quadrant (probe trial) were analyzed. The detailed procedures were summarized in Supplemental Materials.

2.6. Synaptic ultrastructure observation

The synaptic ultrastructure of CA1 region in the hippocampus of rat pups (three in each group) at PND 21 and 56 were observed under a transmission electron microscope (Hitachi 7650, Japan), and the tissue slice were 70 nm. The postsynaptic density thickness and synaptic cleft width were measured with an image analyzing software (NIS-Elements BR 3.2). The detailed methods were conducted as previously described (Yu et al., 2016).

2.7. Western bolt

The hippocampal tissues (six in each group) were lysed using RIPA (Beyotime biotechnology, Shanghai, China) and centrifuged at 4 °C, 12 000 g for 20 min, then protein concentrations were tested by BCA assay kit (Thermo Fisher Scientific, Massachusetts, USA). Aliquots of sample (50 μg per lane) were conducted western bolt analysis as previously described (Yu et al., 2016). Eight percent of SDS-PAGE was used, and the concentrations of first, second antibody were set as NR1 (1:500), NR2A (1:1000), NR2B (1:1000), PSD-95 (1:1000), nNOS (1:500), β-actin (1:2000) and peroxidase conjugated secondary antibodies (1:5000), respectively. PagePuler™ prestained protein ladder (Thermo Fisher Scientific, Massachusetts, USA) was used in this study. The target protein expression was normalized to β-actin.

2.8. Real-time PCR

Trizol was used to extract total RNA of rat pups' hippocampal tissues (six in each group), thereafter, the PrimeScript RT kit was applied to synthetize cDNA. Then, the cDNA templates together with primers were used for real-time PCR amplification with SYBR Premix Ex Taq II and ABI 7500 fast real-time PCR System (California, USA), and the primer details of NR1, NR2A, NR2B, PSD-95, nNOS and GAPDH (as internal control) were summarized in Table 1. Results were analyzed using the comparative Ct method.

2.9. nNOS activity and NO levels

Briefly, hippocampal tissues (six in each group) were homogenized and centrifuged, then the supernatants were collected for assaying nNOS activity by measuring NO produced during the catalysis as the manufacturer's instruction of specific commercial kits. As the short half-life and the rapid diffusion rate, the direct detection of NO in vivo is very difficult. NO can quickly metabolized into nitrite (NO_2^-) and nitrate (NO_3^-) in vivo, which are more stable and can accumulate in the tissues, so we used the NO_2^- and NO_3^- contents to estimate the NO production levels. Nitrate reductase method was used in this study, which used specific nitrate reductase to reduce NO_3^- into NO_2^-, and then test the NO_2^- contents (sum of NO_3^- and NO_2^-) to reflect the NO levels.

2.10. cGMP levels

The hippocampal tissues (six in each group) were homogenized in cell lysis buffer (R&D Systems, Minneapolis, USA). Then the samples were subsequently centrifuged and the supernatants were collected for detecting cGMP concentrations using a commercial ELISA kit according to the manufacturer's instruction.

Table 1
Primer sequences for the target genes.

Gene		Primer sequence (5′-3′)	Length (bp)
NR1	Forward	CAGGCTCAGAAACCCCTCAG	135
	Reverse	GCTGCGCTCTCGTAATTGTG	
NR2A	Forward	CCAGGAGGAGTTTGTGGACC	198
	Reverse	AAGCTGACCAAGGCATCCTC	
NR2B	Forward	GGGTCACGCAAAACCCTTTC	113
	Reverse	CCTTGTTTTTGACGCCCCTG	
PSD-95	Forward	AGATGAAGACACGCCCCCTC	132
	Reverse	CCCTCTGTTCCATTCACCTGC	
nNOS	Forward	AGAGGAGGACGCTGGTGTA	104
	Reverse	GGCGGTTGGTCACTTCATA	
GAPDH	Forward	GCAAGAGAGAGGCCCTCAG	74
	Reverse	TGTGAGGGAGATGCTCACTG	

2.11. Statistical analysis

All data were presented as mean ± standard deviation (SD), and statistical differences among groups were evaluated by One-way ANOVA followed by least significant difference (LSD) multiple comparisons using the SPSS software, version 22.0 (SPSS Inc., Illinois, USA), $p < 0.05$ was regarded as statistically significant.

3. Results

3.1. Embryonic exposure to BPA affected the body and brain weight gain of rats

To explore the effects of exposure to BPA on general growth of the rat pups, the mental health and activities were checked daily, and the results showed that they were in healthy condition with normal diet and drinking, tight fur and normal coloration. The body and brain weight of rats were weighed at GD 20, PND 21 and 56, and results showed that the rats' body and brain weight increased steadily. However, both the body and brain weight of rats in 5 and 50 mg/kg/day BPA groups at GD 20 were significantly lighter than that in control and other BPA groups, whereas no significant difference existed among groups at PND 21 and 56 (Fig. 1A and B).

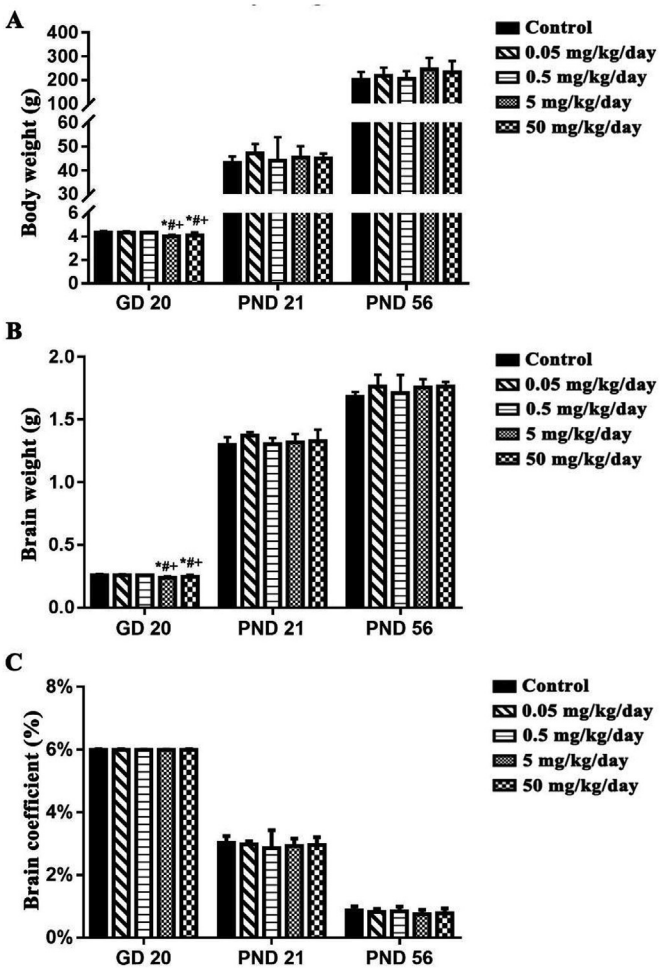

Fig. 1. Changes in body and brain weight of rats from three growth stages after embryonic exposure to BPA. (**A**) Changes in body weight. (**B**) Changes in brain weight. (**C**) Changes in brain coefficient. (GD: gestational day; PND: postnatal day. Data were shown as mean ± SD, n = 6. * denotes comparison with control group; # denotes comparison with 0.05 mg/kg/day BPA group; + denotes comparison with 0.5 mg/kg/day BPA group; $p < 0.05$. One-Way ANOVA followed by LSD tests.)

Compared with control group at GD 20, the body and brain weight of rats decreased by 7.82% and 7.67% in 5 mg/kg/day BPA group and decreased by 6.29%, 6.14% in 50 mg/kg/day BPA group (Fig. 1A and B). Besides, the brain coefficient did not change between the control group and BPA groups at these development stages (Fig. 1C).

3.2. Embryonic exposure to BPA induced spatial learning and memory dysfunction of rats at PND 56

MWM results showed that the escape latency of rats in 50 mg/kg/day BPA group was significantly longer than that in control and other BPA groups on the third and fourth training days. Besides, the escape latency of rats in 5 mg/kg/day BPA group was also significantly longer than that in control and 0.05 mg/kg/day BPA group on the fourth training day. Furthermore, on the fifth training day, the escape latency of rats in 0.5, 5 and 50 mg/kg/day BPA groups was significantly longer than that in control group, in which, the 5 and 50 mg/kg/day BPA groups were also longer than that in 0.05 and 0.5 mg/kg/day BPA groups (Fig. 2A).

As illustrated in Fig. 2B and C, rats in 5 and 50 mg/kg/day BPA groups spent shorter time in the target quadrant (platform previously existed) than control and 0.05 mg/kg/day BPA group in the probe trial and the difference had statistical significance. There also existed significant difference between 0.5 and 50 mg/kg/day BPA groups. However, the distance traveled by rats did not differ between the control group and BPA groups (data shown in Supplementary Fig. 2.) We could also observe that rats in control group mainly swam in the central areas (within the inner and middle ring) of the pool, and searching the platform around the areas where it previously existed (Fig. 2C). While, rats in BPA groups mainly swam in the surrounding areas (between the middle and out ring), and there was no obvious sign for searching the platform (Fig. 2C).

3.3. Embryonic exposure to BPA caused abnormal hippocampal synaptic ultrastructure in CA1 region of rats at PND 21 and 56

As the representative micrographs presented in Fig. 3 showed, for rats in control group both at PND 21 and 56, the hippocampal synaptic ultrastructure in CA1 region was normal with intact cellular membranes, abundant postsynaptic density, together with clearly visible synaptic cleft. However, the postsynaptic density thickness of rats in 5 and 50 mg/kg/day BPA groups thinned obviously than the control, 0.05 and 0.5 mg/kg/day BPA groups (Fig. 3 and Table 2). Nevertheless, the synaptic cleft width of rats in BPA groups showed no significant alteration in comparison with each control group (Fig. 3 and Table 2).

3.4. Embryonic exposure to BPA affected the expression of hippocampal NMDAR subunits

As shown in Fig. 4A, B and C, both the hippocampal NR1 subunit protein and mRNA levels of rats in BPA groups decreased significantly compared with each control group (except for the subunit protein levels of rats at PND 21 in 0.05 mg/kg/day BPA group). Likewise, NR1 subunit protein and mRNA levels of rats at GD 20 and PND 56 in 0.5, 5 and 50 mg/kg/day BPA groups, NR1 subunit protein levels of rats at PND 21 in 50 mg/kg/day BPA group were also

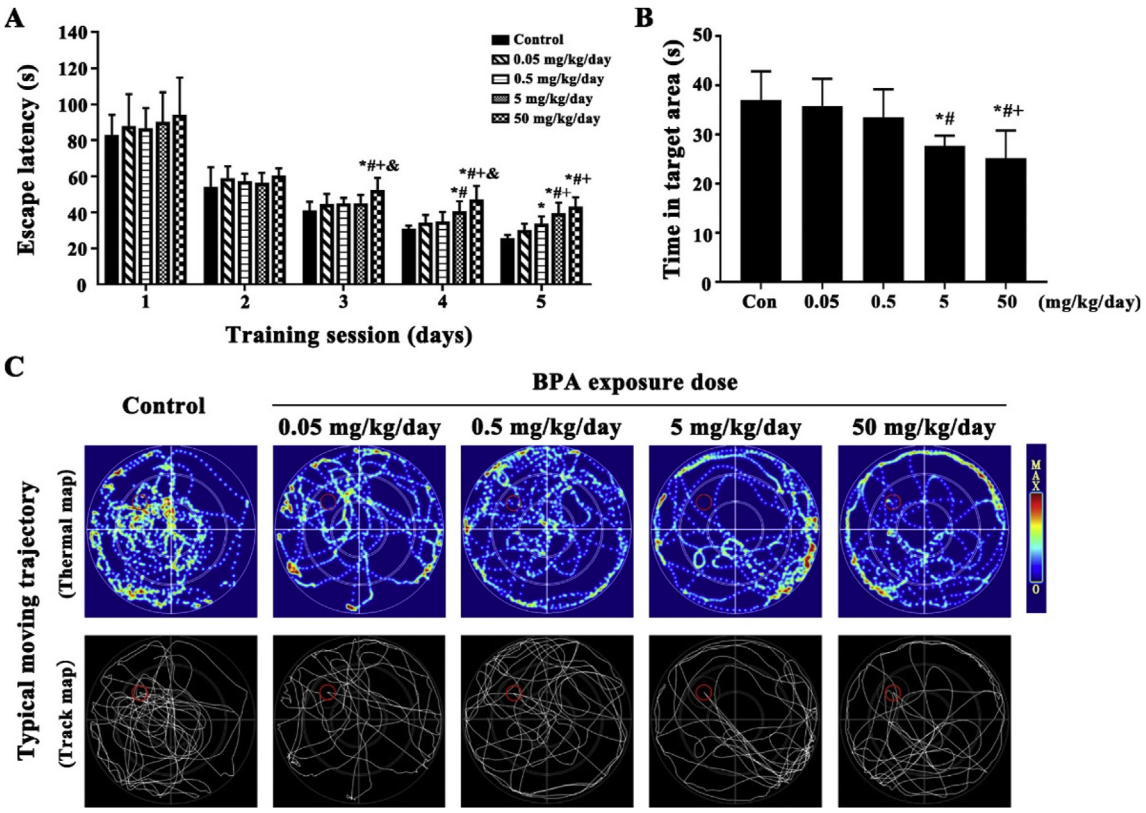

Fig. 2. Changes in spatial learning and memory ability of rats at PND 56 after embryonic exposure to BPA. (**A**) Changes in escape latency of place trial. (**B**) Changes in time spent in the target quadrant in probe trial. (**C**) Representative images of typical moving trajectory in probe trial. The above images were thermal maps and under images were track maps. The red cycle represented the platform former existed areas. (GD: gestational day; PND: postnatal day. Data were shown as mean ± SD, n = 6. * denotes comparison with control group; # denotes comparison with 0.05 mg/kg/day BPA group; + denotes comparison with 0.5 mg/kg/day BPA group; & denotes comparison with 5 mg/kg/day BPA group; $p < 0.05$. One-Way ANOVA followed by LSD tests.). (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

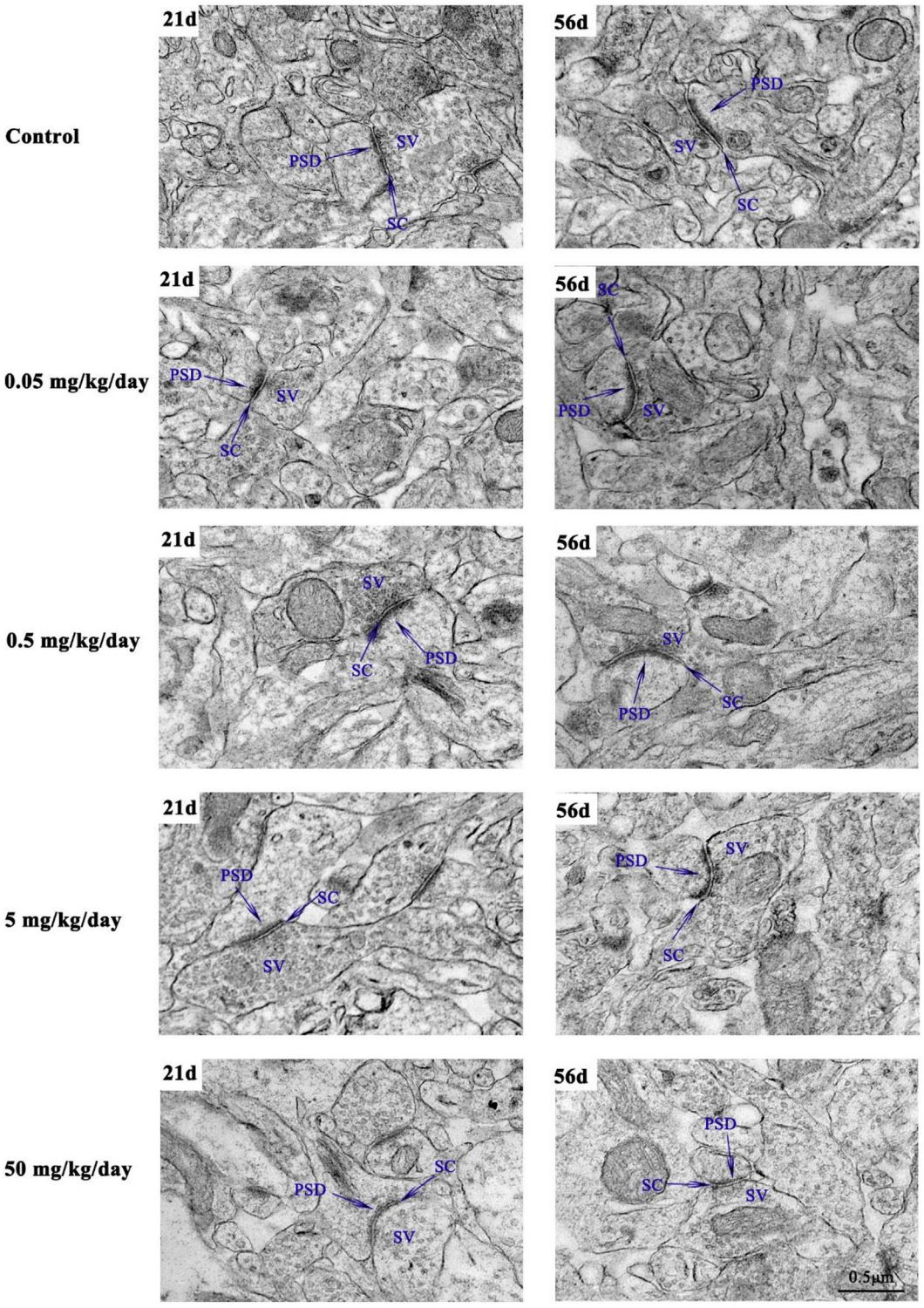

Fig. 3. Effects of embryonic exposure to BPA on the synaptic structure of hippocampal CA1 region in rats at PND 21 and 56. 21d: postnatal day 21; 56d: postnatal day 56. PSD, postsynaptic density; SC, synaptic cleft; SV, synaptic vesicle; Slice thickness = 70 nm. Micrographs were photographed by 30 000 × , n = 3, Scale bar = 0.5 μm.

significantly lower than 0.05 mg/kg/day BPA group. Furthermore, the subunit protein levels of rats at PND 21 and 56 together with the subunit mRNA levels of rats at GD 20 in 50 mg/kg/day BPA group also decreased significantly compared with 0.5 and 5 mg/kg/day BPA groups.

Fig. 4D and E summarized that the hippocampal NR2A subunit protein levels in BPA groups were significantly lower than that in each control group at these growth stages. Otherwise, the NR2A subunit protein expression of rats at GD 20 in 0.5, 5 and 50 mg/kg/day BPA groups, and those of rats at PND 21 and 56 in 50 mg/kg/day BPA groups were significantly lower than 0.05 mg/kg/day BPA group. Besides, those of rats at PND 56 in 50 mg/kg/day BPA group

Table 2
Effects of embryonic exposure to BPA on thickness of postsynaptic density and width of synaptic cleft in hippocampal CA1 region of rats at PND 21 and 56.

Group	Thickness of postsynaptic density		Width of synaptic cleft	
	PND 21	PND 56	PND 21	PND 56
Control	43.0 ± 1.8	48.0 ± 1.7	24.2 ± 2.4	26.1 ± 1.8
0.05 mg/kg/day BPA	42.6 ± 1.9	48.0 ± 1.3	24.7 ± 2.4	25.8 ± 1.6
0.5 mg/kg/day BPA	41.4 ± 2.3	46.5 ± 1.0	25.0 ± 2.6	25.6 ± 2.6
5 mg/kg/day BPA	35.3 ± 2.1$^{*\#+}$	37.4 ± 1.6$^{*\#+}$	25.5 ± 2.3	26.9 ± 1.4
50 mg/kg/day BPA	36.6 ± 3.8$^{*\#+}$	36.7 ± 1.7$^{*\#+}$	25.4 ± 1.5	26.7 ± 2.1

Note: PND: postnatal day. Data were shown as mean ± SD (nm), n = 6.
* Denotes comparison with control group.
# Denotes comparison with 0.05 mg/kg/day BPA group.
+ Denotes comparison with 0.5 mg/kg/day BPA group; $p < 0.05$. One-way ANOVA followed by LSD tests.

also decreased significantly compared with 0.5 and 5 mg/kg/day BPA groups. Similar with the protein changes, the hippocampal NR2A subunit mRNA levels of rats at PND 21 and 56 in BPA groups decreased significantly compared with each control group (Fig. 4F). The NR2A subunit mRNA levels of rats at PND 21 in 50 mg/kg/day BPA group decreased significantly compared with other BPA groups, and those of rats at PND 56 in 0.5, 5 and 50 mg/kg/day BPA groups also decreased significantly over 0.05 mg/kg/day BPA group (Fig. 4F).

As illustrated in Fig. 4G and H, hippocampal NR2B subunit protein levels of rats at GD 20 in 5 and 50 mg/kg/day BPA groups were significantly higher than those in control group and 0.05 mg/kg/day BPA group. Besides, those in 50 mg/kg/day BPA group were also significantly higher than 0.5 and 5 mg/kg/day BPA groups. However, compared with the control group, the hippocampal NR2B subunit protein levels of rats at PND 21 and 56 in BPA groups significantly decreased. Furthermore, those of rats at PND 21 in 5 and 50 mg/kg/day BPA groups were significantly lower than 0.05 mg/kg/day BPA group, and those of rats at PND 56 in 5 and 50 mg/kg/day BPA groups were also significantly lower than 0.05 and 0.5 mg/kg/day BPA groups. Consistent with the protein alterations, hippocampal NR2B subunit mRNA levels of rats in BPA groups increased significantly at GD 20, but decreased significantly at PND 21 and 56 compared with each control group (Fig. 4I). However, the NR2B subunit mRNA levels showed no significant changes among BPA groups at these growth stages (Fig. 4I).

3.5. Embryonic exposure to BPA inhibited the expression of hippocampal PSD-95

Fig. 5A and B summarized that the hippocampal PSD-95 protein expression of rats in all BPA groups decreased significantly over each control group, in addition, those in 0.5, 5 and 50 mg/kg/day BPA groups (except for rats at PND 21 in 0.5 mg/kg/day BPA group) also decreased significantly over each 0.05 mg/kg/day BPA group. Moreover, those were significantly lower in 5 and 50 mg/kg/day BPA groups at GD 20 and PND 21 compared with the 0.5 mg/kg/day BPA group. The hippocampal PSD-95 mRNA expression of rats at PND 21 in 50 mg/kg/day BPA group and rats at PND 56 in all BPA groups were significantly lower than each control group. In which, rats at PND 21 in 50 mg/kg/day BPA group were also significantly lower than 0.5 and 5 mg/kg/day BPA groups (Fig. 5C).

3.6. Embryonic exposure to BPA promoted the expression and activity of hippocampal nNOS

As illustrated in Fig. 6A and B, the hippocampal nNOS protein levels of rats in 5 and 50 mg/kg/day BPA groups at these developmental stages, along with 0.5 mg/kg/day BPA group at PND 21 were significantly higher than each control and 0.05 mg/kg/day BPA group. Moreover, those were significantly higher than 0.5 mg/kg/day BPA group (except for rats at PND 56 in 5 mg/kg/day BPA group), in which, rats at GD 20 and PND 56 in 50 mg/kg/day BPA group also increased significantly compared with 5 mg/kg/day BPA group. The alterations of hippocampal nNOS mRNA expression were shown in Fig. 6C. The nNOS mRNA levels of rats in all BPA groups at GD 20 increased significantly compared with control group, and peaked at 0.05 mg/kg/day BPA group, then reduced as the increase of exposure dose. Moreover, the significant difference existed between 0.5, 5 and 50 mg/kg/day BPA groups with the 0.05 mg/kg/day BPA group, and existed between 50 mg/kg/day BPA group with 0.5 mg/kg/day BPA group. Likewise, the nNOS mRNA levels of rats at PND 21 and 56 in all BPA groups were significantly higher than each control group, and significantly higher in 50 mg/kg/day BPA group at PND 56 than other BPA groups. We also detected the changes in hippocampal nNOS activity, and the results were summarized in Fig. 6D. Similar with the nNOS protein expression, the nNOS activity in BPA groups increased with dose, significant difference existed in 50 mg/kg/day BPA group at GD 20 and PND 21, and all BPA groups at PND 56 when comparing with each control group. In addition, nNOS activity in 50 mg/kg/day BPA group at GD 20 and PND 21 was also higher than each 0.05 mg/kg/day BPA group, and was higher than 0.5 mg/kg/day BPA group at GD 20.

3.7. Embryonic exposure to BPA affected the levels of hippocampal NO and cGMP

As shown in Fig. 7A, comparing with the control group, NO levels decreased significantly in all BPA groups at GD 20, whereas increased significantly at PND 21 and 56 (except for rats in 0.05 mg/kg/day BPA group at PND 21). However, the NO levels in 0.5, 5 and 50 mg/kg/day BPA groups were significantly lower than the 0.05 mg/kg/day BPA group at PND 56.

As illustrated in Fig. 7B, the cGMP levels in 5 and 50 mg/kg/day BPA groups decreased significantly in comparison with control and other BPA groups at GD 20. In contrast to the reduction of cGMP at GD 20, those in 0.5, 5 and 50 mg/kg/day BPA groups at PND 21 and 56 up-regulated significantly comparing with each control group. Moreover, those in 0.5, 5 and 50 mg/kg/day BPA groups at PND 21 and 50 mg/kg/day BPA group at PND 56 were also significantly higher than 0.05 mg/kg/day BPA group.

4. Discussion

Considering that the embryonic stage is important for the development of nervous system, pregnant rats were exposed to BPA from the beginning of implantation (GD 5) to the end of the embryonic stage (GD 19) in this study. The BPA doses were designed as 0.05 mg/kg/day (Tolerable daily intake, TDI), 0.5 mg/kg/day (ten times higher than TDI), 5 mg/kg/day (No observed adverse effect level, NOAEL), and 50 mg/kg/day (Lowest observed adverse effect level, LOAEL). Although no severe poisoning symptoms were observed in the rats under these exposure conditions, the body and brain weight of rats at GD 20 in 5 and 50 mg/kg/day BPA groups decreased markedly. However, the body and brain weight increased in the BPA groups at PND 21 and 56, but the difference did not have statistical significance. Previous animal studies have shown that exposure to BPA in utero can result in the loss of offspring's birth weight (Susiarjo et al., 2015; Troisi et al., 2014), but increase body weight in their later life (Rubin and Soto, 2009; Susiarjo et al., 2007). Our results are in agreement with epidemiological studies that also indicated the increase of amniotic fluid BPA concentration

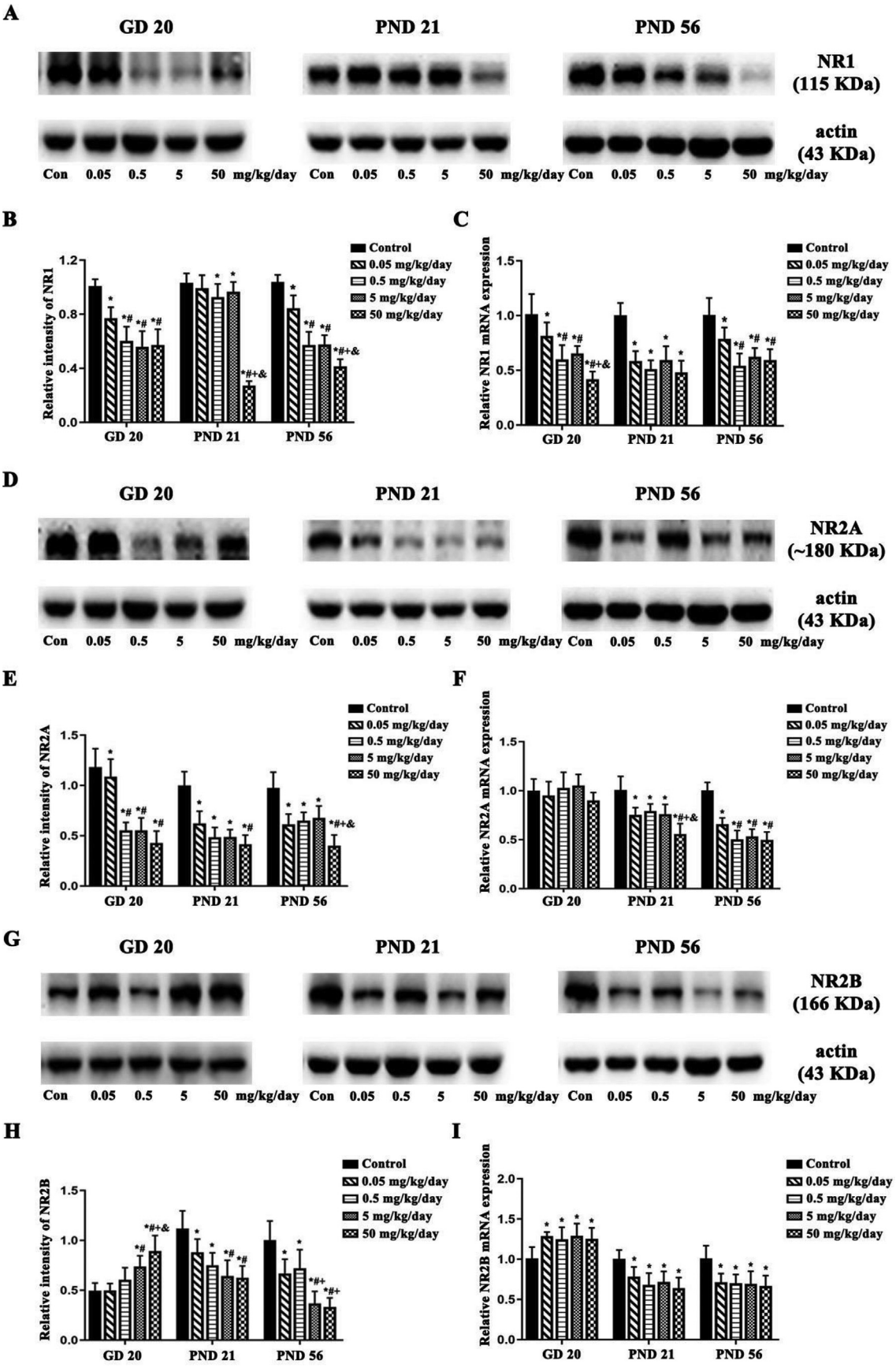

Fig. 4. Effects of embryonic exposure to BPA on hippocampal NMDAR subunits expression of rats from three growth stages. (**A**) Images of WB analysis for NR1. (**B**) Quantitation of WB analysis for NR1. (**C**) Quantitation of real-time PCR analysis for NR1 mRNA. (**D**) Images of WB analysis for NR2A. (**E**) Quantitation of WB analysis for NR2A. (**F**) Quantitation of real-time PCR analysis for NR2A mRNA. (**G**) Images of WB analysis for NR2B. (**H**) Quantitation of WB analysis for NR2B. (**I**) Quantitation of real-time PCR analysis for NR2B mRNA. The protein relative intensities were normalized to β-actin. The gene levels were normalized to GAPDH and presented as fold change with the control group. (GD: gestational day; PND: postnatal day. Data were shown as mean ± SD, n = 6. * denotes comparison with control group; # denotes comparison with 0.05 mg/kg/day BPA group; + denotes comparison with 0.5 mg/kg/day BPA group; & denotes comparison with 5 mg/kg/day BPA group; $p < 0.05$. One-Way ANOVA followed by LSD tests.)

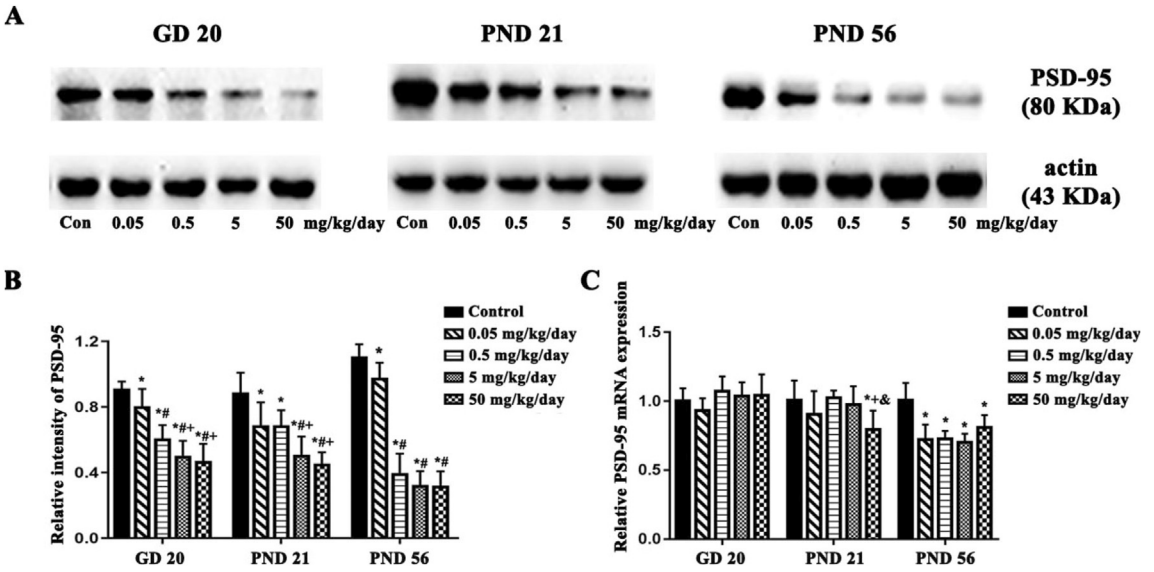

Fig. 5. Effects of embryonic exposure to BPA on hippocampal PSD-95 expression of rats from three growth stages. (**A**) Images of WB analysis for PSD-95. (**B**) Quantitation of WB analysis for PSD-95. The protein relative intensities were normalized to β-actin. (**C**) Quantitation of real-time PCR analysis for PSD-95 mRNA. The gene levels were normalized to GAPDH and presented as fold change with the control group. (GD: gestational day; PND: postnatal day. Data were shown as mean ± SD, n = 6. * denotes comparison with control group; # denotes comparison with 0.05 mg/kg/day BPA group; + denotes comparison with 0.5 mg/kg/day BPA group; & denotes comparison with 5 mg/kg/day BPA group; $p < 0.05$. One-Way ANOVA followed by LSD tests.)

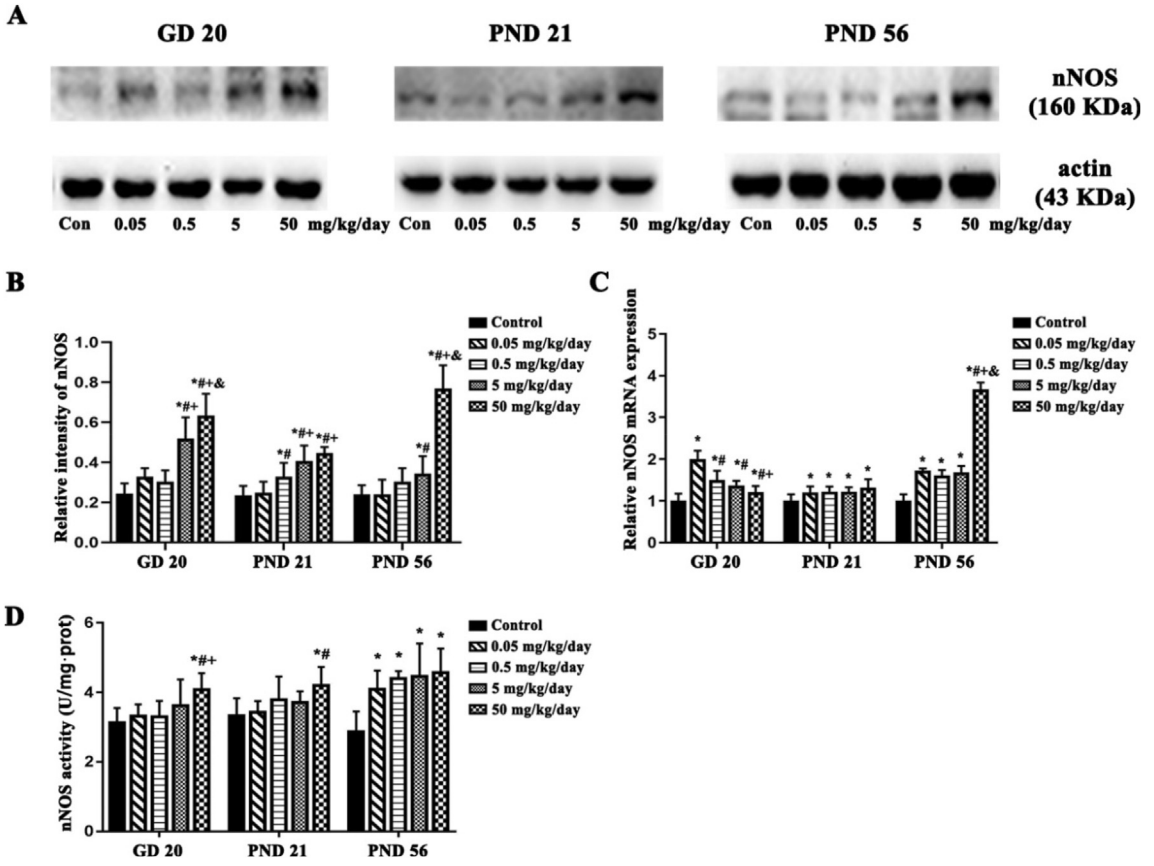

Fig. 6. Effects of embryonic exposure to BPA on hippocampal nNOS expression and activity of rats from three growth stages. (**A**) Images of WB analysis for nNOS. (**B**) Quantitation of WB analysis for nNOS. The protein relative intensities were normalized to β-actin. (**C**) Quantitation of real-time PCR analysis for nNOS mRNA. The gene levels were normalized to GAPDH and presented as fold change with the control group. (**D**) Analysis for nNOS activity. (GD: gestational day; PND: postnatal day. Data were shown as mean ± SD, n = 6. * denotes comparison with control group; # denotes comparison with 0.05 mg/kg/day BPA group; + denotes comparison with 0.5 mg/kg/day BPA group; & denotes comparison with 5 mg/kg/day BPA group; $p < 0.05$. One-Way ANOVA followed by LSD tests.)

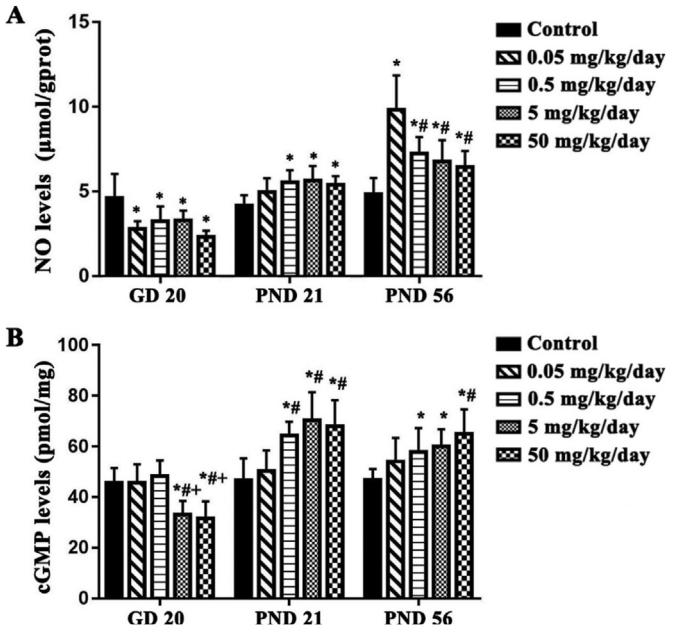

Fig. 7. Changes in hippocampal NO and cGMP levels of rats from three growth stages after embryonic exposure to BPA. (**A**) Quantitation of NO levels. (**B**) Quantitation of cGMP levels. (GD: gestational day; PND: postnatal day. Data were shown as mean ± SD, n = 6; * denotes comparison with control group; # denotes comparison with 0.05 mg/kg/day BPA group; + denotes comparison with 0.5 mg/kg/day BPA group; $p < 0.05$. One-Way ANOVA followed by LSD tests.)

is associated with decreased birth weight in term infants (Pinney et al., 2017). The possible mechanism may be that exposure to BPA in utero can lead compromised placental function, which can manifest with decreased infant body weight. While, exposure to BPA can cause abnormalities in glucose regulation and increased adiposity in the adult stage. Furthermore, the decrease of brain weight of rats in the 5 and 50 mg/kg/day BPA groups at GD 20 may due to the loss of body weight, as the brain coefficient did not change significantly.

To assess the effect of embryonic exposure to BPA on cognitive behaviors of rats at PND 56, MWM test were conducted, which has been the most widely accepted model to evaluate hippocampal-dependent spatial learning and memory in rodents (Jia et al., 2015; Morris et al., 1986; Vorhees and Williams, 2006). The prolonged escape latency in place trial indicated that embryonic exposure to 0.5 mg/kg/day BPA could induce the deterioration of spatial learning ability of rats. The deficits in spatial memory ability were less sensitive to BPA, which a higher dose of embryonic BPA (5 mg/kg/day) could shorten the time staying of rats in the target area. Several investigations have shown that perinatal exposure to BPA (from gestation to lactation) can impair the learning and memory ability of rodents using MWM test (Chang et al., 2016; Gonçalves et al., 2010; Kumar and Thakur, 2014; Xu et al., 2010). Previous study conducted by Xu et al. (2014) have indicated that postnatal exposure to BPA can yield long-lasting behavioral changes in adulthood rats. Behavioral results of this study led a proof that embryonic exposure to BPA can also perturb spatial learning and memory acquisition in the later life, which can confirm those published studies. In addition, our results also revealed that embryonic exposure to BPA had negative impact on the synaptic structure modification, mainly embodied in the thinned PSD in CA1 region of hippocampus and the possible reason will be discussed in the subsequent text. However, the synaptic cleft did not differ, indicating that embryonic exposure to BPA might not affect the expression and function of components existed in the synaptic cleft. Considering that, the synapse is regarded as the research model of learning and memory at cellular level, and there exist a causal relationship between the abnormal structure of synapse with the learning and memory deficiency. Therefore, the results suggested that embryonic exposure to BPA might induce altered synaptic structures, further impair the learning and memory ability.

NMDAR is a key player in learning and memory process, and the abnormal expression of the subunits were well recognized with the NMDAR dysfunction and the related cognitive impairment. Therefore, we explored the NMDAR subunit expression and download signal pathway after MWM test. Our results showed that exposure to BPA at low doses induced the repression of NR1 and NR2 subunits. In addition, the inhibition effects on NR1 were at the transcriptional levels. However, as a fact that the protein levels of NR2A at GD 20 decreased significantly but the mRNA levels were unchanged, we argue that the inhibition effects on NR2A were at the transcriptional and some other post-transcriptional levels. Besides, embryonic exposure to low dose BPA could promote the expression of NR2B at transcriptional levels at GD 20, but inhibit its expression during postnatal period. Several researchers have explored the effects of exposure to BPA on NMDAR subunits expression in vivo and vitro, but the available results were complicated to interpret due to distinct exposure time and dose (Hu et al., 2017; Jardim et al., 2017; Kumar and Thakur, 2014; Xu et al., 2010). Of the studies, Xu et al. (2010) established the first bases of the involvement of NMDAR in the effects of BPA on spatial memory. Two in vivo studies were similar with our BPA dose set. One study conducted in mice indicated that exposure to 5 mg/kg/day BPA from PND 21 to 60 could inhibit the hippocampal NR2A expression but did not influence NR2B levels (Jardim et al., 2017). Another study suggested that exposure to BPA from GD 7 to PND 21 (the embryonic period were included) could reduce the NR1, NR2A and NR2B expression in the hippocampus of mice, in which the NR1 subunit was the most sensitive to BPA, and the NR2B was more sensitive than NR2A (Kumar and Thakur, 2014). Comparison of the above previous studies suggested that embryonic exposure to BPA could have more severe effects on the NMDAR subunits than other exposure period. However, previous studies failed to explore the alterations of NMDAR subunits on the late gestation period, and our research can fill this deficiency. As for the diverse effects of exposure to BPA on the NR2B expression between the prenatal and postnatal stages in our study, the possible reason might be that embryonic exposure to BPA could not only inhibit the expression of NR2B but also could disturb the “NMDAR developmental switch”. The synaptic NMDAR subunit composition changes from predominantly NR2B-NMDAR containing in the prenatal life (Baez et al., 2018; Sans et al., 2000), to NR2A-NMDAR containing at the early postnatal period (Baez et al., 2018; Hoffmann et al., 2000; Monyer et al., 1994), which is known as the “NMDAR developmental switch” (Baez et al., 2018; Sanz-Clemente et al., 2013). This process is important for the refinement and fine-tuning of neuronal circuits, in addition, the balance between NR2A and NR2B composition altered in several neuronal disorders. Our result suggested that embryonic exposure to low dose BPA might decay the NMDAR developmental switch, making the NR2B subunit could not change to NR2A subunit and the un-transformed NR2B formed the increased levels on GD 20.

Furthermore, our results disclosed that embryonic exposure to BPA could inhibit the PSD-95 protein expression at the prenatal and postnatal stages and inhibit the PSD-95 mRNA levels at the adult stage. Previous studies have reported similar down-regulation PSD-95 expression in mice after exposure to BPA (Fang et al., 2017; Wang et al., 2014; Xu et al., 2013a, Xu et al., 2013b). PSD-95 is the most abundant scaffolding protein on the PSD and previous study have indicated that PSD-95 gene knockout mice could not induce LTP

with impaired learning and memory ability (Ehrlich et al., 2007). Taken together, we demonstrated that the down-regulated expression of NMDAR subunits with the PSD-95 might contribute to the BPA-induced synaptic ultrastructural abnormality (thinned PSD), thus impair the learning and memory ability in this study.

Conversely, embryonic exposure to BPA could promote the nNOS expression at the protein and mRNA levels meanwhile increase the nNOS activity at the prenatal and postnatal stages, especially at the adult stage. Only two studies explored the effects of exposure to BPA on nNOS expression before. One study conducted by Chen et al. (2014) has shown that perinatal exposure to 2 μg/kg BPA could promote the hippocampal nNOS protein expression and associated with the antianxiety-like behavior. Another study tested in female pigs also suggested that exposure to BPA could up-regulate the nNOS activity in the gastrointestinal tract (Szymanska et al., 2018). These two researchers supported our results in the increase of nNOS expression. However, the mechanisms in the relationship of down-regulation of NMDAR subunits, PSD-95 with the up-regulation of nNOS need further studies. We speculated that the possible reason might be that embryonic exposure to BPA could affect the intermediate molecular expression like calmodulin to make more nNOS clustered around the PSD-95 to make up the reduction of the PSD-95 and maintain the normal physiological function.

The main biological function of the NMDAR/PSD-95/nNOS is to produce NO, and we also detected the NO levels in the hippocampus. As the biggest source of NO, the expression of nNOS directly related to the amount of NO production, interestingly, even the nNOS expression increased significantly, the results showed embryonic exposure to BPA could inhibit NO generation on GD 20, but promote the NO generation at the postnatal stages. In addition, the newly generated NO can diffuse to the neighboring neurons and make soluble guanylyl cyclase (sGC) activated. Then, the activated sGC can promote guanosine-5′-triphosphate transformed to cGMP. As the important downstream factor of NO and a key second messenger in the learning and memory process, cGMP levels in the hippocampus matter a lot. The results of cGMP levels in our study were similar with the NO contents, of which, exposure to BPA could down-regulate the cGMP levels at GD 20, but up-regulate its levels at postnatal stages. Considering the results of NO and cGMP, we argue that embryonic exposure to BPA may promote the NO and cGMP production and consumption at the same time (GD 20), it is worth noting that the promoting effect on the consumption of them were dominant in the embryonic period. However, owing to the promoting role of BPA on nNOS expression was prolonged, the content of NO and cGMP increased at the postnatal stages. These may be the reasons for the NO and cGMP alterations induced by embryonic exposure to BPA. Nevertheless, the damaging effect of NO in neuronal tissue should not be ignored. The excessive production of NO can combine with superoxide anion to form peroxynitrite, which can attack on the proteins and further elicit mitochondrial damage, microglial activation, oxidative stress and other neurotoxic effects. So, we have a point of view that the BPA-induced alterations of NO and cGMP were connected with neurotoxic roles, rather than the neuroprotective roles in the learning and memory impairment process in our study.

5. Conclusions

In summary, our results suggested that embryonic exposure to BPA could not only suppresses NMDAR subunits and PSD-95 expression in the hippocampus of rats offspring, contributing to abnormal synaptic structure, but also could affect the NMDAR/PSD-95/nNOS–NO–cGMP signaling pathway transmission, these alterations may involve in the learning and memory ability dysfunction in their later life. However, in present study, we failed to address how embryonic exposure to BPA induced these changes and the relationship of down-regulation of NMDAR subunits, PSD-95 with the up-regulation of nNOS. These were the limitations of the current study, and further investigations still needed to explore the precise underlying mechanism.

CRediT authorship contribution statement

Haiyang Yu: Conceptualization, Funding acquisition, Methodology, Writing - original draft, Writing - review & editing, Data curation. **Lin Ma:** Methodology, Software, Writing - original draft. **Di Liu:** Methodology, Validation, Formal analysis. **Yu Wang:** Formal analysis, Data curation. **Xiucong Pei:** Resources, Supervision. **Zhiwen Duan:** Resources, Supervision. **Mingyue Ma:** Writing - review & editing, Visualization. **Yumin Zhang:** Methodology, Validation, Investigation.

Declaration of competing interest

The authors declare that they have no conflict of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.envpol.2020.115055.

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