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Bisphenol-A Exposure Alters Memory Consolidation and Hippocampal CA1 spine formation through Wnt Signaling *In Vivo* and *In Vitro*

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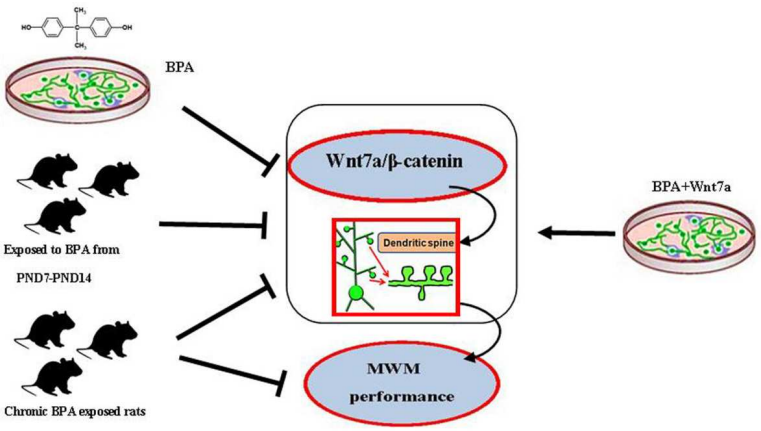
Graphical abstract:

Based on Wnt signaling pathway, this study aims to further mechanistically understand memory alteration after BPA exposure.



Abstract

Bisphenol-A (BPA has been implicated in the impairment of brain function, but the mechanism remains elusive. Our previous work have found that developmental BPA exposure impairs dendritic spine formation in DG area of hippocampus in rats. In the present study, we were to investigate the effects of BPA exposure on memory consolidation and its underlying mechanism, especially focusing on the canonical Wnt signaling pathway. BPA was administered to early developmental male Sprague–Dawley (SD) rat pups by intraperitoneal injection with BPA at the dosages of 50, 250 and 500μg/kg/day for seven days (from postnatal day (PND) 7 to PND 14), as well as chronically exposed to male rats with dosages of 0.15 mg/kg/day and 7.50 mg/kg/day through drinking water containing BPA from prenatal period to 12 weeks old. Further studies were conducted in cultured hippocampal neurons to observe the BPA-induced spine density changes and mechanism *in vitro*. The results showed that BPA exposure significantly impaired spatial memory in adult rats, accompanied by decreased hippocampal CA1 dendritic spine density. Besides, we observed dramatic changes of Wnt related proteins in BPA exposed little pups and cultured hippocampal neurons. Briefly, β-catenin phosphorylation level was significantly increased and Wnt7a, one of its upstream ligand, was significantly decreased following BPA exposure. Additionally, in cultured hippocampal neurons, exogenous Wnt7a application reversed the BPA-induced dendritic spine impairment and the decreased β-catenin phosphorylation level. In summary, this study reported that BPA exposure may produce long-lasting effects and provide a novel mechanism for memory deficits induced by BPA.

Key Words: Bisphenol A, Wnt signaling, dendritic spine, synapse formation

1. Introduction

As a crucial monomer in the production of epoxy resins and polycarbonate products, bisphenol-A (BPA,4,4'-isopropylidene-2-diphenol) ubiquitously exists in numerous products including food containers, dental devices, and thermal paper receipts.[1, 2] In this case, despite its short biological half-life in living systems,[3] BPA still can be detected in fluids of human and animals, such as urine,[4] blood,[5] and breast milk,[6] which increases people's awareness of its impacts on human health. Considering that the developing central nervous system (CNS) is highly and exquisitely regulated by endogenous hormones.[7] BPA, a xenoestrogen, is supposed to disturb learning and memory. Emerging evidence provided by behavioral studies has associated BPA exposure with memory deficits.[8-12] Although the memory impairment induced by BPA exposure has been reported, its underlying mechanism still remains unclear.

Dendritic spines, protrusions that mainly receive excitatory synaptic inputs, have long been considered to provide a morphological and structural basis for synaptic plasticity, one of the important neurochemical foundations of learning and memory. Tehila Eilam-Stock first associated reduced dendritic spine density with memory deficits in adult males after acute BPA administration, suggesting a tight link between dendritic spine changes with memory. In regard to regulation of spine and synapse formation, multiple molecules involved in this process, including Brain-derived Neurotrophic Factor (BDNF),[13] Wnt related proteins,[14] Shank and Homer,[15] small GTP proteins such as Rac1,[16] RhoA[17]. Among these proteins, Wnts drew our attention as synaptic organizers and their key role in promoting the presynaptic assembly.[18] It has been demonstrated that Wnt signaling and BDNF cooperatively promote dendritic spine formation and that Wnt signaling inhibition could reduce dendritic arbor size and complexity and block BDNF-induced dendritic spine formation and maturation in cultured cortical neurons.[19] In canonical Wnt signaling pathway, β-catenin, which is critical in dendritic morphogenesis,[20] is deemed as the core molecule. β-catenin functions by inducing gene transcription through the activation of T-cell factor

(TCF)/lymphoid enhancer factor (LEF) transcription factors. In canonical Wnt signaling pathway, the regulation of β-catenin involves its phosphorylation by AXIN/adenomatous polyposis coli (APC)/glycogen synthase kinase (GSK3β) complex.[21] This phosphorylation targets β-catenin to ubiquitination and degradation by the proteasome system. Besides, as a promoter of Wnt signaling pathway, Wnt7a also plays a key role in spine and synapse formation.[22]

In the present study, we performed Morris water maze (MWM) tests to examine spatial memory alteration upon BPA exposure. We also examined the changes of dendritic spine in hippocampal CA1 area in adult rats, little pups and cultured neurons along with Wnt related proteins, and attempted to throw light on the possible mechanisms lying behind the BPA induced memory deficits.

2. Experimental

2.1. Experimental animals

SD rats were provided by the Laboratory Animal Center, Anhui Medical University, P.R. China, and all experiments were conducted in accordance with the National Institute of Health Guide for the Care and Use of Laboratory Animals. The study was approved by the institutional animal care and use committee at Hefei University of Technology. It is noteworthy that all rats used in the experiments were male to remove estrogen effects on behavior and spine formation.

2.1.1. Chronic BPA exposure animal model

Female SD rats were housed individually after mating and subsequently treated with BPA (0 mg/kg/day, 0.15 mg/kg/day, 7.50 mg/kg/day). Specifically, rats were subjected to distilled water containing a series of concentrations of BPA (0 mg/L, 1 mg/L, 50 mg/L) according to their weight (15ml/100g/day) and rats were weighed weekly. The oral route of BPA administration was chosen to mimic the most likely route of exposure to the compound in human. Only SD male rat pups were recruited in this study. And the rat pups in different groups (12 rat pups in each group) were

exposed to BPA during lactation indirectly through the milk from their mothers and then directly after weaning at the postnatal day 21 (PND21). Young rats were placed into different cages after weaning and subsequently treated with different concentrations of BPA as mentioned above till 12 weeks old. Rats were then subjected to the MWM tests and sacrificed 3 days after the last memory test. Brain tissues were prepared for subsequent experiments.

2.1.2 Developmental BPA exposure animal model

Developmental BPA exposure was performed as follows: 6 male rat pups in each group were intraperitoneal injected with BPA dissolved in DMSO (50, 250 and 500μg/kg/day) or DMSO of equal volumes as a vehicle control at 13:00 per day from PND 7 to PND14. SD pups were killed at PND 15 and brain tissues were prepared for Golgi-cox staining and Western blotting assays.

2.2. MWM tasks

The MWM tests were initiated at 12 weeks of age, during which male SD rats were still exposed to BPA. The experiments were conducted according to previous study[23] with minor modification. Each rat performed four trials daily for 5 days. During which, the rats were allowed to swim to the hidden platform. For each trial, the animals were released from a different position in the water maze. The distance traveled to the hidden platform along with latency and velocity were automatically recorded. On the sixth day, the rats were given a 90 s probe trial in which the platform was removed. The platform crossings and time spent on the platform quadrant were recorded. The experiments were performed in a circular tank with a diameter of 160 cm and depth of 70 cm. During the experiments, the water temperature was controlled at 23±1℃.

2.3. Golgi-Cox staining and spine density assay

Rat pups which sacrificed at PND15 were decapitated quickly. While SD adult rats were deeply anesthetized with CO_2 following with cervical dislocation[24] and then quickly decapitated. The brain was processed by Golgi-Cox staining method as

described by Hu et al[25] with minor modification. Briefly, brains were stored in dark place for two days (37°C) in Golgi-Cox solution, then sectioned at 200μm in the 6% sucrose with a vibratome (VT1000S, Leica, Germany). All hippocampal sections were collected on 2% gelatin-coated slides. Then slices were stained with ammonia for 60 min, washed with water for three times, followed by Kodak Film Fix for 30 min, and then washed with water, dehydrated, cleared, and mounted using a resinous medium. The pyramidal neurons in hippocampus (CA1) were imaged with a Nikon widefield microscope (Eclipse 80i) by using a 40x objective. Then, spine density within those neurons was analyzed using Matlab software. The spines counted in the present study were on 2~3 stretches of the secondary dendrite about 10μm in length. About 10-15 neurons from one animal were selected to quantify the spine density. Generally, brains were longitudinally cut into two halves and the right hemisphere was processed for morphological staining, whereas, the left hemisphere was used to examine special proteins expression.

2.4. Neuronal cultures

Primary hippocampal cultures were prepared from the brains of SD rats at PND 0.[26] Hippocampus (CA1 area) were dissected and dissociated with trypsin (0.03%) for 19 minutes, triturated with decreasing sizes of fire-polished pipets, cultures were then plated (100 cells per mm^2) on dishes precoated with poly-L-lysine (0.5mg/ml) (sigma P-2636) for morphological assay. The next day, culture media was 70% replaced with Neurobasal media supplemented with B27 and Glutamax. Then, half the media was replaced with Neurobasal on DIV (days in vitro)7 with Ara-C (1μl/ml from 4mM stock) (sigma C6645-25MG). Neurons were exposed to BPA (10nM, 100nM, 1μM and 10μM) on DIV14 for 2 hours with or without Wnt7a (100ng/ml, R&D system, USA).

2.5. Transfection and Imaging of Cultured Neurons

Lentiviral vectors for Enhanced GFP (EGFP) gene expression was produced in human embryonic kidney 293FT cells by using the 2nd lentivirus vector generation packaging system. To determine the effects of BPA on dendritic spines in vitro,

hippocampal cultures were lentiviral infected with EGFP at DIV 6 and fixed with 4% paraformaldehyde (PFA) (20 min, room temperature) at DIV 14 after 2 hours BPA exposure with or without Wnt7a treatment (100ng/ml). Z-stacked images were acquired on Olympus FV1000 BX61WI laser scanning confocal microscope. For analysis, about 3-5 dendritic segments were examined in each neuron and about 3-4 neurons were randomly selected from each dish (4-5 dishes per group for each independent experiment). The experiment was repeated at least 3 times from independent cultures. The spine density was expressed as spines/10μm.

2.6. Western Blotting assay

Hippocampus was homogenized and dissolved in ice-cold lysis buffer (PBS; pH 7.4) containing a cocktail of protein phosphatase and protease inhibitors (21μg/ml aprotinin, 0.5μg/ml leupetin, 4.9mM $MgCl_2$，1mM sodium-Meta-vanandante, 1% Triton X-100 and 1mM PMSF) to avoid dephosphorylation and degradation of proteins. All samples were centrifuged at 14000×g at 4□ for 7min. The supernatant was then assayed for total protein concentration. Cultured neurons were directly harvested after BPA exposure with or without Wnt7a. Proteins were separated in 8.5% SDS-PAGE gel, transferred to PVDF membrane, blocked with 5% non-fat dry milk, incubated with primary antibodies (β-actin and Wnt7a were purchased from Abcam), then membranes were washed three times, incubated with secondary antibody and developed using the enhanced chemiluminescence immuno-blotting detection system. All results were normalized against β- actin.

2.7. Statistical analysis

All data were expressed as mean ± SEM. One-way repeated ANOVA was applied to the data of dendritic spine density, Western blot analyses and probe trial in Morris water maze. Two-way ANOVA was used to assess BPA treatment during acquisition training in Morris water maze. Difference between groups was then tested using Fisher's protected least significant difference (PLSD) with 95% confidence. A value of $p<0.05$ was considered to be statistically significant.

3. Results

3.1 BPA exposure induced MWM deficits in adult SD rats

MWM behavioral test is employed to examine the function of spatial memory. In the Morris water maze task, all rats showed a progressive reduction of the average distance and latency to find the hidden platform during the training period of 5 successive days. Two-way ANOVA showed that the main effect of BPA treatment or training day significantly affected latency ($F_{(2, 165)}$ = 21.359, P < 0.001; $F_{(4,165)}$ = 20.313, P< 0.001) and distance travelled to the platform ($F_{(2,165)}$ = 10.889, P < 0.001; $F_{(4,165)}$ = 19.593, P<0.001). The main effect of training day but not BPA treatment significantly affected velocity ($F_{(2,165)}$ = 0.479, P =0.620; $F_{(4,165)}$ = 8.285, P< 0.001) While no significant changes were observed in latency ($F_{(8,165)}$ = 0.834, P = 0.574), distance ($F_{(8,165)}$ = 0.709, P = 0.683) and velocity ($F_{(8,165)}$ = 1.169, P = 0.321) following interactions of BPA treatment×training day. Specifically, exposure to BPA, especially at 7.50 mg/kg/day, significantly extended the latency of rats except on day 3 (day 1，56.65±5.82 s，p<0.01; day 2, 41.16±5.03 s, p<0.05; day 4, 31.56±4.92 s, p<0.01; day 5, 28.17±4.93 s, p<0.01) when compared with the controls (Fig.1A). However,0.15 mg/kg/day BPA exposed rats showed considerable increased latency only on day 1 (52.84±5.70 s, p<0.05) and day 5 (22.85±3.20 s, p<0.05) (Fig.1A). Similarly, 7.50 mg/kg/day BPA exposed rats showed significant extended distance on day 2 (7.25±0.60 m), day 4 (6.11±0.86 m) and day 5 (5.63±0.57 m) when compared with the controls (p<0.01, p<0.05 and p<0.01) (Fig.1B). 0.15 mg/kg/day BPA exposed rats showed significant extended distance only on day 1 (11.20±1.25 m) and day 2 (8.26±0.97 m) when compared with the controls (p<0.05 and p<0.001) (Fig.1B).

Probe tests showed that BPA exposure significantly decreased the number of crossing platform (control，7.86±0.36; 0.15 mg/kg/day BPA, 5.38±0.58，p<0.01; 7.50 mg/kg/day BPA, 5.25±0.80, p<0.01) (Fig.1E). BPA also shortened the time spent in target quadrant. Interestingly, no significant change was observed in 7.50 mg/kg/day exposed rats (time percentage in target quadrant: control, 0.34±0.03; 0.15 mg/kg/day BPA, 0.25±0.02, p<0.05; 7.50 mg/kg/day BPA, 0.30±0.03, p>0.05) (Fig.1D). These data suggested that both acquisition and retention of spatial memory were impaired by BPA exposure.

3.2 Adult Rats with impaired spatial memory displayed decreased dendritic spine density in hippocampal CA1 area following BPA exposure

Since dendritic spine plays an important role in memory, we then examined the alteration of dendritic spine in hippocampal CA1 region. BPA exposure remarkably decreased the dendritic spine density (/10μm) in a dose dependent manner (control, 8.66±0.14; BPA 0.15 mg/kg/day, 7.46±0.19, $p<0.001$; BPA 7.50 mg/kg/day, 6.83±0.16, $p<0.001$) (Fig.2). These results suggested that the decreased dendritic spine density is, at least in part, associated with the impaired spatial memory caused by BPA.

3.3 *Early developmental BPA exposure alters the spine density and Wnt signaling molecules in rat pups*

Then we asked what about the spine morphological changes when exposed to BPA at very early developmental hippocampus? Our previous work showed that BPA impairs hippocampal dentate gyrus spine formation.[27] Here, we wondered whether BPA impaired CA1 dendritic spine formation in the same way. Pups exposed to BPA from PND7 to PND14, as this period was considered as the critical window for rodent nervous system development,[28] were used to do the following experiments. A dosage-dependent decrease of the spine density was observed in BPA exposed groups (50μg/kg/d, 9.85±0.16; 250μg/kg/d, 8.98±0.13; 500μg/kg/d, 8.75±0.16) when compared with the control group (10.26±0.18) (Fig.3). These results suggested that the impairment of CA1 dendritic spine formation by BPA initiated at very early stage of development. Combined with the results in adult rats, this impairment may be long lasting and irreversible.

Wnt signaling pathway, especially the canonical one, plays an important role in embryonic development, dendrite growth and synapse formation.[29] To certify whether Wnt signaling was involved in BPA induced impairment of dendritic spine formation in CA1, we examined the expression of β-catenin, the core molecule of Wnt signaling, and one of its upstream activator, Wnt7a. β-catenin phosphorylation level increased 11.64% ($p>0.05$), 49.41% ($p<0.001$) and 29.55% ($p<0.01$) after BPA treatment (Fig.4A). It indicated that BPA had a contribution to the degradation of β-catenin,

which may directly or indirectly impair the spine formation through inhibiting its downstream molecules expression.

Besides, Wnt7a reduced 9.54% (50μg/kg/day, $p>0.05$), 16.62% (250μg/kg/day, $p<0.05$) and 22.2% (500μg/kg/day, $p<0.01$) compared with the control group. As shown in Fig.4B, 50μg/kg/d BPA failed to significantly affect Wnt related protein expression levels, which corresponded with the spine formation impairment.

3.4 Wnt signaling regulated BPA induced impairments in cultured hippocampal CA1 neurons

It has been demonstrated that application of recombinant Wnt7a conditioned media in cultured hippocampal neurons can lead to an increase of 'active' β-catenin and the activated canonical Wnt/β-catenin signaling could promote synapse formation.[30] Then we asked whether the degradation of β-catenin resulted from reduction of Wnt7a. To address this question, further studies were conducted in cultured hippocampal neurons. Similarly, we first examined the dendritic spine morphology. As shown in Fig. 5, the spine density(/10μm) in BPA exposed groups (10nM, 8.76±0.27; 100nM, 8.58±0.32; 1μM, 7.64±0.29; 10μM, 8.32±0.25) displayed 9.32% ($p<0.05$), 11.18% ($p<0.05$), 20.91% ($p<0.001$) and 13.87% ($p<0.01$) decline when compared with the control group (9.66±0.32).

Then similar results were observed about the alteration of Wnt related proteins. As shown in Fig. 6A, the percentage of phosphorylated β-catenin increased 9.18% ($p > 0.05$), 19.82% ($p<0.05$), 13.71% ($p<0.05$), and 18.00% ($p<0.05$) in these BPA exposed (10nM, 100nM, 1μM, 10μM) neurons, respectively.

The Wnt7a expression level decreased 7.16% ($p>0.05$), 12.88% ($p<0.05$), 13.14% ($p< 0.05$) and 9.65% ($p<0.05$) in BPA poisoned (10nM, 100nM, 1μM, 10μM) neurons compared with the control group (Fig.6B).

3.5. Exogenous Wnt7a attenuated the BPA induced impairments in cultured hippocampal neurons

We then applied exogenous Wnt7a to the cultured hippocampal neurons. As 1μM BPA could produce significant and continuous effect，we then set BPA concentration

at 1μM in our later experiments. The spine density (/10μm) decreased 15.53% (control, 8.13±0.28; BPA 1μM, 6.86±0.53; $p<0.05$) when compared with control and increased to 8.08±0.22 when exogenous Wnt7a was added. Our results showed that Wnt7a abolished the BPA-induced spine density decrease (Fig.7A, B). Furthermore, Wnt7a significantly decreased the phosphorylation level of β-catenin which was up-regulated by BPA exposure (control, 1±0.00; BPA 1μM, 1.22±0.07; BPA1μM+Wnt7a, 1.02±0.04; $p<0.05$) (Fig.7C). Our results demonstrated that Wnt7a played an essential role in spine formation and β-catenin stability.

3. Discussion

BPA is a well known synthetic xenoestrogen that largely used in the manufacturing of plastics and it is also a potential high-risk factor for various health problems. Here we report a decrease in dendritic spine density in hippocampal CA1 of little pups, adult SD rats and cultured CA1 neurons. The alteration of Wnt signaling may be responsible for the decreased spine density and the decreased spine density may be accounted for the impaired spatial memory assessed by MWM.

People all over the world are exposed to detectable levels of BPA[31] due to its pervasiveness and BPA can be accurately measured in human serum and urine.[32] It has been reported that children aged 1.5-6 years have BPA intakes that range from 0.04 to 14.7μg/kg/day, and these values were significantly higher than those for children aged 6-19 years (0.31−0.348μg/kg /day).[33] The estimated daily intakes of BPA in adults from the Unit States are 0.033−0.056μg/kg/day[34] and people in other Asian countries are about 0.037μg/kg/day.[35] Unfortunately, we failed to detect the circulating BPA levels in our present experiments owing to the insensitive approaches we selected or low levels of BPA in serum or other unknown reasons.

In our present study, we employed two different exposure methods: adding BPA to drinking water and intraperitoneal injection. Although oral intake of BPA may undergo first-pass metabolism,[36] we did observe toxic effects similar to that observed in rat pups exposed to BPA by intraperitoneal injection. The doses used in chronic exposed rats (0.15 mg/kg/day, 7.50 mg/kg/day) were much lower than the

no-observed-adverse-effect level (NOAEL; 50mg/kg/day) estimated by the US Environmental Protection Agency for oral exposure to BPA, which would help us to investigate how chronic and low level BPA exposure affect animal behavior. Since developing brains are more vulnerable to BPA, developmental BPA exposure concentrations were set at 50, 250, 500μg/kg /day based on the U.S.E.P.A. reference safe daily limit, 50μg/kg/day.[8, 37]

In this study, we examined the effects of chronic BPA exposure on spatial memory alteration in SD rats. Consistent with other studies, [38] we observed extended latency and distance to find the platform in BPA exposed rats. Besides, the time percentage spent in target quadrant and platform crossings decreased upon BPA exposure.

In exploring the mechanism of impaired spatial memory, we examined the effects of BPA on dendritic spine formation, a process that is believed to be tightly linked to memory.[39, 40] The CA1 subfield of hippocampus plays an important role in this process. Synaptic gene dysregulation within hippocampal CA1 pyramidal neurons has been shown to be associated with cognitive impairment.[41] Disruption of the direct perforant path input to the CA1 impairs spatial working memory and novelty detection.[42] We observed significant decreased spine density in CA1 of BPA exposed adult rats. In addition, rats exposed to BPA during developmental period exhibited the same phenomena. It has been reported that prenatal and neonatal exposure to BPA could lead to memory deficits in adult rats,[12] but the mechanism remains elusive. The decreased spine density observed in little pups may subsequently influence synapse formation and synaptic plasticity in adult rats. Our results may throw light on the possible underlying mechanism. The acute experiments conducted in cultured hippocampal neurons in our experiments also identified the adverse effects of BPA. It is worth noting that 2h BPA administration was sufficient to impair spine formation. It has been shown that BPA can antagonize hormone actions at both estrogen and androgen receptors, and both estrogen and androgen can enhance synaptic density in both the CA1 region and mPFC in rodents.[43] Besides, perinatal exposure to BPA has been found to significantly decrease the expression of estrogen receptor beta (ERβ)

and NMDAR subunits NR1, NR2A, and NR2B in the hippocampus of ICR mice.[10] So it is not clear that whether the current spine density alteration involves the action of BPA on estrogen or androgen receptors.[44] Our results suggest a link between dendritic spine and spatial memory. However, since neurons we observed in this study were not three-dimensionally reconstructed and animals were sacrificed 3 days after the MWM memory test, neuroanalysis may not be directly correlated to memory performance. While, it's well accepted that dendritic spine changes can influence memory.[45-47]

Another goal of this study was to shed light on the possible mechanisms underlying the effects of BPA on dendritic spine density in the hippocampus. Wnt signaling pathway is one of pathways that are extensively studied. Growing evidence indicates that Wnt signaling plays an important role in the formation and maturation of the central nervous system. As the core molecule of canonical Wnt signaling pathway, β-catenin is an attractive candidate that has a responsibility for spine and synapse formation. Generally, Wnt signaling is suggested to inhibit β-catenin phosphorylation, thus leading to the accumulation of cytosolic β-catenin, which will bind to TCF/LEF activating Wnt/β-catenin-responsive genes.[48] Proper regulation of Wnt/β-catenin signaling by inhibiting β-catenin degradation is important for normal embryogenesis and adult tissue homeostasis. Besides, the enhancement of dendritic arborization was induced by overexpression of β-catenin in cultured hippocampal neurons.[49] We then focused on Wnt signaling pathway and tried to figure out whether the signaling pathway was involved in BPA induced spine formation impairment. In our present studies, the phosphorylation level of β-catenin was significantly increased following BPA exposure, suggesting that BPA may promote β-catenin degradation. The mechanism by which BPA promotes β-catenin degradation is not clear, but one thing for sure is that the β-catenin degradation achieved by BPA exposure will affect spine formation to some extent.

Since β-catenin degradation are modulated by multiple factors[50-52] and some Wnt secreted proteins also play partial roles in this process. We then focused on one of Wnt ligands, Wnt7a, for several reasons. Polychlorinated biphenyls (PCBs), which

have been proposed to have a weak estrogenic activity, represses Wnt7a expression in the female reproductive tract.[53] It's reasonable to hypothesize that BPA may also influence Wnt7a expression. PC12 cells, which do not express Wnt7a, can induce the accumulation of intracellular β-catenin when transfected a Wnt7a expression construct, suggesting a role of Wnt7a in β-catenin stability.[54] Besides, Wnt7a signaling can promote dendritic spine growth and synaptic strength.[22] Wnt7a is one of ligands that are representative for canonical Wnt famlily members and Wnt7a acts as a stimulator of the presynaptic activity. In our present studies, BPA significantly decreased Wnt7a expression. Furthermore, the BPA induced spine density decrease in cultured hippocampal neurons was attenuated and the phosphorylation level of β-catenin was significantly decreased after exogenous Wnt7a was applied. These results demonstrated that Wnt7a not only had a promotion effect on spine formation, but also had a role in stabilizing β-catenin.

A remaining caveat of the present study is that nanomolar concentrations of BPA also led to a decrease of spine density in cultured hippocampal neurons, which was not consistent with the results of other groups. The number of filopodia extending from dendrites of hippocampal neurons was markedly increased and the filopodia motility was also significantly enhanced by acute exposure to nanomolar concentrations of BPA for 30 min at DIV7.[55] This difference may be due to the different exposure time or period, even the different cell density.

BPA has been reported to exert different effects on male and female animals.[56-58] In our present study, we mainly observed the influence of BPA on male rats, but how does it affect female rats and what's the mechanism underlying its sex-specific effects. This issue will be further investigated in our future study.

Conclusion

Our results revealed that BPA, regardless of different exposure methods, impaired spine formation in CA1 through Wnt signaling pathway, which may in turn negatively influence memory. Our results provide novel evidence for the underlying biomolecular mechanisms of BPA induced memory deficits.

Conflict of interest

We declare that there is no conflict of interest.

Acknowledgment

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Figure captions

Fig.1. Effects of BPA treatment on Morris water maze tests of adult male SD rats. Latency (A), distance travelled to reach the platform (B) and velocity (C) in control and BPA exposed rats during MWM training tests. Time percentage in target quadrant (D) and platform crossings (E) in control and BPA exposed rats in probe trial. n=12 rats per group.

Fig.2. Dendritic spine alteration of CA1 pyramidal neurons after BPA exposure. Representative Golgi-Cox impregnated dendritic arborization and dendritic spine density (spines/10μm) of CA1 exposed to different dose of BPA in 12-week old rats. Scale bar = 10μm. (*$p<0.05$, **$p<0.01$ and ***$p<0.001$). n=12 rats per group.

Fig.3. Representative Golgi-Cox impregnated dendritic arborization and dendritic spine density (spines/10μm) of CA1 in rats exposed to BPA from PND7 to PND14. Scale bar = 10μm. n=6 rats per group.

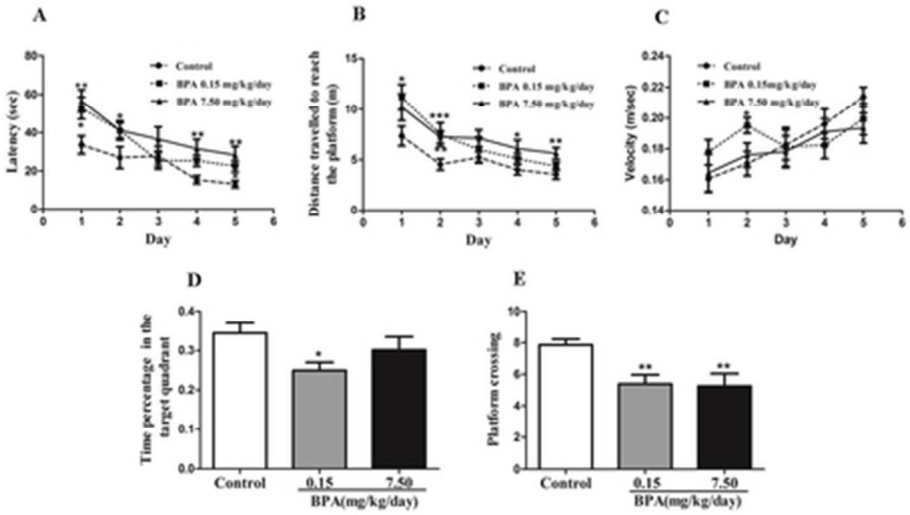
Fig.4. Effects of BPA on the Wnt related proteins of rats exposed to BPA from PND7 to PND14. Representative corresponding densitometric analysis and immunoblot showed the ratio of phosphorylated β-catenin to total β-catenin (A) and Wnt7a (B) expression level in control and BPA treated groups. (*$p<0.05$, **$p<0.01$ and ***$p<0.001$). n=6 rats per group.

Fig.5. Dendritic spine alteration of hippocampal pyramidal neurons after BPA exposure. (A) Representative EGFP-transfected hippocampal cell cultures exposed to BPA. Scale bar =10μm. (B) Representative sections (20μm) of dendritic spines in four groups. (C) Histograms plot showing the alteration of dendritic spine density (spines/10μm) after BPA treatment. n= 40-45 neurons per group. All experiments

were repeated at least three times from independent cultures.. (*$p<0.05$, **$p<0.01$ and ***$p<0.001$ compared to control group).

Fig.6. Effects of BPA on the Wnt related proteins in cultured hippocampal neurons. Representative corresponding densitometric analysis and immunoblot showed the ratio of phosphorylated β-catenin to total β-catenin(A) and Wnt7a (B) expression level in control and BPA treated groups (10nM, 100nM, 1μM, 10μM). (*$p<0.05$).

Fig.7. Effects of BPA and Wnt7a on the spine formation and β-catenin stability. (A) Representative EGFP-transfected hippocampal neurons exposed to BPA(1μM) with or without Wnt7a（100ng/ml）. (B) Histograms plot showed the alteration of dendritic spine density (spines/10μm) after BPA treatment with or without Wnt7a. n= 40-45 neurons per group. (C) Representative corresponding densitometric analysis and immunoblot showed the ratio of phosphorylated β-catenin to total β-catenin in control and BPA treated groups with or without Wnt7a. (*$p<0.05$, **$p<0.01$ and ***$p<0.001$).



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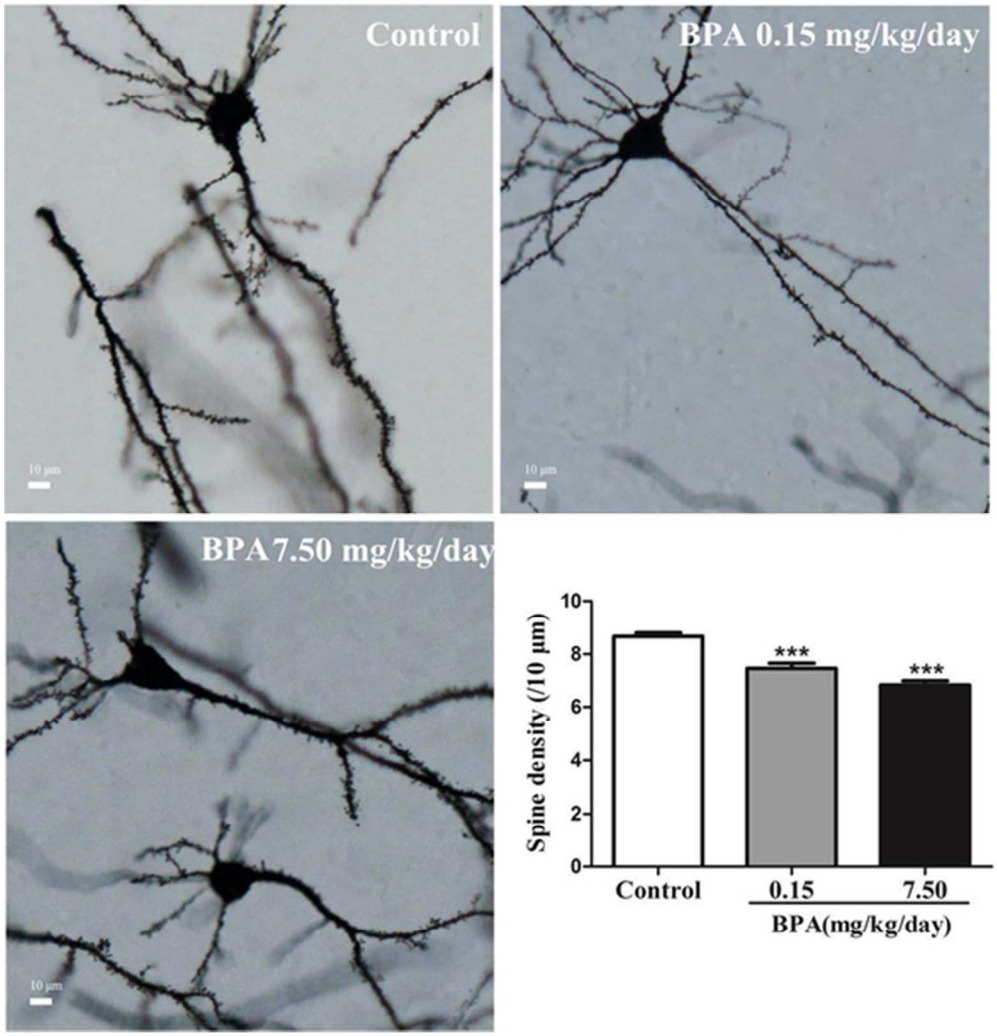

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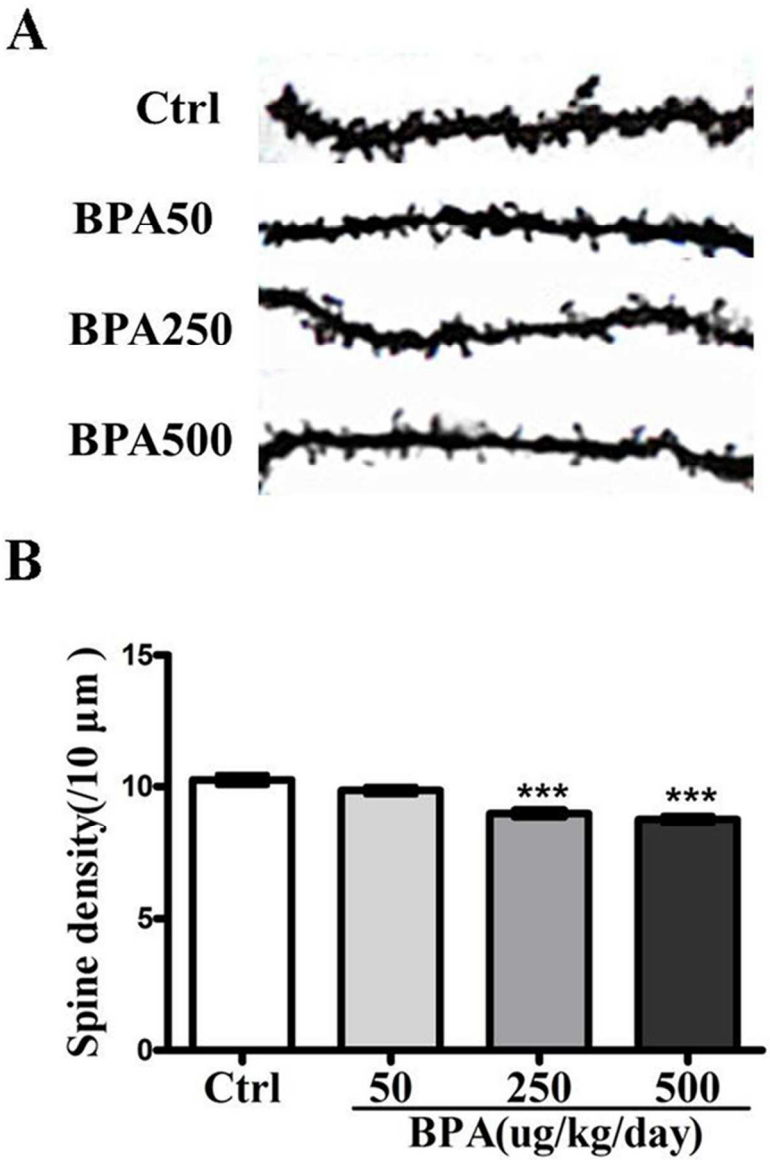

Fig.3. Representative Golgi-Cox impregnated dendritic arborization and dendritic spine density (spines/10μm) of CA1 in rats exposed to BPA from PND7 to PND14. Scale bar = 10μm. n=6 rats per group.
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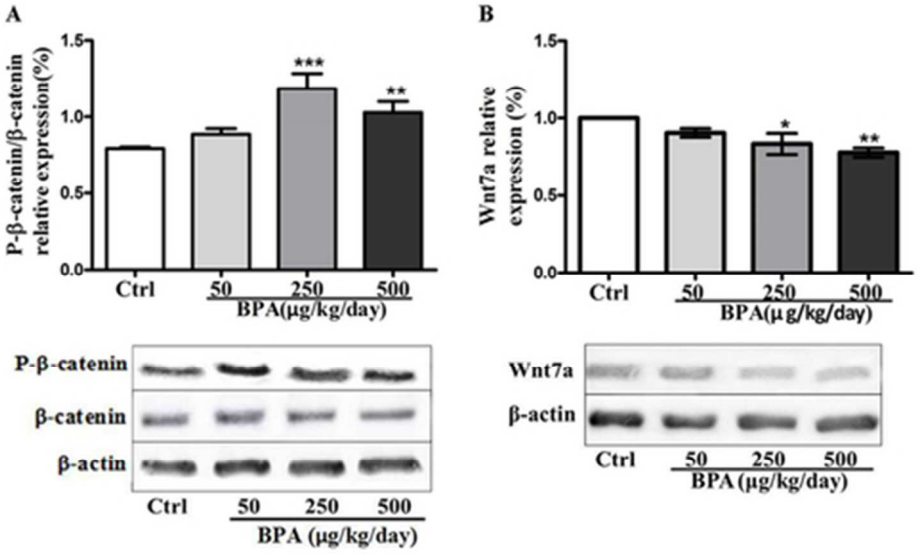

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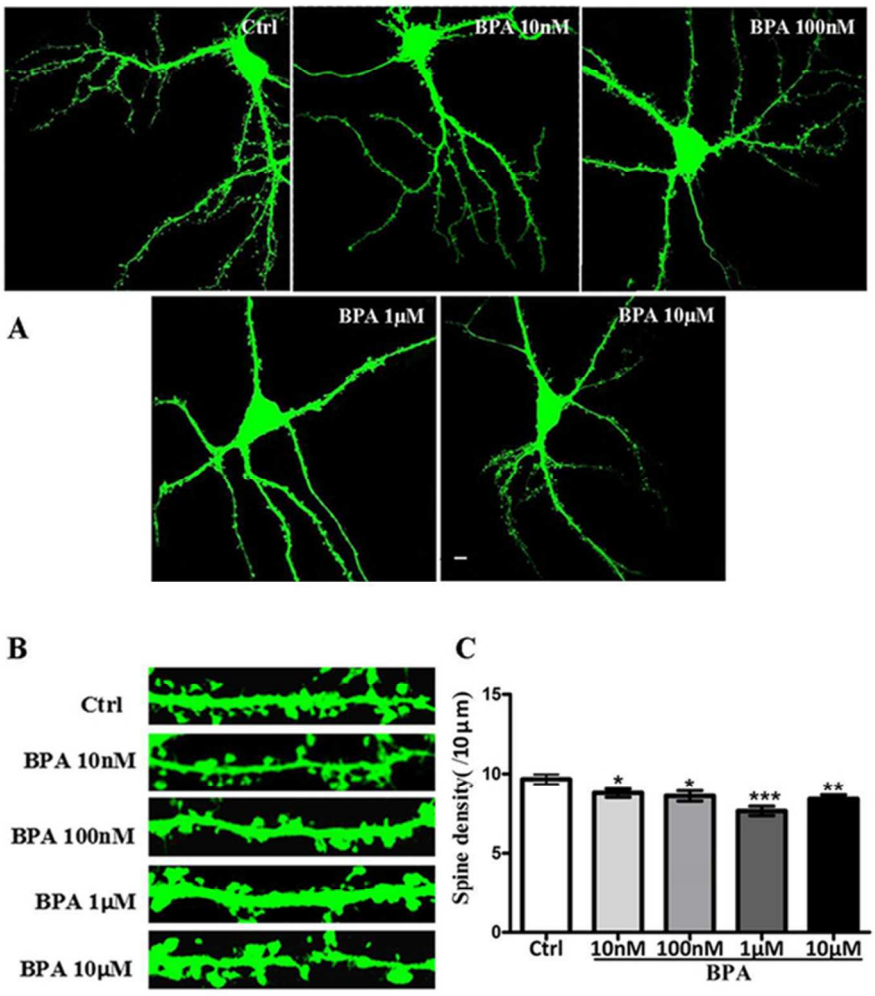

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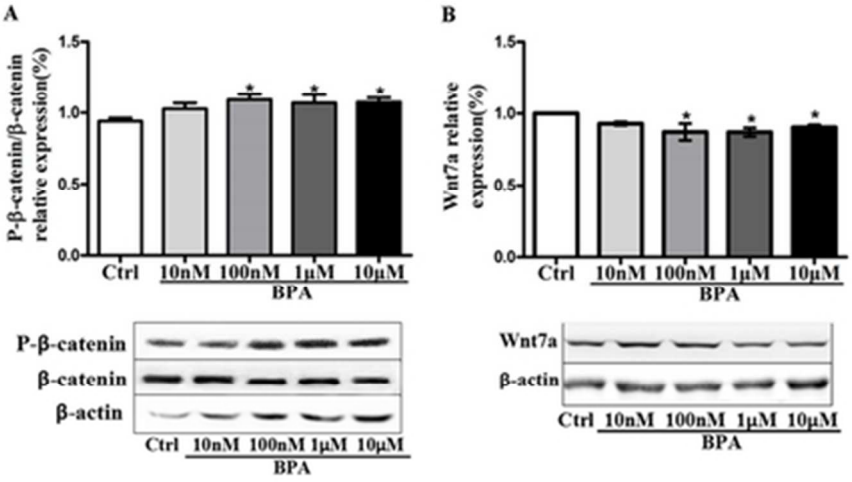

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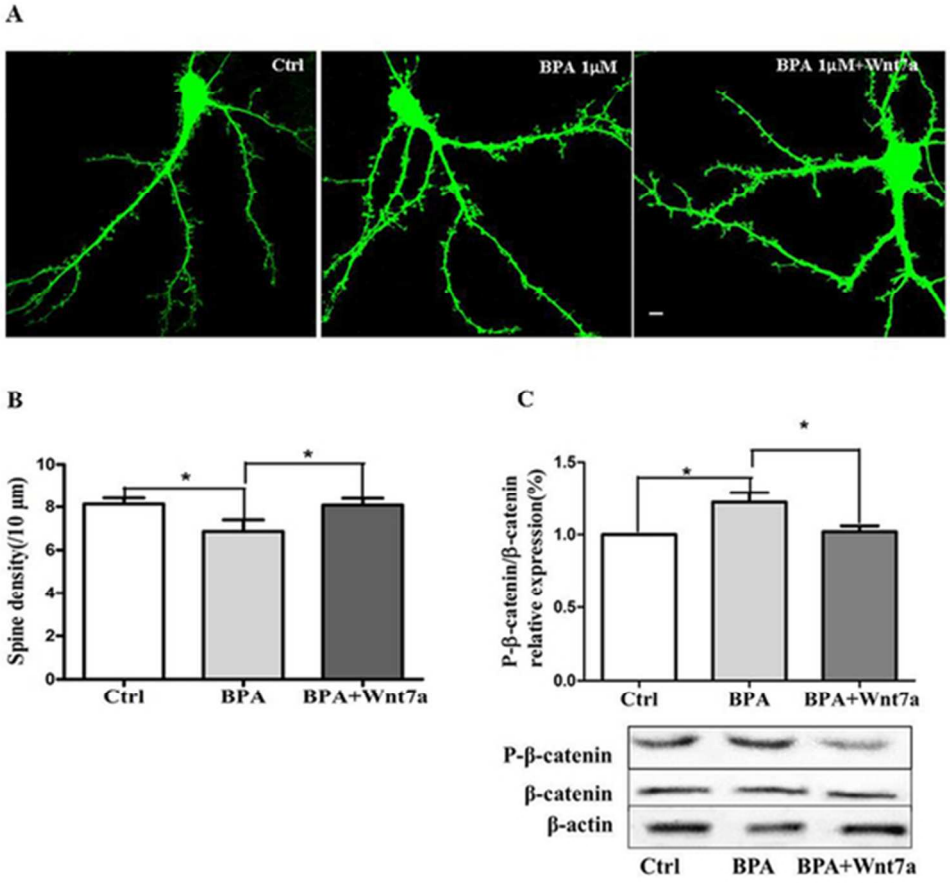

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