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Perinatal exposure to low-dose bisphenol A disrupts learning/memory and DNA methylation of estrogen receptor alpha in hippocampus

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Abstract

Developmental exposure to bisphenol A (BPA) has been indicated to pose long-lasting effects on brain development and behaviors in adulthood. Previous studies have also showed BPA may disrupt epigenetic programming of genes in the brain. Here, we focused on investigating the effects of perinatal exposure to low-dose BPA on learning/memory function and emotional regulation, as well as the associated molecular events. Pregnant Sprague-Dawley (SD) rats were treated with control corn oil or BPA (40 μg/kg/day) throughout gestation and lactation. Morris water maze (MWM) and elevated plus maze (EPM) were used to evaluate learning/memory and anxiety-like behaviors at postnatal day (PND) 60 and 85 respectively. The expression level of mRNA for estrogen receptors (ER), ERα and ERβ, in the hippocampus and serum corticosterone level were determined, as well as the DNA methylation status of ERα gene promoter. Perinatal exposure to BPA prolonged the escape latency independent of gender, and decreased the percentage of time spent in the target quadrant when examined in MWM task. While no substantial alteration was observed in the EPM test, serum corticosterone level was altered in a gender-specific manner. BPA also decreased the expression of mRNA for ERα in the hippocampus, in company with elevated DNA methylation of ERα gene promoter. These results suggest that perinatal exposure to BPA impairs learning/memory function and elevated DNA methylation of ERα gene in hippocampus may be involved.

Keywords: bisphenol A; learning/memory; anxiety-like behavior; corticosterone; estrogen receptor α; DNA methylation

Introduction

Early-life experiences have been suggested to permanently alter gene expression and pose life-long impacts on behaviors. Mounting evidence from both animal studies and human researches has identified a number of risk factors which may alter the normal neurodevelopment trajectories, including prenatal and/or early postnatal exposure to malnutrition, social experiences, maternal care, and environmental chemicals.[1-5] Endocrine disrupting chemicals (EDCs), to which human population are widely exposed, have drawn much attention in terms of its role in altering behavioral development.[6]

Bisphenol A (BPA), an estrogen-mimicking endocrine disruptor, is widely used in the manufacture of polycarbonate plastics and epoxy resins lining food and beverage containers. The majority of human population, including pregnant women and newborn infants, present measureable levels of BPA in both body fluids and tissues.[7-10] Animal studies have shown that developmental exposure to BPA affects brain sexual differentiation, social and anxiety-like behaviors, and learning and memory.[11-15] Emerging evidence from human epidemiological studies has also suggested that prenatal exposure to BPA is associated with alterations in behavioral and emotional regulation in children, especially in girls.[16, 17]

The underlying molecular mechanisms of the neurodevelopmental toxicity and sex-specific effects of BPA are not clear. As an estrogen agonist, BPA has been well documented to be able to interact with estrogen receptor alpha (ERα). Furthermore, it has been hypothesized that BPA may also regulate the expression of ERα through

DNA methylation.[18]

ERα has been implicated to be the potential target for early-life exposure to exert their actions on behavior development (e.g. learning/memory), especially for sex-dimorphic behaviors. ERα-selective agonist propyl pyrazole triol (PPT), rather than ERβ-selective agonist diarylpropionitrile (DPN), induced a key process for learning and memory in the rat hippocampus, which could be blocked by administration of ERα antagonist ICI 182,780.[19] Human studies have also demonstrated that ERα polymorphisms are associated with mood and cognition.[20]

During development, the relative abundance of ERα mRNA in hippocampus was substantially altered during the postnatal development processes.[21] Moreover, ERα has been suggested to be susceptible to DNA methylation and histone modification during early postnatal period in rat models,[22] which may serve as the molecular mechanisms underlying the effects of environmental chemicals on the development of behaviors. Recent study has reported that prenatal (gestational days 0-19) exposure to BPA disrupted the DNA methylation of ERα gene and reduced the ERα expression in a brain region- and sex-specific manner at weaning, which was associated with altered behaviors in adulthood.[23] However, studies involving the DNA methylation of ERα in the hippocampus following perinatal exposure to BPA remain limited.

In the present study, we aim to verify the effects of perinatal exposure to BPA at an environmentally relevant dose on the development of learning/memory and anxiety-like behaviors in adult rat offspring and the expression of ERs in the hippocampus, as well as the regulation role of DNA methylation.

Materials and Methods

Animals and experimental design

Female (250 - 300 g) and male (350 – 400 g) Sprague-Dawley rats (purchased from Vital River laboratory, China) were housed in a special pathogen-free (SPF) condition, and maintained on a 12-h light/dark cycle with ad libitum access to food and water. Rats were fed with a chow diet containing 12.05% fat, 24.93% protein, and 63.02% carbohydrates, with 6 mg folate/kg diet (data provided by Slac laboratory, China). BPA-free polypropylene bottles and cages were used in this study to avoid unnecessary BPA exposure. After acclimatization for 1 week, two females were caged with one male and allowed to mate overnight. The presence of vaginal plug or sperm-positive smear in females defined the gestational day 0 (GD 0). Pregnant rats were randomly assigned to two treatment groups: 40μg/kg/d BPA (Sigma-Aldridge, USA) or vehicle corn oil (Sigma-Aldridge, USA). Reagents were orally administered through gavage to maternal rats throughout gestation and lactation (a total exposure time of 44 days). After delivery, 8 new born pups with an equal number of males and females were kept with every dam in one litter and the rest pups were culled. The final litter numbers in control and BPA group were 12 and 13 respectively. Pups were weaned and separated into 4 sets: each contained one male and one female pup from every litter with males and females separately caged on postnatal day (PND) 21. Only one set of offspring was selected for each test, which made the *n* in each test equal the original litter number. The experimental procedures were reviewed and approved by an institutional committee for animal care and use in Tongji medical college, Huazhong University of Science and Technology, China.

Morris water maze task (MWM)

Male and female (n = 12 and 13 for control and BPA group) offspring were subjected to MWM test at PND 60. The apparatus used in this test was a circular pool filled with water (150 cm in diameter × 70 cm in depth). The pool was geographically divided into four quadrants according to the release points, named south-west, south-east, north-east, and north-west respectively. A black platform with a diameter of 10 cm was placed 1.5 cm beneath the water surface in the middle of the north-west quadrant. Every day before the test was performed, the pool was first filled with fresh water and heated to 23 ± 1 °C, followed by dying to black color with 15 ml ink. Each rat was allowed to perform four trials per day with a 30 min interval between 9:00 and 16:00, and testing order was counterbalanced across the days and treatment groups to minimize circadian effects. Rats were placed into the water facing the sidewalls of the apparatus at different start positions across trials. The trial was stopped when the rat reached the platform within 60 s and was allowed to stay on the platform for 15 s. If the rat failed to find the platform within 60 s, then it was led to the platform by the researcher and was allowed to stay for 15 s to memorize the location. When a rat was performing its test, other test subjects were kept in an outer room to avoid the effects of directional olfactory and auditory cues. All rats were pre-trained for four successive days before taking a probe test in which the platform was removed and the rats were placed into water at a randomly chosen start position. The escape latency, time spent in each quadrant, swimming track and velocity of each trial were recorded

automatically by a tracking video system (EthoVision®, NOLDUS, Netherlands).

Elevated plus maze task (EPM)

Twenty-five days after MWM test (PND 85), exploratory behaviors of both male and female offspring were assessed using EPM. The plus maze consists of a plus-shaped apparatus with two open arms (50 cm × 10 cm) and two closed arms (50 cm × 10 cm × 40 cm), each with an open roof, connected to the central zone (10 cm × 10 cm) to form a cross. The apparatus was elevated to a height of 70 cm from the floor. Rats were placed in the central zone heading to the open arm, and were allowed to explore the maze for 5 min. Entry was defined as both front paws and shoulders entering into an arm. The time spent in the open arm and the number of open arm entries was recorded automatically by a tracking video system (EthoVision®, NOLDUS, Netherlands). After each trial, the maze floor was cleaned thoroughly using 10% ethanol to remove directional olfactory cues. Rats which accidentally fell off the maze during the test were excluded from data analysis.

Serum corticosterone analysis

Animals were sacrificed by decapitation the day after EPM test was done. Serum was collected to determine the corticosterone concentration using a Corticosterone ELISA kit (Enzo Life Sciences, USA) according to protocol provided by the manufacturer. Serum from 6 males and 6 females randomly chosen from each group were subjected to the test. The sensitivity and intra-assay coefficient of variation for the assay was 13.79 pg/ml and 5.6% respectively.

Real-time PCR

Total RNA was extracted from rat hippocampus using a TRIzol® Reagent (Invitrogen, USA), followed by reverse transcribed to cDNA with a RevertAid First Strand cDNA Synthesis Kit (Thermo Fisher Scientific, USA) according to the manufacturer's instructions. Real-time PCR was carried out using FastStart Universal SYBR Green Master (Rox) (Roche, Germany) on an ABI PRISM 7900HT real-time PCR system (Applied Biosystems, Framingham, MA, USA) according to the protocol provided by the manufacturer. Forward primer for ERα amplification was 5′-AATGTCGTGCCTCTCTATG-3′; and the reverse primer was 5′-TTGTAAGGAATGTGCTGAAGT-3′. Forward primer for ERβ amplification was 5′-AACCTCCTGATGCTTCTTTCTCAC-3′ and the reverse primer was 5′-CTTCATGCTGAGCAGATGTTCC-3′. Forward primer for the internal reference control gene GAPDH was 5′-GCGAGATCCCGCTAACATCA-3′; and the reverse primer was 5′ CTCGTGGTTCACACCCATCA-3′. The condition for real-time PCR was as follows: first denaturing at 95°C for 10 min followed by 40 cycles of denaturing at 95 °C for 15 s, annealing and extension at 60 °C for 1 min. Gene expression level was calculated using comparative CT method as previously reported.[24]

Sequenom Massarray for quantitative DNA methylation

Three male and female offspring were randomly chosen from each group to test the methylation status of the promoter region of ERα gene. Genomic DNA was extracted from the hippocampus using the Biospin Tissue Genomic DNA Extraction kit (QiaGen, Germany) according to the manufacturer's instructions. DNA (2 mg) was

treated with sodium bisulfite using the EZDNA Methylation-Gold TM kit (QiaGen, Germany) according to the manufacturer's instructions. Bisulfite-modified DNA (100 ng) was used for Methylation-specific PCR. The primers for ERα are 5′: aggaagagagTTGGAGTTTTTTTTAGGAATGTTGA and 3′: cagtaatacgactcactatagggagaaggctCACAACCTCCTTCTCCAACTAAAAT (Generay, Biotech Co., Ltd., Shanghai). The master mixture (20 ml) consisted of 0.5 ml of 10× PCR buffer, 0.1 μl of 10 pmol/ml forward and reverse primers, 25 mM of dNTP mix, 0.04μl of 5U/μl HotStar Taq (Takara, Dalian, China), 1μl of bisulfate-modified DNA, 0.16μL of 25 mM $MgCl_2$ and 3.06 ul of HPLC grade H_2O. The PCR conditions were as follows: 94°C 4 min; 94°C 20 sec, 56°C 30 sec for 45 cycles; 72°C 4 min; and 4°C forever. SAP incubation was then performed in the following condition: 37°C 20 min, 85°C 5 min, 4°C forever. After Shrimp alkaline phosphatase (SAP) treatment, *in vitro* transcription was performed, the generated transcript was subjected to an enzymatic base specific cleavage. The master mixture (20 ml) of T Cleavage transcription/RNase A cocktail consisted of 3.21 μl RNase-free ddH_2O, 0.89 μl 5x T7 Polymerase Buffer, 0.22 μl T Cleavage Mix, 0.22 μl 100mM of DTT, 0.40 Ml T7 RNA & DNA Polymerase and 0.06 μl RNase A. The procedure of Cleavage transcription/RNase A is as follows: 94°C 30 sec, 94°C 5 sec, 52°C 30 sec 40 cycles, 80°C 5 sec 5 cycles, 72°C 3 min, 4°C forever. The fragment mass is determined by Matrix-Assisted Laser Desorption/Ionization-Time of Flight Mass Spectrometry (MALDI-TOF MS, Sequenom, USA). EpiTYPER software automatically generates a report that contains quantitative information for each analysis.

Statistical Analysis

All the statistical analyses were carried out on SPSS13.0 (IBM, USA). Data was first checked for normality with Shapiro–Wilk test. In case there was any violation to the assumptions of the test, a logarithm or exponential transformation of the data was performed. For the determination of sex-specific effects, data was first subjected to analysis of variance (ANOVA) to determine the interaction between treatment and gender, followed by separately comparison between genders and/or treatments with Bonferroni correction, in case there was significant interactions between treatment and gender. For data obtained from MWM during the training days, three-factor mixed ANOVA with one within-subject variable (time) and two between-subject variables (treatment and gender) was used. Other data were analyzed using two-way factorial ANOVA with treatment and gender treated as independent variables. In case Mauchly's Test of Sphericity was statistically significant ($p < 0.05$), Greenhouse-Geisser correction was applied. Data are presented as mean ± SEM; $p \leq 0.05$ was considered to be statistical significant.

Results

Spatial learning and memory

All the F and p values resulted from the mixed ANOVA were shown in Table 1. During the 4 successive training days, the overall escape latency and swimming distance progressively decreased over time in both groups (Table 1A). For the escape latency, no significant interaction was detected across the three factors (Table 1A, B). A significant main effect of treatment rather than gender was found, revealing that BPA exposure significantly increased the escape latency regardless of gender (Table

1B, Fig. 1A). For the swimming distance, a marginal significance of interaction between treatment and gender was detected (Table 1B), while no significant effects of treatment or gender was detected (Fig. 1B). A significant interaction was detected between treatment and gender in terms of swimming velocity (Table 1B). Post hoc test revealed that BPA significantly increased the swimming velocity in female offspring ($F_{1,46}$ = 7.473, p = 0.009) rather than in male offspring ($F_{1,46}$ = 0.290, p = 0.593; Fig. 1C).

A probe test was conducted to test the memory retention of animals on the fifth day of the test. Two-way ANOVA detected a significant difference in the treatment ($F_{1,46}$ = 4.968, p = 0.031) without significant interaction between treatment and gender ($F_{1,46}$ = 0.566, p = 0.456), revealing that perinatal exposure to BPA significantly decreased the percentage of time spent in the quadrant where the platform was placed before regardless of gender (Fig. 1D).

Anxiety-like behaviors

After excluding rats that accidentally fell off the maze, the final sample size for control male, control female, BPA male and BPA female were 8, 10, 11, and 12 respectively. No significant interaction between treatment and gender was detected. There was a significant decrease in the frequency of entries into the open arms for BPA treated offspring regardless of gender ($F_{1,37}$ = 8.689, p = 0.006, Fig. 2A). However, when we took both entries into the open arms and closed arms into consideration, the percentages of entries into the open arms showed no significant changes ($F_{1,37}$ = 0.781, p = 0.382; Fig. 2B). Neither the time spent in the open arms

($F_{1,37}$ = 2.485, p = 0.123), nor the percentage of time ($F_{1,37}$ = 1.315, p = 0.259) showed significant changes (Fig. 2C and D).

We also assessed the serum corticosterone level after the EPM test. A significant interaction between treatment and gender was revealed ($F_{1,20}$ = 19.341, p < 0.001), suggesting that BPA impacts on serum corticosterone level in a gender-specific manner. The post hoc test showed that serum corticosterone was significantly increased in the female offspring ($F_{1,20}$ = 16.748, p = 0.001), whereas decreased in the male offspring in the BPA exposed group ($F_{1,20}$ = 4.524, p = 0.046; Fig. 2E). Moreover, the gender difference in control group (male > female; $F_{1,20}$ = 8.120, p = 0.010) was inversed in the BPA group (female > male; $F_{1,20}$ = 11.356, p = 0.003).

Expression level of mRNA for ERα and ERβ gene in hippocampus

Fig. 3 shows the expression level of mRNA for ERα and ERβ gene in the hippocampus. A significant interaction between treatment and gender was detected ($F_{1,20}$ = 8.181, p = 0.010), suggesting that BPA also exerts its action on ERα gene expression in a gender-specific manner. The post hoc tests revealed that BPA exposure reduced the expression of ERα gene in both female ($F_{1,20}$ = 39.184, p < 0.001; Fig. 3A) and male hippocampus ($F_{1,20}$ = 4.905, p = 0.039; Fig. 3A). However, the gender difference in ERα expression in control group ($F_{1,20}$ = 5.839, p = 0.025) was diminished in BPA group ($F_{1,20}$ = 2.652, p = 0.119; Fig. 3A). Significant difference was detected in sex ($F_{1,20}$ = 4.471, p = 0.047) but not in treatment ($F_{1,20}$ = 1.181, p = 0.290), without an interaction ($F_{1,20}$ = 0.002, p = 0.968) in the expression of ERβ gene, indicating that there was no significant difference in ERβ expression in hippocampus

between the control and the BPA group irrespective of the gender (Fig. 3B).

DNA methylation pattern of ERα gene

We further assessed DNA methylation status at the promoter region of ERα gene in hippocampus. A schematic diagram of the promoter region of the rat ERα gene was illustrated in Fig. 4A. This region, known as promoter 0/B, is 87% homologous to the promoter C of human ERα gene. No interaction between treatment and gender and main effects of gender was observed across the 17 CpG sites. Perinatal exposure to BPA resulted in a significant increase in DNA methylation at CpG site 3 ($F_{1,8} = 6.946$, $p = 0.030$), 10 ($F_{1,8} = 43.860$, $p < 0.001$), 11 ($F_{1,8} = 43.860$, $p < 0.001$), 12 ($F_{1,8} = 6.328$, $p = 0.036$), 13 ($F_{1,8} = 6.604$, $p = 0.033$), and 16 ($F_{1,8} = 105.091$, $p < 0.001$) across the 17 CpG sites regardless of gender, whereas site 9 ($F_{1,8} = 38.028$, $p < 0.001$) was demethylated (Fig. 4B and C). No significant difference in DNA methylation status was observed between males and females irrespective of treatment.

Discussion

In the present study, we found that perinatal exposure to BPA at an environmentally relevant dose impaired the learning and memory function in adult rats. We also showed that perinatal exposure to BPA reduced the expression level of mRNA for ERα in the hippocampus, which was in parallel with increased DNA methylation of the promoter of ERα gene. We found no substantial change in anxiety-like behaviors in both genders. However, the serum corticosterone level was increased in females while decreased in males in BPA group after the EPM test.

Emerging studies have reported the effects of developmental exposure to BPA on the learning/memory function in adulthood. However, previous studies varied in many

aspects of design, including animal species, exposure doses and periods, which led to the inconsistency of the study results, so as the conclusions. Neonatal (PND 1 to 14) exposure to BPA at 100 μg/kg/d rather than 250 μg/kg/d was able to eliminate the gender difference in acquisition at PND 34-37. On the contrary, BPA at 250 μg/kg/d rather than 100 μg/kg/d significantly lessen time spent in the escape quadrant in female rats in the probe test.[25] Perinatal exposure to BPA at 0.5, 5, and 50 mg/kg/d significantly impaired the learning abilities in MWM, whereas only at 0.5 or 5 mg/kg/d markedly impaired the memory retention in probe test in both PND 21 and 56 male mice.[12] These results indicate that developmental exposure to BPA may have various impacts on learning/memory. In our present study, exposure to BPA (40 μg/kg/d) during gestation and lactation significantly prolonged the escape latency in MWM training and reduced the time spent in the target quadrant in the probe test in PND 60 offspring. The results are, at least in part, consistent with those from previous studies.

Previous studies have also suggested that anxiety-like behaviors are sensitive to the exposure of BPA. Developmental exposure to BPA at 200 μg/kg/d marginally reduced time in the open arms in EPM, while no significant changes were observed in spatial memory in female mice.[26] Furthermore, prenatal urinary BPA concentration correlated with anxiety and depression in boys at seven years old.[27] In the present study, the frequency of entry into the open arms was significantly decreased in BPA treated offspring. However, this difference was diminished when we took both open arm entries and closed arm entries into account. The results from the closed arms suggest

that the alterations in maze activity may be due to changes in overall locomotor behavior. Studies reporting the impairments of locomotion level of offspring following developmental exposure to BPA have been emerging.[28-30] We may need to further verify this hypothesis in the future using an open field test in addition to EPM.

However, we observed an altered serum corticosterone level in the BPA exposed offspring after the EPM test. In the present study, perinatal exposure to BPA significantly increased the serum corticosterone in the female offspring, while decreased it in the male offspring. Corticosterone has been suggested as a potential mediator of the effect of BPA on the emotional control. Similar as our present study, exposure to 40 μg/kg/d BPA throughout pregnancy and lactation induced elevated plasma corticosterone level in female offspring in both basal and Y-maze stressed conditions.[31] And these alterations led to increased anxiety-like behavior and loss of exploration attitude in the BPA treated female offspring.[31] One possibility for the difference from previous studies is the glucocorticoid dependent negative feedback on the hypothalamic–pituitary–adrenal axis. Estrogen has been shown to impair glucocorticoid negative feedback via ERα within hypothalamus.[32] In addition to increased expression of glucocorticoid receptor (GR), some previous studies have also suggested decreased expression of GR after perinatal exposure to BPA.[33, 34] These along with our present results need to be further confirmed.

Estrogen receptors in the hippocampus play vital roles in mediating estrogen effects on memory. Previous studies utilizing estrogen receptor knockout mice and delivery of specific receptor by viral vector have all suggested that the relative expression of

ERα/ERβ in hippocampus interacts with estrogen to determine the effects on memory.[35-38] These studies have driven the authors conceived a frame in which decrease in ERα expression impairs memory and ERβ works as a negative-regulator of ERα mediated transcription in a recently published review.[39] Moreover, BPA has been shown to interfere with the regional estrogen synthesis in hippocampus and ERα has been suggested as potential target.[40] In the present study, we observed a reduction in the transcripts of ERα gene but not the ERβ gene in the hippocampus along with impaired learning and memory, which is in consistent with previous studies suggesting that decrease in the relative expression of ERα/ERβ due to loss of ERα impairs learning/memory. However, Kundakovic and colleague previously described that prenatal exposure to BPA (2-200 μg/kg/d) did not alter the expression of ERα in the hippocampus in both sexes.[23] The difference in exposure paradigm (prenatal vs. perinatal) may account for the difference in the results. Indeed, previous studies have revealed that ERα expression in the hippocampus exhibited a distinct sex-specific pattern at the end of the first postnatal week in mice.[41] In rats, ERα expression peaked at PND 4 and decreased toward the adult level.[21] These dynamic characteristics indicate that postnatal rather than prenatal development may be of particular important for the ERα in hippocampus.

ERα gene expression has been implicated to be sensitive to epigenetic programming via DNA methylation in mouse cortex development.[42] Cross-fostering of offspring to differentially licking/grooming mothers altered the ERα expression in the medial preoptic area in female rats and this was associated with the DNA methylation of ERα

1b promoter.[43] Differentially directed maternal care at different sexes can create sexually dimorphic DNA methylation patterns in ERα gene within the developing preoptic area.[44] However, to our knowledge, little is known about DNA methylation of ERα in rat hippocampus, not to mention the effects of perinatal exposure to BPA. While the mechanisms remain elusive, BPA has been implicated as an epigenetic modulator, especially in DNA methylation.[18] Prenatal exposure to 20 μg/kg/d BPA increased DNA methylation of exon A in the prefrontal cortex in male mice, whereas decreased DNA methylation in the hypothalamus in females.[23] In the present study, we found that perinatal exposure to BPA significantly elevated methylation at six CpG sites in 5′ untranslated exon B of ERα gene in the hippocampus in adult rats, which paralleled the reduction in expression of ERα in this brain region. Compared to previous study, our results suggest that postnatal development of ERα may be particular sensitive to environmental exposures. Indeed, previous study has demonstrated that the sex and hormone-induced differences in DNA methylation of ERα at PND 1 were eliminated at PND 20.[45]

It is worth noting that except for a marginal significant interaction between gender and treatment in terms of swimming distance in MWM, the alterations in swimming velocity, corticosterone level, and ERα expression all exhibited a gender-specific manner. This specific manner of BPA action has been revealed by most previous studies.[23, 46] Given the relatively low sample size in behavioral test, we believe that our results, at least in part, support the idea that BPA exerts its effects on learning/memory in a gender-specific manner.

Conclusions

Therefore, we conclude that perinatal exposure to BPA at a dose below the current reference dose (RfD) induces learning/memory deficits and alters stress-induced secretion of corticosterone in rat offspring. Moreover, perinatal exposure to BPA decreased ERα expression in the hippocampus, which may be attributed to DNA hypermethylation of the promoter of ERα gene. Our study provides evidence for the hypothesis that BPA may exert its effects on brain and behavior development through epigenetic regulation of key genes.

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Conflict of interest

The authors have declared that no conflicting of interest exists.

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Table 1A. The *F* and *p* values for tests of within-subjuects effects.

	Latency		Distance		Velocity	
	$F_{1,138}$	p	$F_{1,138}$	p	$F_{1,138}$	p
Time	27.720	0.000[a]	3.081	0.030[a]	9.575	0.00[a]
Time×Treatment	0.220	0.882	0.900	0.443	0.441	0.600
Time×Gender	0.055	0.983	0.277	0.842	0.499	0.567
Time×Treatment×Gender	1.328	0.268	0.941	0.423	0.870	0.401

[a] $p < 0.05$ versus control.

494

Table 1B. The *F* and *p* values for tests of between-subjects effects.

	Latency		Distance		Velocity	
	$F_{1,46}$	p	$F_{1,46}$	p	$F_{1,46}$	p
Treatment	8.668	0.005[a]	3.351	0.074	2.409	0.128
Gender	0.699	0.407	2.687	0.108	0.048	0.827
Treatment×Gender	0.941	0.337	4.012	0.051[b]	5.355	0.025[c]

495 [a]$p < 0.05$ versus control; [b]marginal interaction between treatment and gender;
496 [c]significant interaction between treatment and gender.
497

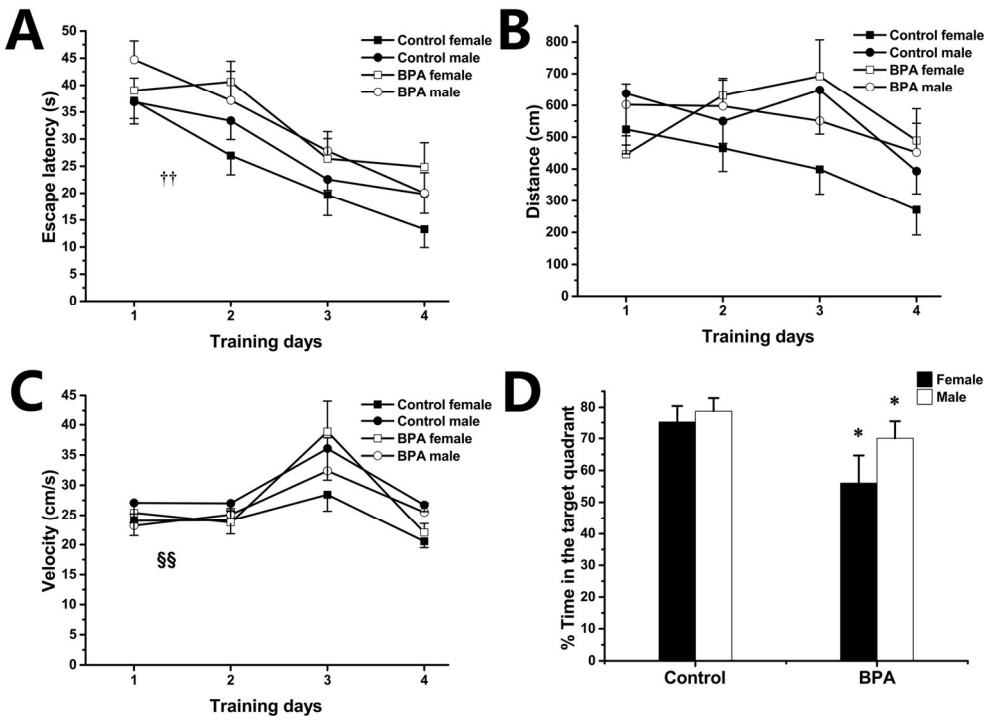
Figure captions

Figure 1. Effects of BPA on the escape latency (A); swimming distance (B); and swimming velocity (C) in the training days of the MWM test. (D) Shows the time spent in the target quadrant in the probe test. Data was presented as mean ± SEM; both control (n = 24) and BPA (n = 26) group have equal numbers of female and male rats; ††, the BPA group differed significantly from the control group regardless of gender ($p < 0.01$); §§, the BPA females differed significantly from the control females ($p < 0.01$). *$p < 0.05$ versus control of the same gender.

Figure 2. Anxiety-like behaviors assessed by the elevated plus maze (EPM). Anxiety-like behaviors were assessed with (A) total entries into the open arms, (B) percentage of entries into the open arms, (C) time spent in open arms, and (D) percentage of time spent in the open arms. Percentages were calculated by dividing the open arm entries and time with the sum of entries and total time. Data was presented as mean ± SEM; n = 18 (10 females, 8 males) and 23 (12 females, 11 males) for control and BPA group respectively; **$p < 0.0$l versus control of the same gender. (E) shows the serum corticosterone level. Data was presented as mean ± SEM; n = 12 with equal numbers of male and female rats; **$p < 0.0$l versus control of the same gender; #$p < 0.05$, ##$p < 0.01$ versus females with the same treatment.

Figure 3. Expression of mRNA for ERα (A) and ERβ (B) in hippocampus. Data was presented as mean ± SEM; n = 12 with equal numbers of male and female rats; *$p <$ 0.05, ***$p < 0.001$ versus control of the same gender; # $p < 0.05$ versus females with the same treatment.

520 **Figure 4. Effects of BPA exposure on methylation status of the promoter region**
521 **of ERα gene in hippocampus.** (A) Schematic illustration of the promoter regions of
522 the human (hER) and rat (rER) gene. Percentages represent the degree of homology
523 between these two species. Red font: position of the PCR primers; Underlined:
524 analyzed CpG sites; +1 and Green font: transcription start site. DNA methylation of
525 17 CpG sites in ERα promoter was examined in female (B) and male (C)
526 hippocampus. Methylation of CpG site 14 was not detected. Data was presented as
527 mean ± SEM; $n = 6$ with equal numbers of male and female rats; *$p < 0.05$, ***$p <$
528 0.001 versus control of the same gender.



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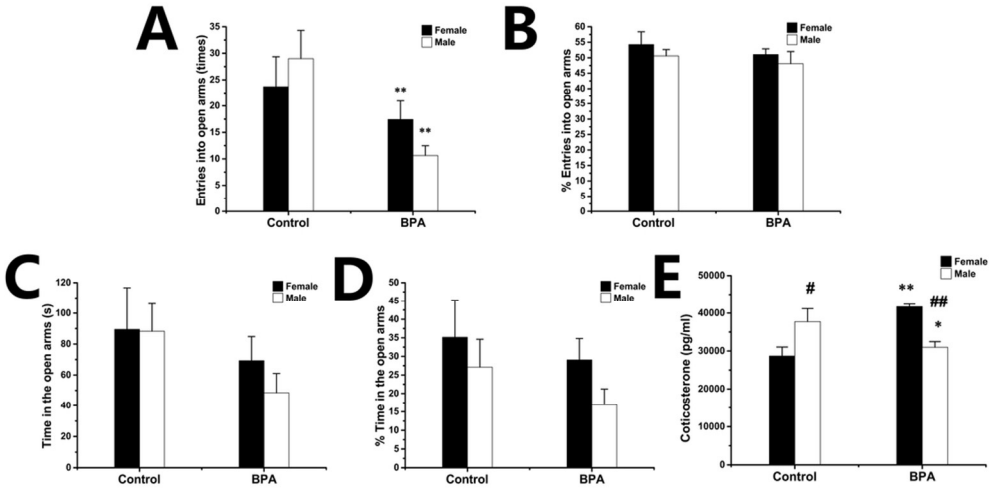

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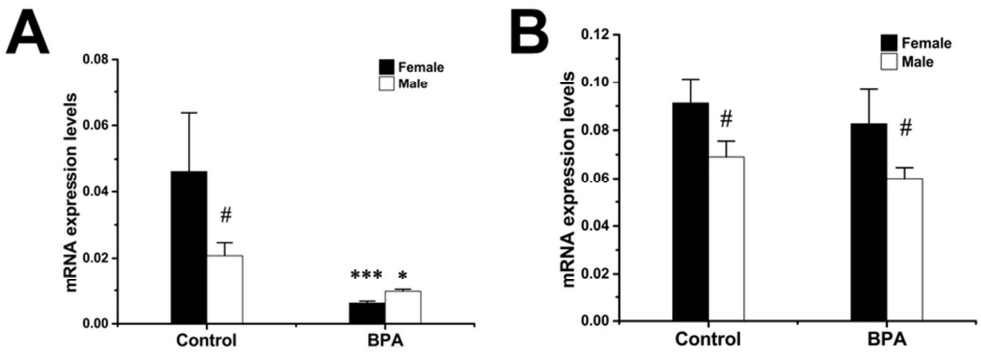

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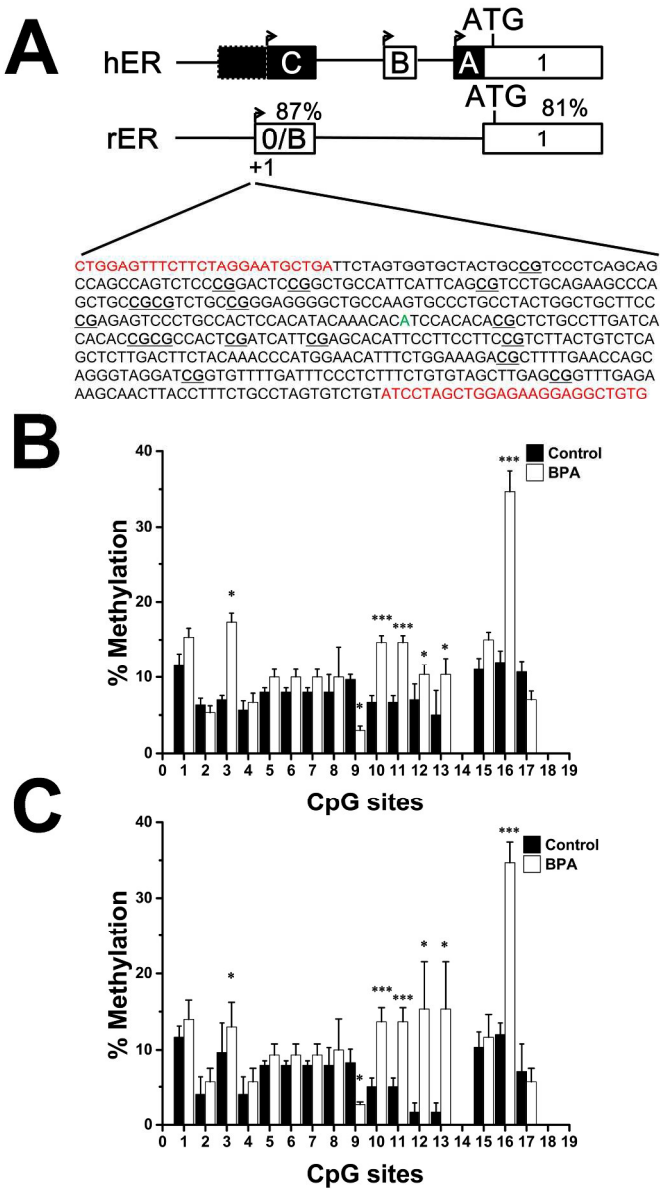

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