Accelerated reduction of serum thyroxine and hippocampal histone acetylation links to exacerbation of spatial memory impairment in aged CD-1 mice pubertally exposed to bisphenol-a

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Abstract Age-related cognitive decline has been associated with changes in endogenous hormones and epigenetic modification of chromatin, including histone acetylation. Developmental exposure to endocrine disrupting chemicals, such as bisphenol-A (BPA) that produces endocrine disruption and epigenetic changes, may be a risk factor for accelerating cognitive deficits during aging. Thus, we exposed CD-1 mice to BPA (0, 1, and 100 mg/l BPA in the drinking water) orally during

puberty (from postnatal days 28 to 56) and investigated whether pubertal BPA exposure exacerbates the age-related impairment of spatial cognition in old age (18 months old) and whether serum sex and thyroid hormones or hippocampal histone acetylation (H3K9ac and H4K8ac) are associated with cognitive effects. A young control group (6 months old) was added to analyze the age effect. Results showed untreated aged mice had marked decline of spatial learning and memory in the novel location recognition and radial six-arm water maze tasks, with decreased levels of these hormones and hippocampal H3K9ac and H4K8ac compared to young controls. The BPA treatment exacerbated age-related spatial cognitive impairment and accelerated the reduction of free thyroxine (FT4), H3K9ac, and H4K8ac, and the 100 mg/l BPA group showed more significant impact. Additionally, correlation analyses revealed that lower levels of FT4, H3K9ac, and H4K8ac were accompanied by decreased spatial memory abilities. We concluded that accelerated reduction of serum FT4 and hippocampal H3K9ac and H4K8ac might be linked to exacerbation of age-related spatial cognitive impairment due to pubertal BPA exposure.

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Introduction

Normal aging is associated with a progressive decline in cognitive functions, particularly in hippocampal-

dependent spatial learning and memory (SLM) (Harada et al. 2013). However, there is marked individual variability in age-related cognitive changes (Nyberg et al. 2012), e.g., some individuals have relatively intact cognitive function, while others develop severe cognitive deficits or even dementias such as Alzheimer's disease (AD). However, the factors and the mechanisms responsible for this variability are unclear.

Developmental exposure to environmental toxic chemicals, known as EDCs—endocrine disrupting chemicals—may be an important risk factor. Among EDCs, bisphenol-A (BPA) is one produced in the highest volumes worldwide. Many findings from animal studies have documented that perinatal exposure to BPA may disrupt neurodevelopment, affect neuronal plasticity in the hippocampus, and thereby lead to impaired learning and memory in offspring (Goncalves et al. 2010; Jasarevic et al. 2013; Kumar and Thakur 2014; Kuwahara et al. 2013; Xu et al. 2010). In recent years, there also has been a growing interest in the negative effects of BPA exposure during puberty, an important period of postnatal neurodevelopment (Schneider 2013). Studies have found that pubertal BPA exposure is associated with poorer cognitive performance in adolescence (Diaz Weinstein et al. 2013) and adulthood (Bowman et al. 2015). However, no studies to date have investigated the association into old age, and the mechanisms for BPA-induced cognitive changes are largely unknown.

Cognitive abilities in the old age can be influenced by endogenous hormones. For example, sex hormones (SHs), including both androgens in men and estrogens in women, have been shown to play a role in preserving cognitive function with aging, whereas age-related depletion of SHs is a well-established risk factor for the development of AD (Drummond et al. 2009; Frick 2009; Hogervorst 2013; Vest and Pike 2013). In addition, studies have demonstrated that thyroid dysfunction possibly implies a risk factor for cognitive decline in the old age (Ritchie and Yeap 2015; Wijsman et al. 2013). As a well-known EDC, BPA can interfere with the synthesis, metabolism, and action of endogenous hormones, affecting the hormonal system via estrogenic, anti-estrogenic, anti-androgenic, and anti-thyroid mechanisms (Negishi et al. 2003; Vandenberg et al. 2012; Zoeller et al. 2005). Since accumulating researches affirm the effects of age-related endocrine dysfunction on cognitive decline, we speculate that BPA-induced cognitive changes may be the result of endocrine disruption.

A number of studies point to the fact that age-related cognitive decline is associated with changed epigenetic modification of chromatin, including altered histone acetylation (Kosik et al. 2012; Peleg et al. 2010; Penner et al. 2010; Stilling and Fischer 2011). Histone acetylation occurs at a variety of lysine positions within the four core histone proteins (H2A, H2B, H3, and H4), and specific acetylation marks (either singly or in combination) produce distinct outcomes. Among the multiple sites of histone acetylation, growing evidence has shown that acetylated histones H3 lysine 9 and H4 lysine 8 (H3K9ac, H4K8ac) are particularly important in regulating hippocampal-dependent learning and memory. For example, one study finds that contextual fear conditioning coincides with increases of hippocampal H3K9ac and H4K8ac (Peleg et al. 2010). New evidence indicates many endocrine disrupting chemicals, such as BPA, are able to disrupt developmental epigenomic programming (Kundakovic and Champagne 2011; Mileva et al. 2014; Vaiserman 2014; Wolstenholme et al. 2011), suggesting epigenetic mechanisms including histone acetylation may underlie the long-lasting effects of these compounds. Therefore, it is possible that BPA affects the expression of the H3K9ac and/or H4K8ac in the hippocampus and hence, influences SLM.

Based on these findings, the present work investigated whether pubertal exposure to BPA exacerbates the age-related impairment of SLM in aged CD-1 mice, and whether serum SHs and thyroid hormones (THs) or hippocampal H3K9ac and H4K8ac are associated with cognitive effects of BPA.

Materials and methods

Animals and general protocol

Three-week-old male and female CD-1 mice were purchased from Beijing Vital River whose foundation colonies were all introduced from Charles River Laboratories, Inc. They were allowed free access to food and water at all times and were maintained on a 12-h light/dark cycle in a controlled temperature (22–25 °C) and humidity (50 ± 5 %) environment. After acclimatization for 1 week, the animals were randomly divided into an old control group (O-con) and two BPA groups, with 16 mice (8 males, 8 females) in each group. The mice in the low-dose BPA (LD-BPA) and high-dose

BPA (HD-BPA) groups received water with BPA (99.9 %, Sigma Chemical Co., St. Louis, MO) dissolved in 0.01 % ethanol in it (1 and 100 mg/l, respectively) as the sole drinking fluid for 4 weeks during puberty (from postnatal day [PND]28 to PND56). The doses of BPA were based on previous toxicological studies (Bodin et al. 2013; Kubo et al. 2003). The oral route of BPA administration was chosen to mimic the most likely route of human exposure. We estimated the mean levels of BPA consumed daily by pubertal mice from the drinking water at approximately 0.25 mg/kg/day (low dose) and 25 mg/kg/day (high dose), which are both lower than no-observed-adverse-effect level (50 mg/kg/day) of BPA (Kubo et al. 2003).

When the mice reached old age, they underwent cognitive testing at 18 months of age. Any animals with movement disorders, hair removal, or any visible tumor were excluded before the tests. The final number of mice was 11 in the O-con (6 males, 5 females), 14 in the LD-BPA (7 males, 7 females), and 13 in the HD-BPA (7 males, 6 females) groups. Moreover, in order to analyze the age effect, 12 mice (6 males, 6 females) aged 6 months undertook the same tests as young controls (Y-con). The animals were treated in accordance with the guidelines published in the NIH Guide for the Care and Use of Laboratory Animals.

Behavioral tests

Novel location recognition

Novel location recognition (NLR) is widely used to test spatial memory based on the inherent preference of rodents to objects in new locations (Murai et al. 2007). Testing took place in dim light using a rectangle black open-field box (40 cm × 30 cm × 40 cm). Identical plastic columns (height 4 cm, diameter 4.5 cm) were used as objects. A video camera (SONY SSC-DC488P) was mounted above the apparatus so that the activities of mice can be captured and recorded. In the acclimation phase, the mice were allowed to freely explore the apparatus for 5 min every day for 3 days without object. On the fourth day, the mice were placed in the empty apparatus for a 1-min free exploration before the formal trial. In the sample phase, two identical objects were linearly located in two corners. Each mouse was placed in the open-field arena and exposed to the objects for 5 min. Choice phases were conducted with both a 10-min delay (10-min phase; to test short-term memory)

and a 24-h delay (24-h phase; to test long-term memory). During 10-min or 24-h phase, the mice were placed in the box to explore for 5 min with one object displaced to a new location, while the other object was not moved. The apparatus and objects were thoroughly cleaned between trials with 70 % ethanol. It was considered exploration if an animal directed its nose to the objects at a distance of no longer than 1 cm and/or touched it with its nose. For each trial, the exploring total time (Tt) and the time spent exploring the object at new location (Tn) within the first 1 min during the choice phases were recorded by analyzing the video using software (XNote Stopwatch 1.39). The preferential index (PI) for novel location was defined as $Tn/Tt \times 100 \%$.

Radial six-arm water maze

Radial six-arm water maze (RAWM) was used to assess the SLM abilities (Chen et al. 2004). The apparatus and the procedure were as previously described (Cao et al. 2013). A black circular tank (diameter 100 cm, depth 21 cm) was filled with 20–21 °C water and placed on a steel rack (30 cm in height). The tank had six swimming alleys (30.5 cm × 19 cm × 21 cm) radiating out from a 40-cm-diameter center area. A black escape platform (diameter 10 cm) submerged 1.0 cm below the water surface was placed at the peripheral end of one swimming alley. A white cloth curtain surrounded the tank 35 cm apart from the wall of tank, and three black cardboards of different shapes (circle, triangle, and square) were hung equidistantly on the interior of the curtain served as spatial cues. On each day, the mice underwent four consecutive acquisition trials (trials 1–4) and one memory retention trial (trial 5) for 10 days. During trials 1–4, mouse was released into water from four random entry alleys, expect for the alley containing the platform and its opposite alley. In each trial, mouse was allowed to find the platform within 60 s and stay there for 30 s. Upon entering an incorrect alley with its whole body or failing to select any alleys within 10 s, the mouse was gently dragged back to the start alley and the attempt was recorded as an error. After trial 4 was completed, the mouse was placed in the home cage for 30 min and then returned to the maze from the start alley of trial 4 to complete trial 5. The number of errors and the escape latencies (time from entering water to locating the platform) were recorded, and the data in the daily learning period were averaged for statistical analysis.

Serum and tissue preparation

To avoid the impact of behavioral manipulations on the protein measurements, the mice were anesthetized with halothane and killed 15 days after the behavioral tests. The blood samples were obtained by quickly removing the eyeball from the socket and centrifuged at $12,000\times g$ for 10 min immediately. The serum was collected and quickly frozen at $-80\text{ }^{\circ}\text{C}$ until use. After decapitation, the brain tissues were rapidly removed from the skull and bisected in the mid-sagittal plane, fixed with 4 % paraformaldehyde at $4\text{ }^{\circ}\text{C}$ for 3 days, and embedded with paraffin into blocks for immunohistochemistry. Coronal sections were serially cut at a $6\text{-}\mu\text{m}$ thickness from tissue paraffin blocks by a Leica Microtome (Leica RM 2235, Germany).

Enzyme-linked immunosorbent assay

Serum concentrations of SHs [testosterone (only in males) and estradiol (only in females)] and THs [free triiodothyronine (FT3) and free thyroxine (FT4) in all mice] were measured with enzyme-linked immunosorbent assay (ELISA) using special kits (Shanghai Jianglai Biological Technology Co., Ltd., China) according to the manufacturer's instructions. Absorbance was read at 450 nm, and the hormone concentrations were quantified by comparing the absorbance of the samples to the standard curve.

Immunohistochemistry

The streptavidin-biotin-peroxidase complex (SABC) method was employed as described previously (Nagashima et al. 1992). Briefly, after dewaxing and hydration in graded alcohol solutions, sections containing the dorsal hippocampus were treated with periodate inactivated enzyme to quench the endogenous peroxidase activity, followed by antigen retrieval in a microwave for 20 min with citrate buffer (0.01 mol/l , pH 6.0). After washing with phosphate-buffered saline (PBS), the sections were treated with 5 % bovine serum albumin in PBS for 10 min at $37\text{ }^{\circ}\text{C}$ to block non-specific staining, and then incubated overnight at $4\text{ }^{\circ}\text{C}$ with primary antibodies (diluted 1:1000) including polyclonal rabbit anti-H3K9ac (ab10812, Abcam, USA); anti-H4K8ac (ab15823, Abcam, USA); anti-total H3 (AH433, Beyotime, China), and anti-total H4 (ab10158, Abcam, USA). As a negative control, some

sections were reacted with normal rabbit IgG (A7016, Beyotime, China) instead of the primary antibodies. The following day, the sections were rewarmed at $37\text{ }^{\circ}\text{C}$ for 40 min and successively treated with a secondary antibody (biotin-labeled goat anti-rabbit) and SABC (Wuhan Boster Bioengineering Co., China) and then visualized by diaminobenzidine.

Images of the complete dorsal hippocampus (4×10) and the three hippocampal subfields, i.e., dentate gyrus (DG), cornu ammonis (CA)1, and CA3 (20×10), were captured by a digital camera (Nikon, Japan) equipped with optical microscopy (Olympus). The histones are located within the cell nuclei; therefore, the average optical density (AOD) of immunoreactivity in the cellular layer of each hippocampal subfield was measured using Image-Pro Plus 6.0 image analysis software following the semi-quantitative method introduced by Xavier et al. (2005). Briefly, subtraction of background staining was performed by the intensity calibration function prior to analysis. For the images of each sample, the intensity of background staining was measured and the mean value was calculated and used to calibrate the incident light for minimizing the effect of background staining. In the cellular layer in different hippocampal subfields, the areas of interest (AOIs) were selected randomly and the optical density values of immunoreactive products were measured. The AOD was calculated according to optical density/AOI to represent the relative level of specific protein. Image analysis was performed following the double-blind principle.

Statistical analysis

All results were expressed as the mean $\pm$ means of standard error. For the performances in the RAWM, analysis was performed using a three-way repeated-measure analysis of variance (3w-rm. ANOVAs) with day, group, and sex as independent variables. Two-way ANOVAs with sex and group as independent variables was performed to analyze the NLR data, serum THs levels, and the relative protein levels from immunohistochemical staining. The serum SHs levels were analyzed by one-way ANOVA with group as independent variable. The followed multiple comparisons was performed by Dunnett's post hoc test with the O-con set as the control, so age effect or BPA effect can be shown in the comparison of the Y-con or the two BPA groups to the O-con, respectively. The Bonferroni post hoc test was used to reveal any difference between the BPA

groups. In the NLR task, besides the analysis of age and BPA effect, one-sample *t* test was used to determine whether the PIs in each group were different from chance performance (50 %). Pearson's correlations with Bonferroni's correction for multiple correlations were used to analyze the relationships between cognitive performances and results of serum and immunohistochemical tests. All analyses were conducted using SPSS® 16.0 for Windows.

Results

Behavioral tests

Novel location recognition

In the sample phase, all mice spent an equal amount of time exploring each of the two identical objects, indicating an equivalent exploration preference. Figure 1a, b shows the Tt and PI of each group in both 10-min and 24-h choice phases. One-sample *t* test showed that the PIs of young and old controls were significantly higher than 50 % ($P_s < 0.05$), suggesting a preferential exploration of the displaced object when compared to the non-displaced object. However, the PIs of the old BPA-treated mice were not different from 50 % ($P_s > 0.05$).

During the 10-min phase, there were significant group effects on the Tt [$F_{(3,42)} = 5.815$, $P = 0.002$] and

PI [$F_{(3,42)} = 6.410$, $P = 0.001$]. The O-con exhibited significantly lower Tt and PI than the Y-con ($P = 0.017$; $P = 0.031$) and similar Tt and PI with the old BPA-treated mice ($P_s > 0.05$). The Bonferroni post hoc test showed that the two BPA groups were not significantly different ($P_s > 0.05$). Neither sex nor interactions of group $\times$ sex had significant effect on the Tt [$F_{(1,42)} = 0.685$, $P = 0.413$; $F_{(3,42)} = 0.321$, $P = 0.810$] and PI [$F_{(1,42)} = 0.176$, $P = 0.677$; $F_{(3,42)} = 0.489$, $P = 0.692$].

During the 24-h phase, the group effect was significant on the PI [$F_{(3,42)} = 16.103$, $P < 0.001$], but not on the Tt [$F_{(3,42)} = 1.906$, $P = 0.143$], with lower PI in the O-con compared to the Y-con ($P = 0.021$). In addition, both the old HD-BPA and LD-BPA groups exhibited significantly lower PIs than the O-con ($P = 0.015$; $P = 0.011$), with insignificant difference between BPA groups ($P > 0.05$). The effects of sex and interaction of group $\times$ sex on the Tt [$F_{(1,42)} = 0.445$, $P = 0.508$; $F_{(3,42)} = 0.178$, $P = 0.911$] and PI [$F_{(1,42)} = 0.020$, $P = 0.889$; $F_{(3,42)} = 1.000$, $P = 0.402$] were insignificant.

Radial six-arm water maze

Learning phase The numbers of errors [$F_{(9,378)} = 26.832$, $P < 0.001$] and latency [$F_{(9,378)} = 33.506$, $P < 0.001$] progressively decreased with days for all mice combined [see Fig. 2a, b], suggesting that the mice were able to learn the task. The 3w-

Fig. 1 The total exploring time (a) and preferential index for novel location (b) in both 10-min and 24-h choice phases of the NLR task. All values are means $\pm$ S.E.M. # $P < 0.05$ denotes

significant age effect (O-con versus Y-con). * $P < 0.05$ denotes significant BPA effect (HD-BPA or LD-BPA versus O-con, respectively)

Fig. 2 Performance in the RAWM in the CD-1 mice. Number of the errors (a) and latency (b) during the learning phase; and number of the errors (c) and latency (d) during the memory phase. All values are means $\pm$ S.E.M. # $P < 0.05$, ## $P < 0.01$ denote

significant age effect (O-con versus Y-con). * $P < 0.05$ denotes significant BPA effect (HD-BPA or LD-BPA versus O-con, respectively)

rm. ANOVAs results revealed a significant group effect on the errors [$F_{(3,42)} = 12.738$, $P < 0.001$] and latency [$F_{(3,42)} = 8.669$, $P < 0.001$]. Dunnett's post hoc test showed that the O-con exhibited significantly more number of errors ($P = 0.006$) and longer latency ($P = 0.038$) than the Y-con. The old HD-BPA but not LD-BPA group had significantly more errors ($P = 0.038$), but not latency, than the O-con. The Bonferroni post hoc test showed no significant difference between two BPA groups ($P_s > 0.05$). The effects of sex and interactions of group $\times$ sex, group $\times$ day, sex $\times$ day, and group $\times$ sex $\times$ day were insignificant for the errors and latency ($P_s > 0.05$).

Memory phase The errors [$F_{(9378)} = 6.583$, $P < 0.001$] and latency [$F_{(9378)} = 10.278$, $P < 0.001$] also decreased over days [see Fig. 2c, d] for all mice combined. The effect of groups was significant for the errors [$F_{(3,42)} = 14.582$, $P < 0.001$] and the latency [$F_{(3,42)} = 7.723$, $P < 0.001$]. The post hoc test indicated that the errors and latency were significantly more ($P = 0.007$) or marginally longer ($P = 0.091$) in the O-con compared to the Y-con. The old HD-BPA group exhibited significantly more errors ($P = 0.017$) and marginally longer latency ($P = 0.069$) than the O-con. However, there were no significant differences in both memory parameters between the O-con and the old LD-

BPA group, and both BPA groups ($P_s > 0.05$). Sex and the interaction of group $\times$ sex, group $\times$ day, sex $\times$ day, and group $\times$ sex $\times$ day insignificantly affected the errors and latency ($P_s > 0.05$).

Serum hormone levels

Figure 3a, b show the serum concentrations of estradiol in females and testosterone in males. The one-way ANOVA revealed significant group effect on the serum levels of estradiol [$F_{(3, 20)} = 3.283$, $P = 0.042$] and testosterone [$F_{(3, 22)} = 5.637$, $P = 0.005$]. Further, the O-con had significantly lower levels of estradiol ($P = 0.019$) and testosterone ($P = 0.016$) than the Y-con in the corresponding sex. However, there were no significant differences in the estradiol and testosterone levels between the O-con and the old BPA-treated groups in the same sex ($P_s > 0.05$).

For the serum concentrations of FT3 and FT4 in all mice (Fig. 3c), the two-way ANOVAs revealed significant group effects on the levels of FT3 [$F_{(3, 42)} = 4.722$, $P = 0.006$] and FT4 [$F_{(3, 42)} = 12.372$, $P < 0.001$]. Further, the O-con showed significantly or marginally lower levels of FT3 ($P = 0.006$) and FT4 ($P = 0.090$) than the Y-con. In addition, the serum FT4 levels significantly reduced in both the old HD- and LD-BPA groups compared to the O-con ($P = 0.016$; $P = 0.023$), but FT3 did not ($P_s > 0.05$). Neither the sex nor the interaction of group $\times$ sex had significant effects on the levels of FT3 [$F_{(1, 42)} = 0.058$, $P = 0.811$; $F_{(3, 42)} = 1.193$,

$P = 0.324$] and FT4 [$F_{(1, 42)} = 0.239$, $P = 0.628$; $F_{(3, 42)} = 0.129$, $P = 0.942$].

Levels of H3K9ac, H4K8ac, total H3, and total H4 in different hippocampal subfields

As shown in Fig. 4, the immunohistochemical staining for the expressions of H3K9ac, H4K8ac, total H3, and total H4 were primarily observed in the cellular layer of the dorsal hippocampus, while no staining was found in sections that were reacted with normal rabbit IgG as a negative control. Figure 5a–d show the relative levels of H3K9ac, H4K8ac, total H3, and total H4 in DG, CA1, and CA3. The two-way ANOVAs revealed that the group effect was significant on the levels of H3K9ac [$F_{(3, 42)} = 12.678$, $P < 0.001$; $F_{(3, 42)} = 9.627$, $P < 0.001$; $F_{(3, 42)} = 3.009$, $P = 0.041$] and H4K8ac [$F_{(3, 42)} = 13.159$, $P < 0.001$; $F_{(3, 42)} = 11.285$, $P < 0.001$; $F_{(3, 42)} = 3.415$, $P = 0.026$], but insignificant on the levels of total H3 [$F_{(3, 42)} = 0.531$, $P = 0.664$; $F_{(3, 42)} = 0.674$, $P = 0.573$; $F_{(3, 42)} = 0.264$, $P = 0.851$] and total H4 [$F_{(3, 42)} = 0.603$, $P = 0.617$; $F_{(3, 42)} = 0.409$, $P = 0.747$; $F_{(3, 42)} = 0.303$, $P = 0.823$] in DG, CA1, and CA3 subareas. Dunnett's post hoc test showed that the O-con had significantly lower levels of H3K9ac ($P = 0.041$; $P = 0.036$; $P = 0.016$) and H4K8ac ($P = 0.003$; $P = 0.012$; $P = 0.007$) in DG, CA1, and CA3 than the Y-con. In addition, the old HD-BPA group showed significantly lower levels of H3K9ac ($P = 0.031$; $P = 0.032$) and H4K8ac ($P = 0.031$; $P = 0.036$) in DG and CA1 compared to the O-con.

Fig. 3 The serum levels of estradiol in females (a), testosterone in males (b), FT3 and FT4 in all mice (c) in the four-group mice. All values are means $\pm$ S.E.M. # $P < 0.05$ denotes significant age effect

(O-con versus Y-con). * $P < 0.05$ denotes significant BPA effect (HD-BPA or LD-BPA versus O-con, respectively)

Fig. 4 Effects of age and BPA exposure on the immunohistochemical staining with H3K9ac, H4K8ac, total H3, and total H4 in the dorsal hippocampi. Representative low-magnification images

from the Y-con, O-con, LD-BPA, and HD-BPA groups are shown. As a negative control, some sections were reacted with normal rabbit IgG instead of the primary antibodies. Scale bar 400 μ m

The old LD-BPA group also had significantly decreased H3K9ac level in DG ($P = 0.038$) relative to the O-con. Sex and the interaction of group $\times$ sex insignificantly affected the levels of H3K9ac, H4K8ac, total H3, and total H4 in each subfield ($P_s > 0.05$).

Relationships between spatial performances and serum hormone levels

For all the mice combined (see Table 1), Pearson's correlations showed that only serum FT4 level correlated positively with the PI ($r = 0.538$, $P < 0.001$) in the 24-h phase of the NLR task after Bonferroni's corrections. Moreover, the cognitive performances in the different tasks were not correlated to testosterone for males combined or estradiol for females combined (see Table 2). When each group was analyzed separately, no correlations were found between cognitive performances and serum hormone after Bonferroni's corrections (see Supplementary Tables 1 and 2).

Relationships between spatial performances and histone acetylation levels in hippocampal subfields

For all the mice combined (see Tables 3 and 4), Pearson's correlations showed that DG-H3K9ac level correlated positively with the PI ($r = 0.599$, $P < 0.001$) in the NLR 24-h phase, as well as negatively with the number of errors ($r = -0.454$, $P = 0.001$) in the RAWM memory phase after Bonferroni's corrections. Negative

correlations were also found between the CA1-H3K9ac level and the errors in the RAWM learning ($r = -0.434$, $P = 0.002$) and memory ($r = -0.432$, $P = 0.002$) phases. Meanwhile, DG-H4K8ac correlated negatively with the learning errors ($r = -0.456$, $P = 0.001$) and latency ($r = -0.420$, $P = 0.002$) in the RAWM. CA1-H4K8ac correlated positively with the PI ($r = 0.456$, $P = 0.001$) in the NLR 24-h phase, and negatively with the errors and latency in the learning ($r = -0.554$, $P < 0.001$; $r = -0.460$, $P = 0.001$) and memory ($r = -0.540$, $P < 0.001$; $r = -0.431$, $P = 0.002$) phases of the RAWM task. There were no correlations between spatial performances and the CA3 levels of H3K9ac and H4K8ac after Bonferroni's corrections. When the analysis was completed according to each group (see Supplementary Tables 3 and 4), no correlations were found between cognitive performances and the levels of H3K9ac and H4K8ac in different hippocampal subfields after Bonferroni's corrections.

Discussion

Pubertal bisphenol-A exposure exacerbated impairment of spatial learning and memory in aged mice

Aging is accompanied by a decline in several aspects of cognitive function, with SLM being especially sensitive to aging effect. In order to evaluate SLM in rodents, various types of behavioral tasks have been designed.

Fig. 5 The relative protein levels of H3K9ac (**a**), H4K8ac (**b**), total H3 (**c**), and total H4 (**d**) in different hippocampal subfields (DG, CA1, and CA3) indicated by average optical density (AOD) of immunohistochemical staining. All values are means $\pm$ S.E.M.

$^{\#}P < 0.05$, $^{##}P < 0.01$ denote significant age effect (O-con versus Y-con). $^{*}P < 0.05$ denotes significant BPA effect (HD-BPA or LD-BPA versus O-con, respectively)

Table 1 Correlations between spatial performances and serum thyroid hormone levels

Tasks	Phases	Measures	FT3 r (P)	FT4 r (P)
RAWM	Learning	Errors	-0.234 (0.102)	-0.235 (0.100)
		Latency	-0.228 (0.112)	-0.214 (0.135)
	Memory	Errors	-0.075 (0.603)	-0.351 (0.012)
		Latency	-0.036 (0.805)	-0.228 (0.111)
NLR	10 min	PI	0.149 (0.300)	0.195 (0.174)
	24 h	PI	0.276 (0.052)	0.538 (<0.001)*

RAWM radial six-arm water maze, NLR novel location recognition, PI preferential index, FT3 free triiodothyronine, FT4 free thyroxin

* $P < 0.0041$ level of significance after Bonferroni's correction of 6×2 test

Table 2 Correlations between spatial performances and serum sex hormone levels

Tasks	Phases	Measures	All females Estradiol <i>r</i> (<i>P</i>)	All males Testosterone <i>r</i> (<i>P</i>)
RAWM	Learning	Errors	−0.271 (0.200)	−0.265 (0.191)
		Latency	−0.170 (0.427)	−0.263 (0.194)
	Memory	Errors	−0.339 (0.106)	−0.312 (0.121)
		Latency	−0.289 (0.171)	−0.212 (0.299)
NLR	10 min	PI	0.293 (0.164)	0.155 (0.449)
	24 h	PI	0.320 (0.127)	0.286 (0.157)

RAWM radial six-arm water maze, NLR novel location recognition, PI preferential index

* $P < 0.0083$ level of significance after Bonferroni's correction of 6×1 test

One common method is through the use of exploration-based memory tasks such as the Morris water maze and RAWM (Paul et al. 2009). Our previous study demonstrated that RAWM was more sensitive for displaying impaired SLM abilities than the Morris water maze task (Chen et al. 2004), especially in old age (Yang et al. 2015). Another commonly tested type is recognition memory, and the most common way to assess spatial memory in mice is through the use of the NLR task (Murai et al. 2007). NLR task seems to be advantageous for detecting spatial cognitive function because it is not physically demanding and it induces little stress (Wimmer et al. 2012). In our previous study, CD-1 mice showed a decreased SLM ability in the RAWM task at 12 months of age and further deterioration thereafter

(Cao et al. 2013). In another study, aged CD-1 mice also exhibited the spatial memory deficits in the NLR task (Murai et al. 2007). Therefore, we simultaneously used RAWM and NLR tasks to evaluate SLM abilities.

In the present study, the aged (18 months old) CD-1 mice displayed deficits of both short-term (10-min delay) and long-term (24-h delay) recognition memories in the NLR test relative to the young mice (6 months old). For the RAWM task, the aged mice had significantly more learning errors, longer learning latency, and more memory errors than the young mice. These results suggested that aged CD-1 mice have definite SLM impairment, in agreement with previous findings (Cao et al. 2013; Chen et al. 2011; Ennaceur et al. 2008; Li et al. 2016; Murai et al. 2007).

To the best of our knowledge, the present study for the first explore whether pubertal exposure to BPA exacerbates SLM impairment in the aged CD-1 mice. Our results showed that pubertal exposures to not only high-dose (100 mg/l) but also low-dose (1 mg/l) BPA significantly decreased PIs in the NLR 24-h phase during old age when compared to the O-con. However, in the RAWM, only the HD-BPA group had significantly more errors in the learning and memory phases than the O-con. Compared to the NLR, the RAWM used negative (water immersion) reinforcers and inflicted more stress on the animals, which can affect spatial performance and lead to inconsistent results (Murai et al. 2007; Wimmer et al. 2012). These findings suggested that pubertal exposure to BPA exacerbated age-related impairment of SLM in the aged CD-1 mice, especially in higher-dose BPA exposure.

Table 3 Correlations between spatial performances and H3K9ac in each hippocampal subfield

Tasks	Phases	Measures	H3K9ac		
			DG <i>r</i> (<i>P</i>)	CA1 <i>r</i> (<i>P</i>)	CA3 <i>r</i> (<i>P</i>)
RAWM	Learning	Errors	−0.344 (0.015)	−0.434 (0.002)*	−0.243 (0.089)
		Latency	−0.347 (0.014)	−0.388 (0.005)	−0.170 (0.237)
	Memory	Errors	−0.454 (0.001)*	−0.432 (0.002)*	−0.247 (0.084)
		Latency	−0.397 (0.004)	−0.404 (0.004)	−0.110 (0.447)
NLR	10 min	PI	0.255 (0.074)	0.227 (0.112)	0.064 (0.659)
	24 h	PI	0.599 (<0.001)*	0.384 (0.006)	0.169 (0.242)

RAWM radial six-arm water maze, NLR novel location recognition, PI preferential index, DG dentate gyrus, CA cornu ammonis

* $P < 0.0027$ level of significance after Bonferroni's correction of 6×3 test

Table 4 Correlations between spatial performances and H4K8ac in each hippocampal subfield

Tasks	Phases	Measures	H4K8ac		
			DG <i>r</i> (<i>P</i>)	CA1 <i>r</i> (<i>P</i>)	CA3 <i>r</i> (<i>P</i>)
RAWM	Learning	Errors	−0.456 (0.001)*	−0.554 (<0.001)*	−0.088 (0.543)
		Latency	−0.420 (0.002)*	−0.460 (0.001)*	−0.076 (0.600)
	Memory	Errors	−0.396 (0.004)	−0.540 (<0.001)*	−0.081 (0.574)
		Latency	−0.394 (0.005)	−0.431 (0.002)*	0.036 (0.806)
NLR	10 min	PI	0.369 (0.008)	0.398 (0.004)	−0.044 (0.760)
	24 h	PI	0.400 (0.004)	0.456 (0.001)*	0.068 (0.639)

RAWM radial six-arm water maze, NLR novel location recognition, PI preferential index, DG dentate gyrus, CA cornu ammonis

* $P < 0.0027$ level of significance after Bonferroni's correction of 6×3 test

Pubertal bisphenol-A exposure accelerated the reduction of serum-free thyroxine in aged mice

In the current study, age significantly decreased serum level of testosterone in the males and estradiol in the females, while pubertal BPA exposure did not alter SHs levels in old age. However, age did affect serum THs levels, i.e., age-related decrease. Additionally, the aged BPA-treated mice at both doses showed a markedly reduced FT4 but not FT3 level compared to the O-con. This result was consistent with a recent BPA exposure study in pregnant ewes showing lower circulating total and/or free T4 levels in pregnant and newborns compared to controls (Viguie et al. 2013) and a study in rats showing a transient decrease in the FT4 concentration in the early postpartum period (PND0–PND7) in dams treated with 0.1 mg/l BPA during pregnancy (Xu et al. 2007). Conversely, another study in rats shows that perinatal exposure to BPA causes increase in serum total T4 in pups on PND15 (Zoeller et al. 2005). Epidemiologic studies in humans have also demonstrated that BPA can affect thyroid function, although study results are limited and conflicting. One study, using data from National Health and Nutrition Examination Survey 2007–2008, reports a suggestive inverse relationship between urinary BPA concentrations and total T4 concentrations in adults (Meeker and Ferguson 2011). In another study, data from the fourth Thai National Health Examination Survey 2009, suggests that a significantly negative correlation occurs between serum BPA and FT4 levels among the male adults (Sriphrapradang et al. 2013). However, a study of BPA-exposed workers in China showed positive association between urinary

BPA concentrations and FT3 but not FT4 concentrations (Wang et al. 2012). These inconsistent results may be attributable to differences in the BPA exposure timing, dose, and method. For the relationships between serum hormones and cognitive function, our results showed a positive correlation existed between the THs and spatial memory for all mice combined, indicated as FT4 positively correlated with the PI in the NLR 24-h phase ($r = 0.538$, $P < 0.001$). These findings suggested that the dysfunction of THs was, at least partially, associated with impaired spatial memory in the old mice, including normal aging and BPA-treated animals.

Pubertal bisphenol-A exposure accelerated the reduced levels of H3K9ac and H4K8ac in the aged hippocampi

From the epigenetic perspective, recent studies have clarified BPA's ability to change DNA methylation patterns (Kim et al. 2014; Kundakovic and Champagne 2011; Yeo et al. 2013), but there is very little information available concerning the effect of BPA on histone acetylation. Here, we focused on alterations in H3K9ac, H4K8ac, total H3, and total H4 in the DG, CA1, and CA3 of dorsal hippocampus. The immunohistochemical staining showed that both age and BPA treatment insignificantly affected the core histones H3 and H4 expression in each hippocampal subfield. However, both age and treatment did affect the H3K9ac and H4K8ac levels. We found that the O-con had lower H3K9ac and H4K8ac levels across all subfields of the hippocampus compared to the Y-con. Furthermore, we observed that pubertal exposure to high-dose (100 mg/l) BPA could accelerate the age-related decreases of H3K9ac and

H4K8ac in the DG and CA1. Low-dose (1 mg/l) BPA accelerated the age-related decline of H3K9ac in the DG either.

Currently, evidence reveals that histone acetylation is of vital importance to normal cognitive function and, in turn, deregulation of histone acetylation may participate in the pathogenesis of cognitive impairment (Peixoto and Abel 2013). Our results indicated that lower H3K9ac and H4K8ac levels in the DG and CA1 were accompanied with decreased SLM abilities. Similarly, several other studies also suggest that cognitive function is closely related to alterations of the H3K9ac or H4K8ac in the central nervous system. For example, one study found that memory impairment caused by brain iron overload is associated with reduction of dorsal hippocampus H3K9ac (Silva et al. 2012). Another study reports that fetal brain samples of prenatal arsenic-exposed mice have a global decrease of H3K9ac, while adult exposure exhibited memory deficits (Cronican et al. 2013). Recent studies show that a marked increase of hippocampal H4K8ac induced by histone deacetylase inhibitors, such as sodium butyrate (NaB) and pentyl-4-yn-VPA, is relevant to improving memory (Foley et al. 2014; Itzhak et al. 2013). In another study by Intlekofer et al. (2013), the effect of exercise and NaB on transforming a subthreshold learning event into long-term memory is associated with increased H4K8ac of brain-derived neurotrophic factor promoter. Our recent studies also find that chronically oral administration of acarbose and 1-deoxynojirimycin can alleviate the age-related impairment of SLM and the alleviation tended to be associated with alleviated reduction of H4K8ac (Chen et al. 2015; Yan et al. 2015). Taken together, existing findings suggest that accelerating the reduction of H3K9ac and H4K8ac may be associated with the effect of pubertal BPA exposure on accelerating age-related decline in SLM.

There are a few limitations in our study. Firstly, because the immunohistochemical data could only indicate a relative level of proteins, confirmation of these findings with immunoblotting or quantitative mass spectrometry is needed on future studies. Secondly, as this is a preliminarily explorative study, we only focused on the two most classic sites of histone acetylation (H3K9ac, H4K8ac), but we did not assess acetylation in other lysine sites. Whether there are changes in the levels of any of the other histone modifications in our model is now unknown. We will do further exploration in our future study. Additionally, we also did not detect

the downstream mechanism of histone acetylation. The acetylation of histone is associated with transcriptional activation. The reduction of H3K9ac and H4K8ac may lead to a general decrease in gene transcription. The regulation of synaptic plasticity and the formation of long-term memory need new protein synthesis. Increasing evidence supports that the H3K9ac and H4K8ac are vital in regulating learning and memory. However, which gene has its transcription affected is unknown. Therefore, we cannot come to a cause and effect conclusion that the histone acetylation mediates the BPA-related memory aging. Further study with intervention of specific site of histone acetylation may be needed.

Summary

The aged CD-1 mice had marked decline of SLM, with decreased levels of serum sex and thyroid hormones and hippocampal H3K9ac and H4K8ac. Pubertal exposure to BPA exacerbated the age-related SLM impairment and accelerated the reduction of serum FT4 and hippocampal H3K9ac and H4K8ac in aged mice with a greater effect in the HD-BPA group. Meanwhile, there were significantly negative correlations between spatial memory decline and the levels of serum FT4 and hippocampal H3K9ac and H4K8ac. These results suggested that accelerated reduction of serum FT4 and hippocampal H3K9ac and H4K8ac might be involved in exacerbation of age-related SLM impairment due to pubertal BPA exposure. This study identifies pubertal BPA exposure is a prominent environmental risk factor for accelerating cognitive deficits during aging. The potential mechanism that environmental EDCs exposures during vulnerable developmental time windows affect the aging process needs further study.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no financial conflicts of interest.

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