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Effects of subchronic exposure to benzo[a]pyrene (B[a]P) on learning and memory, and neurotransmitters in male Sprague–Dawley rat

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ABSTRACT

The harmful effects of the environmental carcinogen, benzo[a]pyrene (B[a]P), on mammalian neurodevelopment and behavior as yet remain unclear. Several studies have suggested that B[a]P impairs learning and memory. In the present investigation, we investigated the effects of subchronic exposure to B[a]P on rats. Male rats received daily injection of B[a]P (0, 1.0, 2.5, and 6.25 mg/kg, i.p.) or vehicle for 13 weeks. Employing the Morris water maze (MWM) test, we observed that rats exposed to either 2.5 mg/kg or 6.25 mg/kg B[a]P had modified behavior compared to controls as indicated by the increased mean latencies, the decreased number of crossing platform and the decreased swimming time in the target area. B[a]P treatment decreased the levels of malondialdehyde (MDA), nitric oxide (NO), nitric oxide synthase (NOS), superoxide dismutase (SOD), acetylcholine (ACh), choline acetyltransferase (ChAT), and increased the activity of acetylcholinesterase (AChE). Endogenus monoamine levels, norepinephrine (NE), adrenaline (A), dopamine (DA) and 5-hydroxytryptamine (5-HT) and their selected metabolites dihydroxyphenylacetic acid (DOPAC) and 5-hydroxyindoleacetic acid (5-HIAA) in hippocampus were measured using high performance liquid chromatography (HPLC). B[a]P at both doses, 2.5 and 6.25 mg/kg, increased NE, DA, DOPAC and 5-HT content in the hippocampus. Our results suggested a close link between the modified levels of neurotransmitters in the hippocampus and the impaired behavioral performance, indicating that B[a]P is a potential neurotoxic pollutant.

1. Introduction

Benzo(a)pyrene (B[a]P), a typical polycyclic aromatic hydrocarbon (PAH), is formed during incomplete combustion of organic matter and is a prevalent environmental pollutant. The levels of B[a]P in ambient air range from 0.1 to 66 ng/m^3 (ATSDR, 1995), occupational exposures during industrial and other domestic activities can increase these levels to 49 μg/m^3 (Szczeklik et al., 1994). B[a]P has been detected in food ranging from <0.1 to 7.2 μg/kg (ATSDR, 1995), drinking water ranging from 0.2 to 1000 ng/l (IARC, 1983). The routes of human exposure to B[a]P are the ingestion of contaminated food and water and the inhalation of particulates in the ambient air and cigarette smoking (ATSDR, 1995).

Experimental data demonstrate that similar to most lipophilic compounds, B[a]P and/or B[a]P-metabolites can reach the brain tissues by crossing the blood–brain barrier and thereby gains direct access to the central nervous system (Das et al., 1985; Grova et al., 2008; Saunders et al., 2006; Moir et al., 1998). Epidemiological data report that neurological abnormalities such as cognitive impairment, learning difficulties, parasympathetic dysregulation and loss of short-term memory, were associated with exposure to B[a]P (Kilburn and Warshaw, 1995; Dayal et al., 1995; Majchrzak et al., 1990). In animal models, B[a]P induced a variety of behavioral deficits including decreased motor activity, neuromuscular, physiological and autonomic abnormalities, and decreased responsiveness to sensory stimuli (Saunders et al., 2001, 2002, 2003). Gestational exposure to B[a]P reduced learning and memory abilities in the F1 generation mice (Wormley et al., 2004a,b). Subacute exposure to B[a]P (0.02 and 0.2 mg/kg) in adult mice induced learning and memory deficits (Grova et al., 2007). Additionally, malignant transformations of fetal mouse cells originating from whole brain and cortex were observed after in vitro B[a]P exposure (Markovits et al., 1979).

The brain may be extremely susceptible to attack by reactive oxygen species derived from B[a]P and/or B[a]P metabolism due to its characteristics such as high oxygen consumption, high iron and lipid content and low level of antioxidants (Lebel, 1991). B[a]P-induced acute neurobehavioral toxicity through oxidative stress

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has been observed (Saunders et al., 2006). Oxidative stress can alter brain activity including neurotransmission and cause neuronal cell death (Lebel, 1991; Cardozo et al., 1999). Alterations in the dopaminergic and serotoninergic systems throughout the brain have been reported after single administration of B[a]P (Stephanou et al., 1998; Jayasekara et al., 1992). Acetylcholinesterase (AChE) inhibition has also been found in vitro when B[a]P was administered (Jett et al., 1999). These findings support the hypothesis that PAHs can alter cellular constituents and cause tissue damage in the CNS (Andersson et al., 1998). The hippocampus is essential for the regulation of spatial learning and memory processes in animals (O'Keefe, 1999). Thus, the alternation in the neurotransmitter systems including cholinergic and monoaminergic system, and oxidative stress in this region may contribute to the impairment of learning and memory.

Differences in Morris water maze (MWM) performance were found between male and female rodents with male animals showing an advantage in spatial learning (Bucci et al., 1995). Furthermore, the male animals appeared to be less tolerance to B[a]P in blood elements and organs than the female animals (Knuckles et al., 2001). Therefore, in the present study male Sprague–Dawley rats were chosen to exposed to subchronic doses of B[a]P to test the hypothesis that B[a]P induces spatial learning and memory impairment, and that these may be associated with oxidative stress and consequent changes in the levels of neurotransmitters in hippocampus. B[a]P was administrated in doses (0, 1.0, 2.5, and 6.25 mg/kg) that are relevant of the toxic range of B[a]P (IPCS, 1998; Grova et al., 2008).

2. Materials and methods

2.1. Animals

Male Sprague–Dawley rats (4 weeks old) purchased from a Laboratory Animal Center (Chongqing Medical University) [certificate: SCXK (YU) 2007-0001] were used in this study. All animal procedures were approved by the Institutional Animal Care and Use Committee of Chongqing Medical University and in accordance with the policy of the Ethical Committee, State Key Laboratory of Reproductive Biology, Institute of Zoology, Chinese Academy of Sciences. The rats were housed in an environmentally controlled room (temperature 23 ± 1 °C; 12 h light/12 h dark cycle, light on at 6 PM and off at 6 AM) in groups of two or three per cage with rat chow and water ad libitum. Rats were 5 weeks of age at the onset of experiments. Rats were anaesthetised with pentobarbital-sodium after Morris water maze and were decapitated for sample collections.

2.2. Chemicals

B[a]P was purchased from Sigma–Aldrich Co. (St. Louis, MO, USA). Biochemical kits used for the determination of MDA, NO, SOD, NOS, ACh, AChE and ChAT, were purchased from Nanjing Jiancheng Bioengineering Institute (Nanjing, China). All standard substances for HPLC to analyze monoamine were obtained from Sigma–Aldrich Co. (St. Louis, MO, USA). Other reagents were analytical grade and obtained from Chongqing Medicines, Co., LTD (Chongqing, China).

2.3. Benzo(a)pyrene administration

B[a]P stock solution was dissolved in DMSO and subsequently diluted in corn oil. Rats were randomly assigned to one of the following four treatment groups ($n = 8$/dose): 0, 1.0, 2.5, and 6.25 mg/kg of B[a]P. The control group received vehicle (DMSO and corn oil). B[a]P was administered intraperitoneally (i.p.) in an injection volume of 1 ml/kg body weight and control group receiving injections of vehicle alone in the same volume as the B[a]P treated group. The treatment group at the B[a]P dose of 0 mg/kg did not received injection. Rats were treated once daily in the morning with B[a]P or vehicle for a 13 weeks (90 days) treatment period.

2.4. Morris water maze (MWM)

Rats of five groups were tested on hidden platform version of the Morris water maze before exposure to B[a]P. One week following a 13 weeks treatment period, rats were trained in the Morris water maze. The water maze consisted of a circular pool of water with a diameter of 130 cm. The water was maintained at 21–23 °C and was made opaque by the addition of non-toxic black paint. The target platform onto which mice were trained to escape was 9 cm in diameter and was submerged 1 cm under the surface of the water. The pool was located in a small, white room with VCR equipment and the experimenter to serve as cues.

Behavioral testing occurred over six consecutive days and consisted of a brief pretraining protocol in which rats were trained to escape to a hidden platform, twenty hidden training trial during which the escape platform was in a fixed pool location and a probe trial during which the escape platform was removed and the rat was allowed to swim for 120 s. An automated tracking system Morris Maze Experimental Assistant System (Institute of Materia Medica, Chinese Academy of Medical Sciences, Beijing, China) was used to measure the length of the swim path during training trials and the expression of spatial memory during the probe trial.

2.4.1. Pretraining

The rats were exposed to the water maze and trained to swim to a hidden platform. Each rat was initially placed on the hidden platform for 30 s after which it was removed from the platform, placed in the pool at one of four start locations (N, S, E and W), and was allowed to swim back to the platform. If the mouse did not escape within 120 s, it was gently guided to the platform by the experimenter.

2.4.2. Hidden platform MWM

Twenty-four hours after pretraining, rats were trained over a course of four trials using the hidden platform fixed in the center of one of the four quadrants. The location of the platform remained constant and in each trial the rat was allowed to swim for 120 s or until it found the platform. Each rat performed four consecutive trials, with an inter-trial interval of 60 s, per day. For each rat, the entire session of twenty trials was completed in five consecutive days. Each trial began by placing the rat in the water at the edge of four starting locations (N, E, S, and W). The sequence of starting locations was maintained across all rats. The swim path and the time for the rats to find the platform (escape latency) were recorded for each trial.

2.4.3. Probe trial

After the last hidden platform trial, rats were returned to water and were tested with a single 120-s probe trial during which the target platform was removed from the pool. Swimming patterns during the probe trial were analyzed with respect to a target annulus consisting of a 20-cm diameter circular area centered at the previous target location. The swim path of each rat was recorded, the percentage of time spent in the target annulus and the number of times the target annulus was crossed during the probe were recorded for each rat.

2.5. Preparation of tissue samples

Rats were anaesthetised with pentobarbital-sodium after the MWM. Rat brains were removed and forty hippocampus tissues were obtained. Each rat's left hippocampus tissue was homogenized in normal saline, using glass homogenizers. The homogenate was

centrifuged at 3000 rpm for 15 min at 4 °C to prepared supernatants and the supernatants were stored at −20 °C until use. In addition, each rat's right hippocampus tissue was rapidly removed and snap-frozen in liquid nitrogen, and stored at −80 °C until assay.

2.6. Biochemical analysis

2.6.1. Determination of MDA and NO level

The concentration of MDA in hippocampus homogenates was determined using a MDA kit based on the method described by Ohkawa et al. (1979). In this assay, thiobarbituric acid reaction (TBAR) method was used to determine the MDA content. Briefly, the tissue homogenate was mixed with TCA–TBA–HCl solution, butylated hydroxytoluene (BHT) and diethylenetriaminepentaacetic acid (DETAPAC). The mixture was heated for 60 min in a boiling water bath to generate a stable colored product. After centrifugation, the absorbance was recorded at 532 nm using 1,1,3,3-tetraethoxypropane as standard. MDA content in hippocampus homogenates was expressed as nanomoles per milligram of hippocampus protein (nmol/mg Prot).

NO content in hippocampus homogenates was determined using a NO kit. Total nitrate and nitrite concentration was determined to estimate the level of NO in the sample. This method was based on the fact that nitrate reductase catalyzes the enzymatic conversion of nitrate to nitrite and determines total nitric oxide. This step was followed by the colorimetric measurement of nitrite as an azo dye product of the Griess reaction (Stuehr et al., 1989). A two-step diazotization reaction occurred during Griess reaction, where in acidified nitrite produced a nitrosating agent, which reacts with sulfanilic acid to produce the diazonium ion. It was then coupled with N-(1-naphthyl) ethylenediamine to form the chromophoric azo-derivative, the intensity of which absorbs at 550 nm. NO content in hippocampus homogenates was expressed as micromoles per milligram of hippocampus protein (μmol/mg Prot).

2.6.2. Determination of SOD and NOS activity

SOD activity in hippocampus homogenates was measured with a SOD kit based on its ability to inhibit the oxidation of hydroxylamine by the xanthine–xanthine oxidase system (Oyanagui, 1984). Briefly, the reaction was initiated by mixing an appropriate homogenate supernatant with 0.5 mM hypoxanthine, 0.5 mM hydroxylamine and 0.01 U xanthine oxidase in the buffer, consisting of 104 mM potassium phosphate, 78 mM sodium borate and 0.025 mM EDTA (pH 7.0) at 37 °C for 40 min in a reaction volume of 1.0 ml. The reaction was terminated by adding 2.0 ml of 16% (v/v) acetic acid solution containing 2.6 mM sulfanilic acid and 38.6 μM naphthyl ethylenediamine. The absorbance at 550 nm was recorded for the calculation of SOD activity. Under the conditions, 1 U of SOD activity was calculated as that inhibiting 50% of the oxidation of hydroxylamine without an enzyme source. SOD activity in hippocampus homogenates was expressed as units per milligram of hippocampus protein (U/mg Prot).

Total NOS activity in hippocampus homogenates was measured with a NOS kit based on the conversion of L-[^{14}C]-arginine to L-[^{14}C]-citrulline (Louin et al., 2004). 50 μl supernatant was added to a reaction mixture containing 50 mM HEPES and 200 μM nicotinamide adenine dinucleotide phosphate (β-NADPH), 1 mM $CaCl_2$, 50 μM tetrahydrobiopterin (BH4) and 1 μCi/ml L-[^{14}C]-arginine. The reaction was stopped by adding 1 ml of ice-cold 50 mM HEPES (pH 5.5). Sample was applied to Dowex AG50W-X8 column to remove L-[^{14}C]-arginine. The column was then washed with 0.5 ml of distilled water and L-[^{14}C]-citrulline was quantified using a liquid scintillation spectrophotometer. 1 U of total NOS activity was defined as picomoles of L-[^{14}C]-citrulline produced per minute per microgram protein. NOS activity in hippocampus homogenates was expressed as units per milligram of hippocampus protein (U/mg Prot).

2.6.3. Determination of ACh level

ACh level in hippocampus homogenates was measured using the method of Hestrin with slight modification (Hestrin, 1949). 800 μl supernatant was mixed with 1.4 ml distilled water followed by adding 0.2 ml 1.5 mM calabarine sulfate and 800 μl 1.84 mM trichloracetic acid. The mixture was centrifuged at 5000 rpm for 5 min. 1.0 ml the ultimate supernatant was added to 1.0 ml alkaline hydroxylamine hydrochloride (equal volumes of 0.2 M hydroxylamine hydrochloride and 3.5 N sodium hydroxide), incubated at room temperature for 15 min, then was reacted with 0.5 ml 4 M HCl and 0.5 ml 0.37 M ferric chloride. The intensity of brown ferric complex was read at 540 nm. ACh content was expressed as micromoles per milligram of hippocampus protein (μmol/mg Prot).

2.6.4. Determination of AChE and ChAT activity

AChE activity in hippocampus homogenates was determined with an AChE kit according to the method of Ellman et al. (1961). Briefly, 10 μl supernatant was added into the reaction mixture that contained 1 mM sodium phosphate (pH 8.0) 150 μl, 50 μl 2% DTNB. The mixture was preincubated at 37 °C for 6 min. 90 μl 2 mM acetylcholine iodide was added and then the mixture was incubated for 3 min at 37 °C. The reaction was terminated by adding 10 μl of 6 mM neostigmine and the absorbance was measured at 412 nm. 1 U of AChE activity was defined as the number of hydrolyzed micromoles of acetylthiocholine iodide per min per microgram of protein. AChE activity in hippocampus homogenates was expressed as units per milligram of hippocampus protein (U/mg Prot).

ChAT activity in hippocampus homogenates was determined with a ChAT kit according to the spectrometric method (Wolfgram, 1972). The reaction mixture containing 0.5 M sodium phosphate, 6.2 mM acetylcoenzyme A, 1 M choline chloride, 0.76 mM methyl neostigmine sulfate 3 M sodium chloride, 1.1 mM EDTA, 20 μl of 0.5 M creatine HCl was preincubated at 37 °C for 5 min. 100 μl supernatants were added and then the mixture was incubated at 37 °C for 20 min. The reaction was terminated by boiling for 5 min and then adding 400 μl oxygen-free distilled water. After cooled to room temperature, the denatured protein was removed by centrifugation and 10 μl 3 mM 4-PDS was added to 0.5 ml of the mixture for producing 4-TP, the intensity of which absorbs at 324 nm. One unit of ChAT activity was defined as the number of hydrolyzed nanomoles of acetylcholine per min per microgram of protein. ChAT activity in hippocampus homogenates was expressed as units per milligram of hippocampus protein (U/mg Prot).

2.6.5. Protein assay

Protein in hippocampus homogenates was determined by Lowry et al.'s (1951) method using bovine serum albumin (BSA) as a standard.

2.7. Measurement of monoamine by HPLC

The right hippocampus tissues stored at −80 °C were thawed and weighed. Tissues (100 mg wet weight) were homogenized in 1 ml of perchloric acid and centrifuged at 15,000 rpm at 4 °C for 15 min. 50 μl the ultimate supernatant was removed and filtered (0.2 mm pore size; Whatman, UK), and 20 μl filtrate was injected into ESA CoulArray 5600A high-performance liquid chromatography (HPLC) with electrochemical detection (USA) and an analytical column ESA MD-150 (USA). The mobile phase consisted of 60 mM NaH_2PO_2, 1.5 mM 1-octanesulfonic acid sodium salt, 50 μM EDTA, 9% acetonitrile (pH 3.5). The flow rate was maintained at 0.6 ml/min. Chromatograms were recorded and integrated, and neurotransmitter concentrations were calculated from standard curves generated for each analyte. Tissue concentrations were determined for NE, A, DA and its metabolite DOPAC, 5-HT and its

metabolites 5-HIAA. All the values are expressed as nanogram per gram of tissues wet weight (ng/g wet weight). The ratios of DOPAC/DA and 5-HIAA/5-HT were calculated as estimates of DA and 5-HT turnover rates, respectively.

2.8. Statistical analysis

The quantitative data were expressed as mean ± SEM. Data analyses were performed with SPSS v.10.0. The data were analyzed using analysis of variance (ANOVA). Data obtained over training days from the hidden platform trial were analyzed by two-way ANOVA. Escape latency was the dependent variable, day was the within-subjects variable, and the group was the between-subjects variable. Remaining data were analyzed by one-way ANOVA. When appropriate, post hoc comparisons were assessed using the Least Significant Difference (LSD) test (equal variances assumed) or Dunnett's T3 test (equal variances not assumed). A P-value <0.05 was considered statistically significant for all analyses.

3. Results

3.1. Morris water maze

3.1.1. Hidden platform MWM

There were no differences in weight among animals of five groups, before or after the administration of B[a]P (Supplemental Fig. S1). The MWM was used to assess the spatial performance, and rats learned to find the hidden platform and escape onto it. Escape latencies in five groups were comparable before exposed to B[a]P (Supplemental Fig. S2). Whereas, after 13-week B[a]P administration, there were significant main effects of group ($F_{(4,175)} = 30.403$; $P < 0.001$) and day ($F_{(4,175)} = 213.554$; $P < 0.001$), but no significant

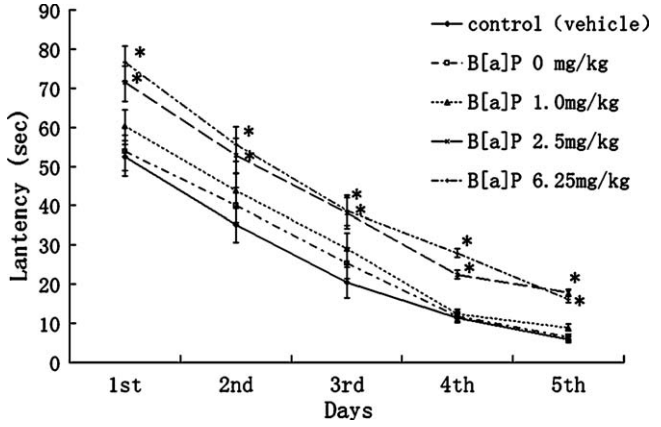

Fig. 1. The escape latencies in the hidden platform MWM after exposure to B[a]P. Data are shown as mean ± SEM, $n = 8$, $^*P < 0.05$ versus control (two-way ANOVA followed by LSD).

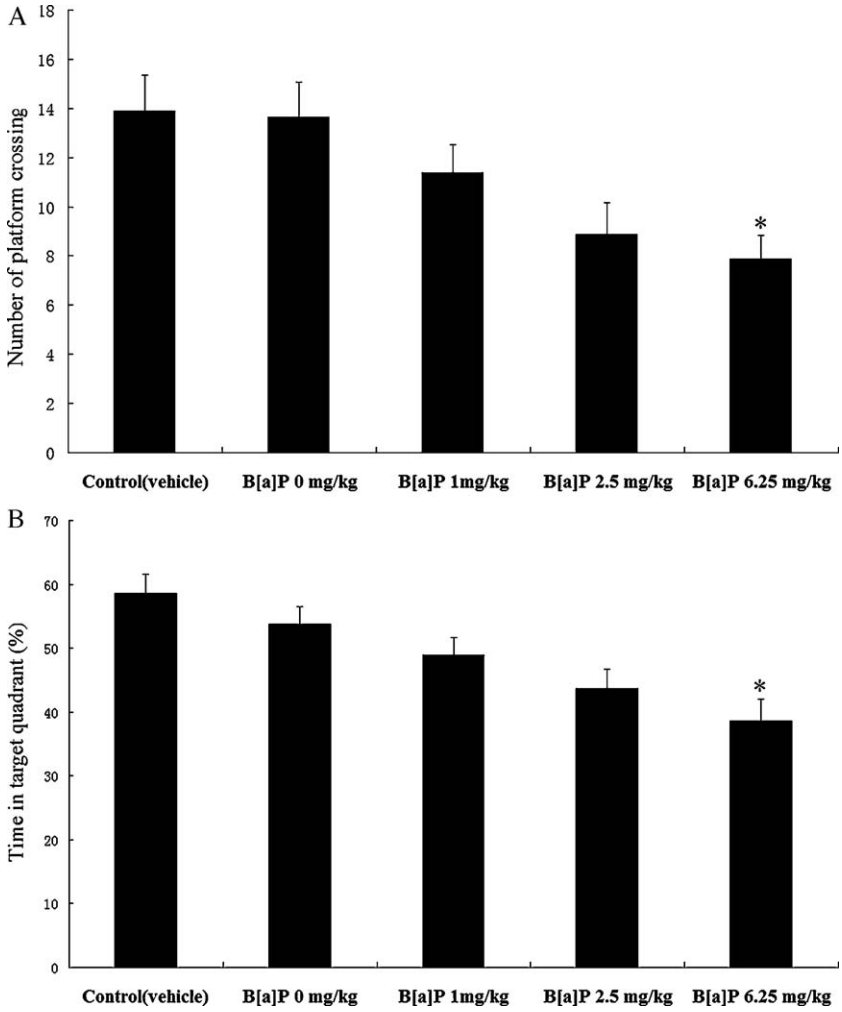

Fig. 2. Effects of subchronic exposure to B[a]P compared to vehicle on the behavior of rats in the probe trial. (A) The number of rat crossing platform in the target area. (B) Time in target quadrant of rats (% of 120 s). Data are shown as mean ± SEM, $n = 8$, $^*P < 0.05$ versus control (one-way ANOVA followed by LSD).

group and day interaction ($F_{(16,175)} = 0.566$; $P > 0.05$) on the escape latencies. The escape latencies for the treatment groups (0, 1.0, 2.5, and 6.25 mg/kg) decreased in a day-dependent manner, similar with the control group pattern, but two-way ANOVA showed the effect of treatment did not depend on day. The post hoc comparisons revealed that the treatment groups (2.5 and 6.25 mg/kg) had longer latencies to find the platform than the control group over the five testing days ($P < 0.05$; Fig. 1).

3.1.2. Probe trial

The spatial memory formation was measured by the percentage of time in the target annulus, and the number of times crossed over the target annulus during probe trial. The probe trial showed that B[a]P decreases the number of platform crossings and time in target annulus in a dose-dependent manner ($F_{(4,35)} = 4.565$ and $F_{(4,35)} = 6.918$, $P < 0.05$) (Fig. 2A and B). Furthermore, rats exposed to 6.25 mg/kg B[a]P spent less time in the target annulus than the control group ($P < 0.05$) (Fig. 2A and B). Rats of the treatment groups (2.5 and 6.25 mg/kg), swam aimlessly in the pool, and rats in control swam in the target quadrant or near this quadrant (Fig. 3).

3.2. Effect of B[a]P on MDA, NO content and SOD, NOS activity in hippocampus

Following the behavioral tasks, the oxidative stress and antioxidant status were analyzed to determine the role of oxidative

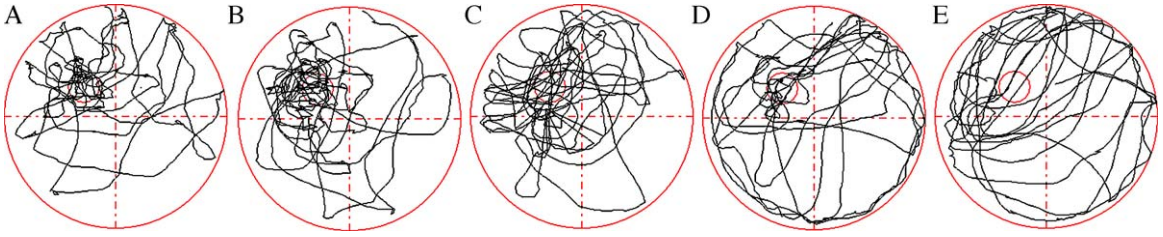

Fig. 3. Representative photographs of rat swimming path in probe trial when exposure to B[a]P. (A) The control. Path length: 1212.31 ± 29.98 cm (B), B[a]P (0 mg/kg). Path length: 1338.21 ± 41.92 cm (C), B[a]P (1.0 mg/kg). Path length: 1204.35 ± 33.86 cm (D), B[a]P (2.5 mg/kg). Path length: 1398.97 ± 116.88 cm. Swimming path shows rat swam aimlessly in the pool. (E) B[a]P (6.25 mg/kg). Path length: 1516.63 ± 112.29 cm. Swimming path shows rat swam aimlessly in the pool and spent approximately same time in four quadrants. Data are shown as mean ± SEM, $n = 8$ (one-way ANOVA followed by Dunnett's T3).

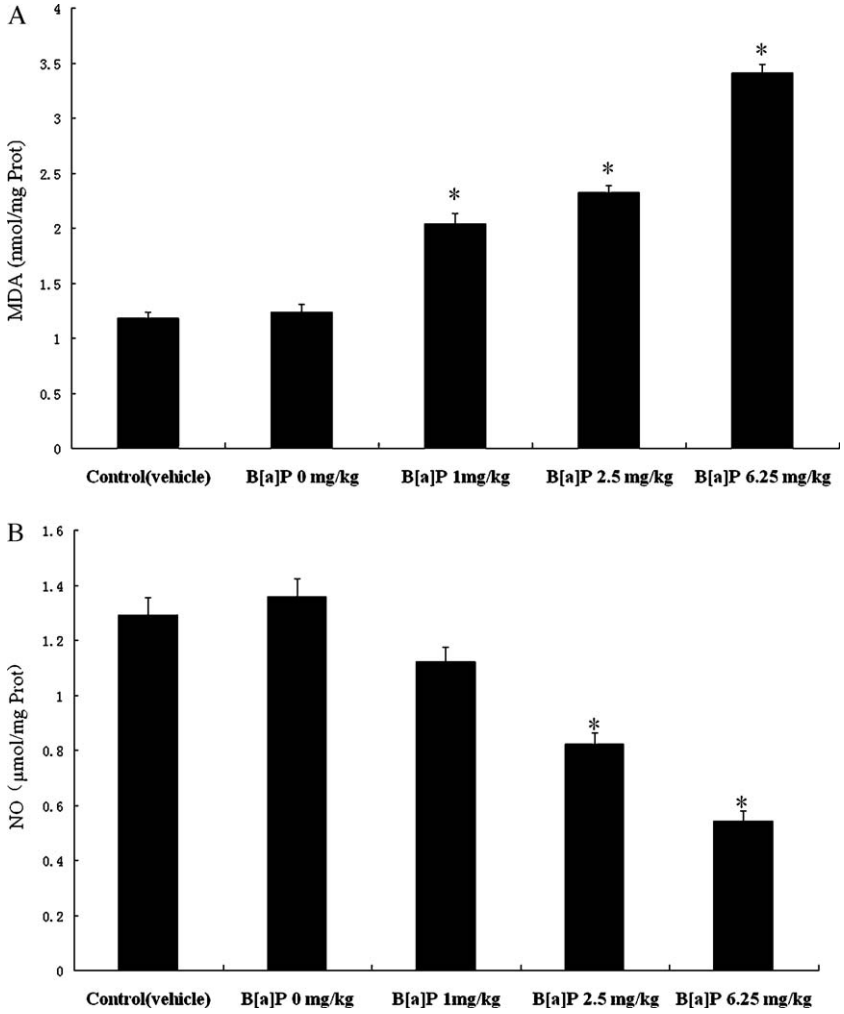

Fig. 4. Effects of subchronic exposure to B[a]P compared to control on MDA and NO levels in rat hippocampus. (A) MDA content. (B) NO content. Data are shown as mean ± SEM, $n = 8$, $^*P < 0.05$ versus control (one-way ANOVA followed by LSD).

damage induced by B[a]P. In addition, the gaseous messenger-NO and NO synthase were measured. The one-way ANOVA indicated that MDA, NO levels and activities of SOD, NOS in rat hippocampus could be significantly changed in the treatment groups by subchronic B[a]P treatment (MDA: $F_{(4,35)} = 150.71$, $P < 0.001$; NO: $F_{(4,35)} = 40.45$, $P < 0.001$; SOD: $F_{(4,35)} = 37.78$, $P < 0.05$; NOS: $F_{(4,35)} = 10.53$, $P < 0.05$) (Figs. 4A and B and 5A and B). Administration of B[a]P at the dose of 1.0, 2.5, and 6.25 mg/kg significantly increased MDA level and decreased SOD activity when compared to the control group ($P < 0.05$) (Figs. 4A and 5A). NO level and NOS

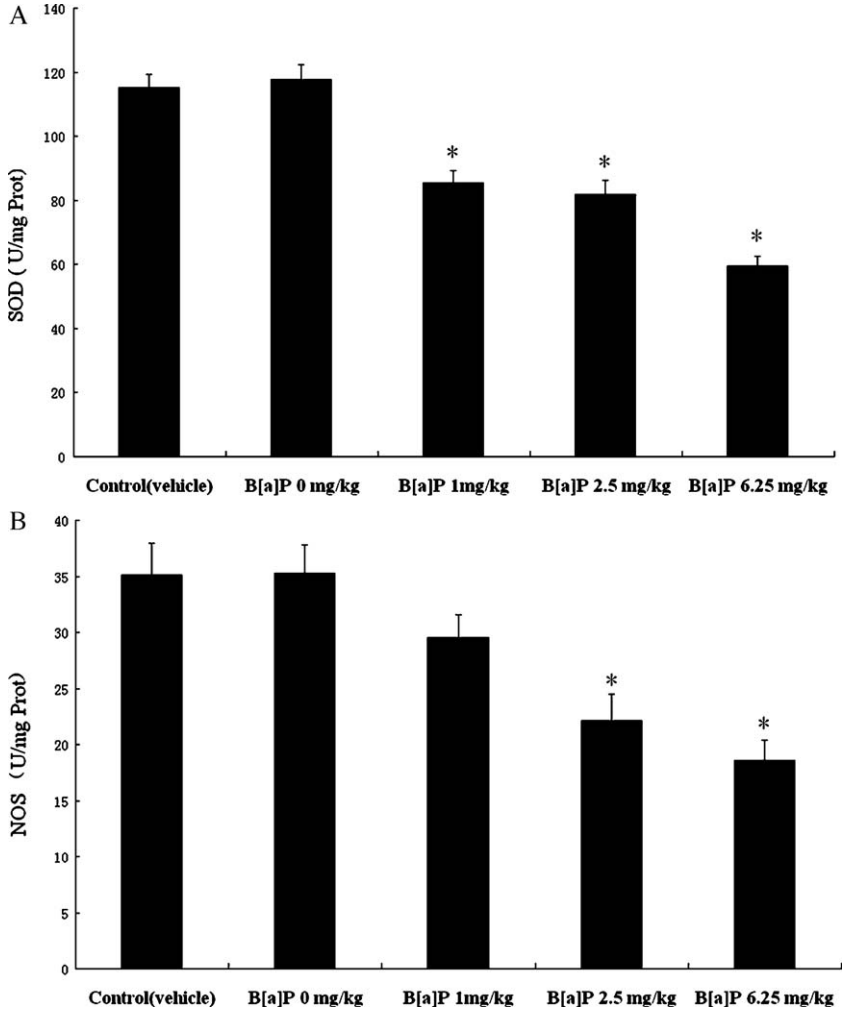

Fig. 5. Effects of subchronic exposure to B[a]P compared to control on the activity of SOD and NOS in rat hippocampus. (A) SOD activity. (B) NOS activity. Data are shown as mean ± SEM, $n = 8$, $^*P < 0.05$ versus control (one-way ANOVA followed by LSD).

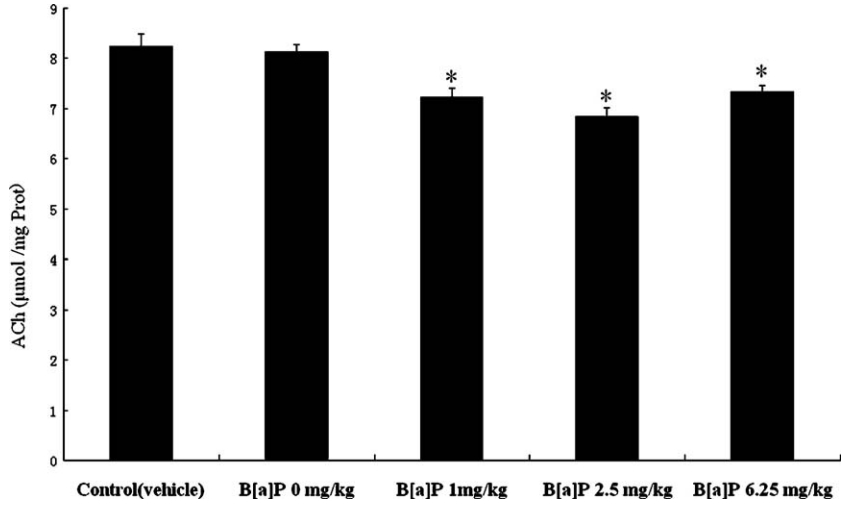

Fig. 6. Effects of subchronic exposure to B[a]P compared to control on the ACh content in rat hippocampus. Data are shown as mean ± SEM, $n = 8$, $^*P < 0.05$ versus control (one-way ANOVA followed by LSD).

activity were decreased in B[a]P (2.5 and 6.25 mg/kg) groups as compared to the control group ($P < 0.05$) (Figs. 4B and 5B).

3.3. Effect of B[a]P on ACh content and AChE, ChAT activity in hippocampus

The enzymes of cholinergic system and ACh content were measured in rat hippocampus after MWM test. There was a significant difference in ACh level and activities of AChE, ChAT among all experimental groups after exposure to B[a]P (ACh: $F_{(4,35)} = 11.78$, $P < 0.001$; AChE: $F_{(4,35)} = 31.37$, $P < 0.001$; ChAT: $F_{(4,35)} = 13.78$, $P < 0.001$) (Figs. 6 and 7A and B). ACh level and activity of ChAT were lower while activity of AChE in rat hippocampus was higher in the dose of 1.0, 2.5, 6.25 mg/kg treatment groups than those in the control group ($P < 0.05$) (Figs. 6 and 7A and B).

3.4. Effect of B[a]P on monoamine content in hippocampus

To detect the effect of B[a]P on monoaminergic system, monoamine neurotransmitters in rat hippocampus were analyzed. B[a]P did not significantly alter A or 5-HIAA concentrations in the hippocampus (A: $F_{(4,25)} = 1.406$, $P = 0.261$; 5-HIAA: $F_{(4,25)} = 2.574$, $P = 0.062$). There was a significant effect of B[a]P on NE, DOPAC, DA and 5-HT concentrations and DA, 5-HT turnover rates in the hippocampus (NE: $F_{(4,25)} = 171.71$, $P < 0.05$; DOPAC: $F_{(4,25)} = 56.82$, $P < 0.001$; DA: $F_{(4,25)} = 292.72$, $P < 0.001$; 5-HT: $F_{(4,25)} = 85.88$, $P < 0.001$; DA turnover rate: $F_{(4,25)} = 13.84$, $P < 0.001$; 5-HT turnover rate: $F_{(4,25)} = 42.25$, $P < 0.001$) (Fig. 8A and B). Post hoc analysis revealed that the administration of B[a]P for 90 days significantly increased DOPAC and 5-HT concentrations in treatment groups (1.0, 2.5, and 6.25 mg/kg) ($P < 0.05$), as well as NE and DA concentrations (2.5 and 6.25 mg/kg) ($P < 0.05$), when compared to control group (Fig. 8A). Both 5-HIAA/5-HT and DOPAC/DA ratios were modified, increased 5-HT turnover rate in treatment group (1.0, 2.5, and 6.25 mg/kg) and decreased DA turnover rate (1.0 and 2.5 mg/kg) as compared to the control group ($P < 0.05$) (Fig. 8B).

4. Discussion

The present study suggests that subchronic exposure to B[a]P has the adverse effects on the learning and memory in male SD rat. As indicated by MWM which are widely used in rodents to assess spatial learning and memory (Morris et al., 1982; Morris, 1984; Stewart and Morris, 1993), rats treated with B[a]P at both doses, 2.5 and 6.25 mg/kg, had worse learning and memory performance than the control group. This is in agreement with previous studies that the B[a]P has adverse effect on neurobehavioral function of animals (Saunders et al., 2001, 2002, 2003). B[a]P can cause

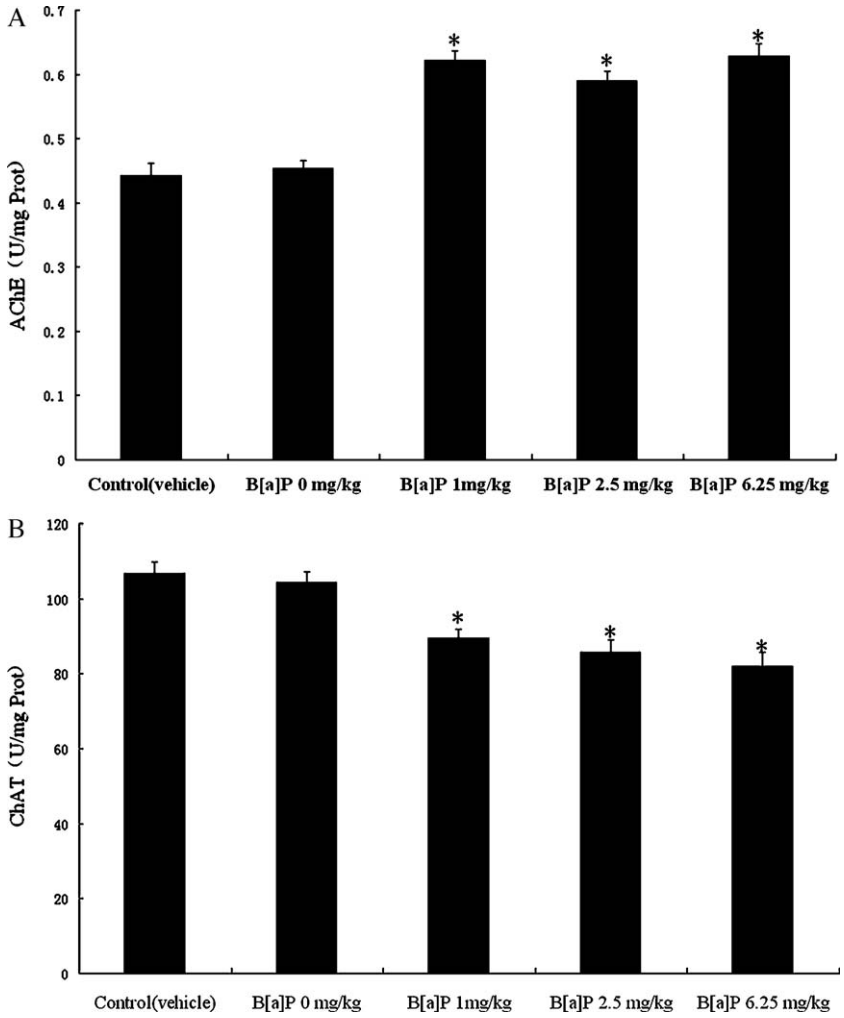

Fig. 7. Effects of subchronic exposure to B[a]P compared to control on the activity of AChE and ChAT in rat hippocampus. (A) AChE activity. (B) ChAT activity. Data are shown as mean ± SEM, $n = 8$, $^*P < 0.05$ versus control (one-way ANOVA followed by LSD).

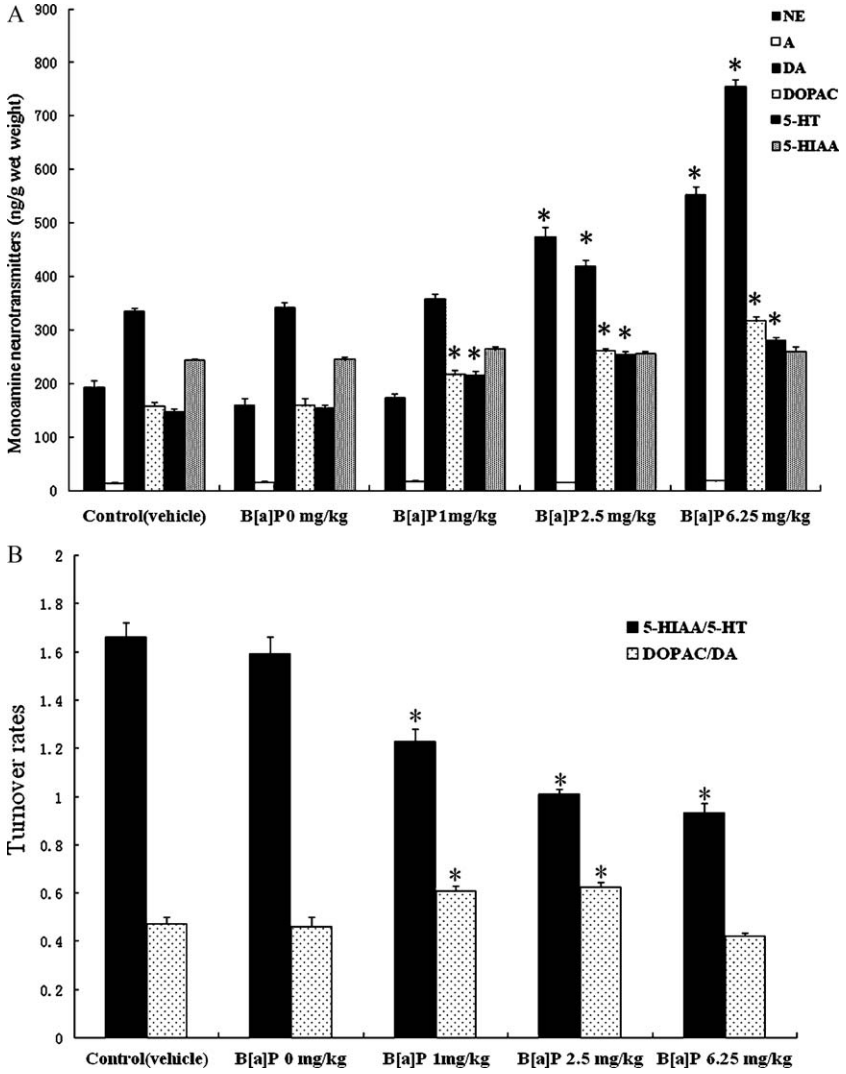

Fig. 8. Effects of subchronic exposure to B[a]P compared to control on the levels of monoamine neurotransmitters and the turnover rates in rat hippocampus. (A) The levels of monoamine neurotransmitters. (B) The ratios of DOPAC/DA and 5-HIAA/5-HT. Data are shown as mean ± SEM, $n = 6$, $^*P < 0.05$ versus control (one-way ANOVA followed by LSD).

behavioral toxic effects on F-344 rats, including reduction of nocturnal motor activity and alterations in neuromuscular, autonomic and physiological functions (Saunders et al., 2001, 2002, 2003). Treatment with 0.05 and 0.1 g/kg B[a]P for 3 days, resulted in significant decrease of motor nerve conduction velocity in mice (Liu et al., 2002). With the intraperitoneal administration of B[a]P, motor activity of mice reduced as indicated by low swimming score (Tu et al., 2002). Subacute exposure to B[a]P (0.02 and 0.2 mg/kg) in adult mice, B[a]P impaired short-term learning and spatial memory capacities in Y maze and MWM (Grova et al., 2007). B[a]P induced the impairment of spatial learning and memory functions on rats after intracerebroventricular administration (Nie et al., 2008). Gestational exposure to B[a]P aerosol attenuated the capacity for long-term potentiation (Wormley et al., 2004a,b) and impaired learning and memory abilities in the F1 generation (Wu et al., 2003). These data strongly suggest that B[a]P can cause alterations in nervous system function. Although our results suggest that B[a]P disrupts spatial learning and memory, it is possible that the long latency in finding the hidden platform could have been a result of nervous system impairment in rat, rather than a result of only spatial learning and memory impairment. B[a]P produced a variety of behavioral effects on mice that are specific for nervous system dysfunction including decreased motor activity, neuromuscular, physiological and autonomic defects and decreased responsiveness to sensory stimuli (Saunders et al., 2001, 2002, 2006; Bouayed et al., 2009). B[a]P also had effects on emotionality causing hyperactivity/hyperarousal state in mice they are faced with a new and unknown situation in learning and memory tests (Grova et al., 2007) and mice treated with B[a]P were less anxious than controls (Boussel et al., 2006; Grova et al., 2008). In the current study, the result of Morris water maze showed that the longer latencies to locate the platform, the less number of platform crossing and the shorter swimming time in the target area were observed in the 6.25 mg/kg B[a]P group compared with the control groups. It suggested spatial learning and memory impairment in rats induced by exposure to B[a]P, furthermore the performance impairment might be an indication of nervous system dysfunction.

The hippocampus is a particularly vulnerable and sensitive region of the brain that is also very critical for spatial learning and memory and supporting cognitive abilities (O'Keefe and Nadel, 1978; Morris et al., 1982; Eichenbaum, 1997; O'Keefe, 1999; Moser et al., 2008). Thus, the oxidative stress and modulation of the neurotransmitters in this region may contribute to the impairment of learning and memory observed in the present study. MDA as an index of oxidative tissue damage, a by-product of lipid peroxidation induced by free radicals, increased concentration of which in intra- and extra-cellular membranes results in damage to the cells,

tissues and organs (Cini et al., 1994). Antioxidants are critical in combating oxidative stress in neurons by way of scavenging free radicals (Saunders et al., 2006). SOD, endogenous antioxidant enzyme, can scavenge free radicals and prevent B[a]P-induced toxicity in human mammary epithelial cells (Leadon et al., 1988). MDA level was significantly increased in hippocampus and the activity of SOD was found to turn down significantly in the hippocampus after exposure to B[a]P (1.0, 2.5, and 6.25 mg/kg), when compared to the control group. B[a]P administration induced increasing lipid peroxidation of hippocampus tissues might be a consequence of inhibiting SOD activity. Therefore, B[a]P-induced neurobehavioral toxicity may occur through oxidative stress due to inhibition of the brain antioxidant scavenging system (Saunders et al., 2006).

Also, B[a]P induced alterations in the dopaminergic and serotoninergic systems in the brain may lead to behavioral alterations (Stephanou et al., 1998). While there are no studies to date that examined whether there is any relationship between neurobehavioral effects of B[a]P and the cholinergic neurotransmitter and NO. The cholinergic system has been implicated in several neuropsychic functions such as learning and memory and is modulated by ACh (Gold, 2003; Everitt and Robbins, 1997; Mohapel et al., 2005; Muir et al., 1993; Whishaw and Tomie, 1987). ChAT and AChE, markers of cholinergic function, are charge of acetylcholine synthesis and degradation (Levey et al., 1983). There is a positively correlation in rat hippocampus between increased levels of ACh and improved spatial memory performance in a maze task (Chang and Gold, 2003; Fadda et al., 2000; Molinengo and Ghi, 1997). The ACh level declined when subchronic treatment with B[a]P (1.0, 2.5 and 6.25 mg/kg) as a consequence of the increase of AChE activity and decrease of ChAT activity. Similar to the ACh level, NO level was reduced in hippocampus after administration of B[a]P (2.5 and 6.25 mg/kg), concomitant to decreased NOS activity. Biosynthesized by NOS, NO is a new class of neurotransmitters in the central nervous system (Snyder, 1992; Vincent, 1994), and a messenger molecule necessary for memory processing (Edwards and Rickard, 2007). Increased NO ameliorates learning and memory, whereas a decrease of NO attenuates spatial behavioral performance in a maze task (Myslivecek et al., 1994, 1996; Estall et al., 1993). For this reason, the concomitant variations occurring in NO levels and ACh level in hippocampus might be associated with learning and memory impairments in B[a]P treatment rats.

Neurotransmitters of the central monoaminergic systems, such as noradrenaline, dopamine, and serotonin play roles in the learning and memory of animals (Rolls, 2000; Al-Zahrani et al., 1996; Prado-Alcala et al., 2003). B[a]P treatment increased the levels of NE, DA, DOPAC and 5-HT in the hippocampus, the result consistent with previous study (Jayasekara et al., 1992; Konstandi et al., 2007), whereas inconsistent with the monoamine levels when acute exposure to B[a]P (Stephanou et al., 1998). The memory processes involves interactions among different neurotransmitters, consistently with the complexity of brain systems (Castellano et al., 1996). The "inverted U-shaped dose-effect curve" (IUSDEC) in learning and memory has been reported when the effects of increasing dosages of a given compound appear to increase up to a maximum, and then the effects decrease (Baldi and Bucherelli, 2005). Hence the IUSDEC is a small part of the complexity of brain systems. Early investigations showed that systemically administered A in normal rats affected retention in an inverted U-shaped dose-effect curve, in which memory is enhanced at moderate doses and impaired at high doses (Gold and van Buskirk, 1975, 1976, 1978; Gold et al., 1977). Optimal increasing levels of DA, A and NE are associated with the enhancement of memory consolidation (Gold and van Buskirk, 1975, 1976; Gold et al., 1977; Introini-Collison et al., 1992; Castellano et al., 1996), but excessive NE and DA release lead to amnesia and memory deficits (Huber et al., 1989; Liang et al., 1990; Canal et al., 2007; Morice et al., 2007; Qi and Gold, 2009). Although subchronic administration of B[a]P do not alter A level, increased DOPAC levels and DA turnover rates in the same region, an indication of increased metabolism of this amine and impairment of spatial working memory (Murphy et al., 1996). Data on the effects of 5-HT on learning and memory are inconsistent, inhibition of 5-HT synthesis in rodents impeded learning (Brody, 1970; Valzelli and Pawlowski, 1979), but other data to the contrary (Wetzel et al., 1980; Altman and Normile, 1986). Despite the inconsistencies, the preponderance of evidence shows that increase of 5-HT in the brain impairs learning and memory (McEntee and Crook, 1991). Although the 5-HIAA level is not changed after exposure to B[a]P, the 5-HT/5-HIAA rates decreased. Reduced 5-HT turnover is associated with impaired long-term memory functioning and cognitive flexibility (Mössner et al., 2000). Therefore, B[a]P impair rat behavioral performance in MWM might be linked with increasing NE, DA, 5-HT levels and 5-HT turnover rate, as well as modified DA turnover rate.

In conclusion, this study suggested that impaired behavioral performance induced by B[a]P in SD male rat may be due to alternation of the neurotransmitters levels and oxidative stress in the hippocampus, indicating that B[a]P is a potential neurotoxic pollutant. Future studies will focus on conducting chronic exposures, which are more relevant to human exposure conditions and consequently may explore a link between learning and memory performance and expression of gene related to the learning and memory.

Conflict of interest statement

The authors declare that there are no conflicts of interest.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.neuro.2010.12.015.

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