Original Article

Modulation of Behavior and Glutamate Receptor mRNA Expression in Rats after Sub-chronic Administration of Benzo(a)pyrene*

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Abstract

Objective The present study aimed to test whether exposure to benzo(a)pyrene [B(a)P] affects spatial learning and short-term memory by modulating the expression of the *Gria1* and *Grin2a* glutamate receptor subunit genes in the hippocampus.

Methods Thirty-six 21–24-day-old, male rats were randomly assigned into high-, medium-, and low-dose toxin exposure groups (6.25, 2.5, and 1 mg/kg, respectively) and a control group, each containing nine rats. The behavioral performance of adult rats exposed to sub-chronic administration of B(a)P was monitored by learning and memory tests (Morris water maze). Real-time PCR assays were used to quantify *Gria1* and *Grin2a* gene expression in the hippocampus.

Results At medium and high doses, B(a)P impaired spatial learning performance. The crossing-platform-location frequency and the time spent swimming in the platform area, which both relate to short-term memory, were significantly decreased in B(a)P-treated rats compared with controls. The level of *Gria1* mRNA increased 2.6–5.9-fold, and the level of *Grin2a* mRNA increased 10–14.5-fold, with a greater fold increase associated with higher doses of B(a)P.

Conclusion We demonstrated that sub-chronic administration of B(a)P inhibits spatial learning and short-term memory, and increases *Gria1* and *Grin2a* expression in the hippocampus. This suggests a relationship of B(a)P exposure levels with *Gria1* and *Grin2a* expression and impairment of short-term and spatial memory.

Key words: Benzo(a)pyrene; *Gria1* ; *Grin2a*

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INTRODUCTION

Benzo[a]pyrene [B(a)P] is a potentially genotoxic and cytotoxic environmental pollutant[1]. Studies have documented the neurotoxic effects on learning and memory that result from exposure to B(a)P. The findings of Saunders et al.[2-3] demonstrated that B(a)P induced acute neurobehavioral toxicity in F344 rats including neuromuscular, autonomic, sensorimotor, and physiological symptoms. A more recent experiment reported that prenatal exposure to B(a)P impairs cortical neuronal function in later life[4].

Glutamate receptors are critical for the activity-dependent synaptic plasticity and long-term changes in synaptic strength involved in hippocampal and cortical learning and memory consolidation[5]. The ionotropic glutamate receptors comprise three groups: the kainate, dL-a-amino-3-hydroxy-5-methylisoxazole-4-propionic

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acid (AMPA), and N-methyl-D-aspartate (NMDA) receptors[6]. Hippocampal NMDA receptors (NMDAR) are involved in the induction of long-term potentiation (LTP) and long-term depression (LTD) in principal neurons and interneurons[7-9]. It has been shown that blockade of either NMDA or AMPA glutamate receptors in the prefrontal cortex disrupts spatial delayed alternation[10].

B(a)P exposure may be associated with spatial learning disabilities, whereas chronic exposure to B(a)P modulates the expression of the NMDAR1 subunit gene *GRIN1*, which is particularly involved in cognitive function[11]. The present study aimed to extend these findings by determining whether B(a)P exposure interferes with hippocampal neuronal responses, as reflected by deficits in memory and learning, mediated by increases in the gene expression of glutamatergic receptor subunits. We found that sub-chronic administration of B(a)P results in impairment of short-term memory and spatial learning. This impairment corresponds with a significant up-regulation of expression of the *Grin2a* and *Gria1* genes in the hippocampus.

MATERIALS AND METHODS

Rats

36 21–24-day-old, clean, male Wistar rats (67-71 g), from the Experimental Animal Center of Chongqing Medical University, were maintained in plastic cages and acclimatized to the animal facility for 1 week with a controlled 12-h light/12-h dark cycle at 24±4 °C and 40%±10% relative humidity. Water and food were available *ad libitum*. 9 rats were randomly assigned to each experimental group, receiving 1, 2.5, or 6.25 mg/kg of B(a)P (98% purity; Sigma, St. Louis, MO). Each animal received a daily intra-peritoneal injection of B(a)P solubilized in vegetable oil for 14 weeks (98 days). Control animals received vegetable oil alone. The rats' capacity for learning and memory was measured using the Morris water maze; the animals were then sacrificed and the hippocampi were rapidly dissected according to the method of Glowinski and Iversen[12]. The samples were frozen in liquid nitrogen and kept at -80 °C until the RNA extraction. Expression of the *Gria1* and *Grin2a* subunit genes was measured by quantitative reverse transcription (RT)-PCR. Animals were handled and cared for in accordance with the Guide for the Care and Use of Laboratory Animals (National Research Council, 1996) and all efforts were made to minimize animal suffering and reduce the number of animals used.

Morris Water Maze

The Morris water maze was made of stainless steel (diameter 180 cm; walls 70 cm in height), and was filled with water (22 °C). Black pigment was added to the water so that the platform was not visible. The pool was situated in a room with an extra-maze visual cue. The pool was separated into four areas, and a platform was placed in one quadrant, 33 cm away from the wall. Each rat was sequentially placed into the pool in a random position. If it failed to reach the escape platform within 120 s, it was retrieved from the pool and placed on the platform for 10 s. The next day, the same procedure was repeated once. The animals were videotaped throughout each trial, and the time to locate the platform (the escape latency) was measured. After the last trial, the platform was removed and the rats were placed into the pool and allowed to move freely for 120 s; the frequency with which they crossed the former platform location and the time spent swimming in the target quadrant were calculated.

Expression of the Gria1 and Grin2a Genes

Total RNA was isolated using Trizol reagent (Invitrogen, Carlsbad, CA), following the manufacturer's instructions. Briefly, the frozen tissues were ground into a powder and added to the Trizol reagent. After sample homogenization, the organic and aqueous phases were separated, and total RNA was precipitated with isopropyl alcohol. The RNA pellets were washed with 70% ethanol, air-dried and dissolved in RNase-free water. The purity of the total RNA was determined spectrophotometrically using the 260-nm absorbance and the 260/280-nm absorbance ratio. RNA integrity was checked by visual examination of the two ribosomal RNA bands (28S and 18S) on a 1% agarose gel stained with 0.5 mg/mL ethidium bromide.

Total RNA was reverse transcribed using 100 U of Exscript™ RT Reagent Kit (TaKaRa Biotechnology Co., Ltd. Dalian Economic and Technical Development Zone). The final concentrations of the reagents in the 40-μL RT reaction were: 1× PrimeScript™ buffer, 2 μL of each of PrimeScript RT Enzyme Mix I, 25 pmol oligo d(T) primer, and 50 pmol random hexamers, and 250 ng/μL total RNA. The reaction conditions were 37 °C for 15 min then 85 °C for 5 s to inactivate the reverse transcriptase. RNA samples were tested for genomic DNA

contamination by including a no-enzyme RT control, and for reagent and aerosol contamination by including two no-template controls (one tube with a closed lid and one with an open lid during template addition). A control for the reagents was performed under the same conditions but omitting the template. All samples and standards were analyzed in duplicate.

Standard curves were generated using five 10-fold dilutions of purified PCR product previously obtained by conventional PCR. Standard curves were used to assess the amplification efficiency of each PCR assay. Gene expression was normalized to the respective expression levels of glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*). To minimize inter-plate variation, the target genes were amplified in a single 96-well PCR plate. PCR amplification was performed on 2 µL of cDNA template in a total volume of 25 µL, using SYBR® Green Premix Ex Taq™ (TaKaRa) and 10 µmol/L each primer (primers were designed by TaKaRa Corp.; oligonucleotide sequences are listed in Table 1). PCR was performed on an iQ™5 instrument (Bio-Rad, Hercules, CA) with the following cycling conditions: 30 s at 95 °C, followed by 40 cycles of 5 s at 95 °C, and 30 s at 60 °C, and completion with a dissociation (melt) curve analysis of 72 °C for 3 min, 95 °C for 1 min, and 55 °C for 1 min. Fluorescence levels were detected at the annealing stage of each cycle.

Table 1. Primer Sequences

Target Gene	Forward Primer(5′–3′)	Reverse Primer(5′–3′)	Amplicon Size(bp)
Gria1	ccaggtgtcttctcctttcttg	ctcgtccctcttcaaactcttc	148
Grin2a	gacagcaagaggagcaagtctc	ctcaaggatgaccgaagatagc	170
GAPDH	gtggacctcatggcctacat	tgtgagggagatgctcagtg	127

Statistical Analysis

Statistical analysis was performed using SPSS 11.5 for Windows (SPSS Inc., 233 South Wacker Drive, 11th Floor, Chicago, IL 60606-6412). Body weight, *Gria1* and *Grin2a* subunit expression were reported as the mean±SEM and compared by one-way analysis of variance followed by planned contrasts with the Student-Newman-Keuls correction. The behavioral data deviated significantly from a normal distribution and the variances of the different test groups were not equal. Therefore, non-parametric statistical procedures were used. The median and quartile values of the controls were compared with those of the B(a)P-exposed animals using a modified Mann-Whitney procedure. Where appropriate, a Friedman test for within-groups analyses, followed by a *post-hoc* modified Wilcoxon test, was applied. For all analyses, differences were considered significant at $P<0.05$.

RESULTS

Body Weight

There was no significant difference in body weight between B(a)P-treated and control rats (Figure 1).

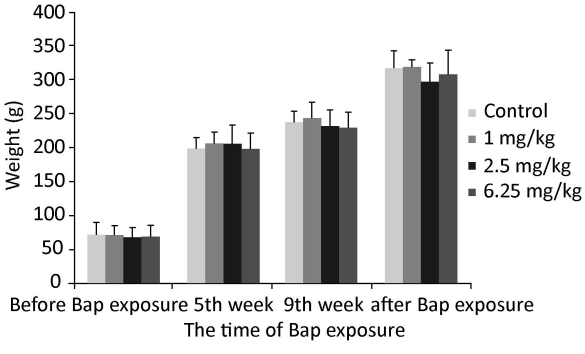

Figure 1. Body weight in benzo(a)pyrene-treated rats. The data are expressed as the mean±SEM of nine rats per group. $P>0.05$, no significant difference between experimental and control animals (Student-Newman-Keuls *t*-test for multiple comparisons).

Water Maze Performance

The escape latency of both the control animals and the B(a)P-exposed groups decreased significantly from the first to the fifth day (Table 2), indicating that all the animals learned to find the platform. Rats in the 2.5 and 6.25 mg/kg B(a)P-treated groups were significantly slower at finding the platform compared with the controls. Within one day, rats injected with 2.5 and 6.25 mg/kg of B(a)P showed a significant 29%

and 32% increase, respectively, in the escape latency compared with controls. This suggests that B(a)P exposure may be associated with spatial learning disabilities. Moreover, in the trials conducted after the platform was removed, the crossing-platform-location frequency and the time spent swimming in the platform target area of B(a)P-exposed rats were both significantly decreased compared with controls (Table 3). This suggests that sub-chronic administration of B(a)P has an inhibitory effect on short-term memory. A significant 42% reduction in the crossing-platform-location frequency was apparent in the 6.25 mg/kg B(a)P-treated group compared with the controls.

Table 2. Escape Latencies of Benzo(a)pyrene-treated Rats in the Morris Water Maze

Group	Day 1 (s)	Day 2 (s)	Day 3 (s)	Day 4 (s)	Day 5 (s)
Control	53.83±11.67	40.11±12.62	25.30±11.00	11.74±3.35	6.50±2.57
1 mg/kg	60.42±12.27	43.76±10.36	29.11±9.14	12.29±7.73	8.93±1.72[b]
2.5 mg/kg	71.53±9.84[a]	52.80±11.40[a]	38.09±11.02[a]	22.48±4.48[a]	17.90±7.71[ab]
6.25 mg/kg	76.68±12.94[a]	55.70±11.42[a]	38.78±9.30[a]	27.94±3.85[a]	16.20±4.74[ab]

Note. The data are expressed as the median and quartile values of nine rats per group. [a]*P*<0.05, significant difference between the experimental and control groups (Mann-Whitney U-test modified for multiple comparisons), [b]*P*<0.05, significant difference between the first and last trials (Wilcoxon W-test modified for multiple comparisons).

Table 3. Crossing-Platform-Location Frequency and Time Spent Swimming in the Target Area of Benzo(a)pyrene-treated Rats in the Morris Water Maze (Platform Removed)

Group	Crossing-platform-location Frequency (*n*)	Time Spent Swimming in the Target Area (%)
control	13.63±4.10	53.73±5.86
1 mg/kg	11.38±3.25[a]	48.95±6.17
2.5 mg/kg	8.87±3.64[b]	43.64±10.56[ab]
6.25 mg/kg	7.89±2.64[b]	38.60±4.35[ab]

Note. The data are expressed as the median and quartiles of nine rats per group. [a]*P*<0.05, statistically significant difference between this group and the other experimental animals (Kruskal Wallis-test for multiple comparisons), [b]*P*<0.05, statistically significant difference between experimental and control animals (Mann-Whitney U-test modified for multiple comparisons).

Expression of the Gria1 and Grin2a Genes

We performed real-time quantitative PCR to test whether B(a)P exposure resulted in modulation of *Gria1* and NMDA receptor subunit expression in the hippocampus. The amplification efficiencies of the target and the reference were approximately equal (Figures 2 and 3). The relative expression of *Gria1* and *Grin2a* in injected rats was determined by normalizing to *GAPDH* levels in the same sample. In the hippocampus, the level of *Gria1* mRNA increased 2.6–5.9-fold, and the level of *Grin2a* mRNA increased 10–14.5-fold; greater fold increases were observed with higher doses of B(a)P (Figure 4).

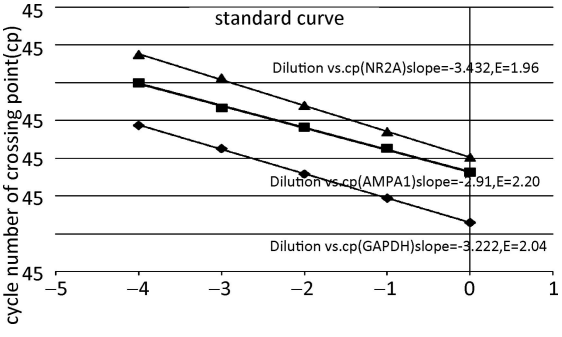

Figure 2. Standard curves for quantitation of gene expression. Determination of real-time PCR efficiencies of the reference gene (*GAPDH*), target gene 1 (*Grin2a*), and target gene 2 (*Gria1*). CP (cycle number of crossing point) *versus* cDNA dilution graphs were plotted to calculate the slope ($\bar{x} \pm s$; n=3). The corresponding real-time PCR efficiencies were calculated according to the equation: E= 10[–1/slope].

DISCUSSION

The present study showed that sub-chronic administration of B(a)P induces learning and memory deficits, as measured by two different tests. It is possible that the impairments are related to up-regulation of *Gria1* and *Grin2a* mRNA expression.

The deficits in spatial learning and short-term memory do, in fact, correlate with an increase in *Grin2a* and *Gria1* subunit expression.

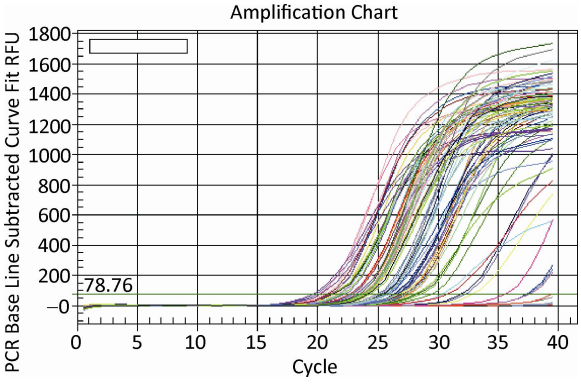

Figure 3. Amplification curve displaying the high amplification efficiency of the PCR reactions.

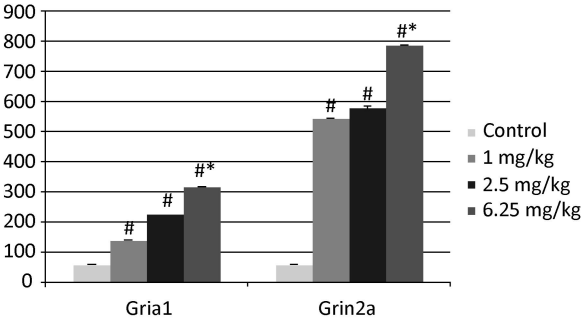

Figure 4. Expression of *Grin2a* mRNA and *Gria1* mRNA in benzo(a)pyrene-treated rats. The data are expressed as the mean±SEM of three rats per group. $^{\#}P<0.01$, significant difference from controls (independent samples *t*-test for multiple comparisons). $^{*}P<0.01$, significant difference from the other B(a)P-treated groups (Student-Newman-Keuls *t*-test for multiple comparisons).

B(a)P is a prototypic polycyclic aromatic hydrocarbon (PAH). Biomonitoring studies have found notable levels of benzopyrene in foods (broiled (grilled)/smoked meats; average intake estimated at 120-2 800 ng/day), in vehicle exhaust and in cigarettes (10 ng/cigarette)[13-15]. While the carcinogenicity of PAHs is well established[16], their neurotoxic effects have received less attention, and at present, the mechanism of B(a)P-induced impairment of cerebral function remains unclear.

There was no significant difference in the body weight between B(a)P-treated and control rats (Figure. 1) Grova et al.[11] found that mice exposed to 0.02 or 0.2 mg/kg B(a)P gained weight more rapidly than controls or mice exposed to 20 mg/kg B(a)P, but this conflicting result may be caused by differences in the species used.

The Morris water maze was designed in the 1980s by English psychologist Morris to study learning and memory[17]; the memory formed in this experiment is referred to as spatial reference memory. Our data revealed that the same animals required less time to locate the platform after five days, and that B(a)P impairs short-term memory and spatial learning capacity. These results were consistent between the platform-present and platform-removed paradigms. In the latter, the frequency of crossing the former platform location and the time spent swimming in the target area were both significantly decreased in B(a)P-treated rats compared with controls.

Previous studies, using different exposure regimens in rodent models, have documented neurotoxic effects on learning and memory resulting from exposure to PAHs. Moreover, prenatal or sub-acute B(a)P exposure has been shown to cause neurotoxicity as evidenced by deficits in learning and memory (assessed by the Y-maze, fixed-ratio operant conditioning, two-lever reversal conditioning, and Morris water maze performance)[11,18-19]. Normal functioning of the hippocampus and/or S1 cortex, as measured by decreases in behavioral learning, was impaired for at least 100 postnatal days in offspring exposed to B(a)P *in utero*[18-19]. Taken together, these results suggest a relationship between changes in behavior and exposure to organic micropollutants[18,20-21]. These deficits are consistent with the short-term memory and spatial learning impairments that we observed using the Morris water maze (Table 2).

Hippocampal NMDARs are involved in the induction of LTP and LTD in principal neurons and interneurons[22]. LTP of synaptic transmission is a well-characterized form of activity-dependent plasticity likely to play important roles in learning and memory[23], and disrupting NMDAR function prevents LTP and leads to changes in learning and memory in mice[24-25] or *Aplysia*[26-27]. The synaptosomal-associated protein, 25kDa (SNAP25) protein in the hippocampal CA1 region is involved in memory consolidation for contextual fear conditioning and spatial water-maze training[28], and it also plays a critical role in the trafficking of NMDARs to the plasma membrane[29-30]. LTP or increased activity of calcium/calmodulin-dependent protein kinase II

(CaMKII) induced delivery of tagged AMPARs into synapses[31]. It has been suggested that spatial learning may be associated with differential expression of NMDAR subunits[32]. Grova et al.[11] reported that the spatial learning deficits observed at low doses (0.02-20 mg/kg) of B(a)P could be explained by the level of NR1 (Grin1) overexpression in the hippocampus.

The present study shows that sub-chronic administration of B(a)P induces learning and memory deficits. This could be explained by *Gria1* and *Grin2a* overexpression in the hippocampus, a cerebral area strongly implicated in the regulation of learning- and emotion-related behaviors. In the hippocampus, the level of *Gria1* mRNA increased 2.6–5.9-fold, and the level of *Grin2a* mRNA increased 10–14.5-fold, with a greater fold increase associated with higher doses of B(a)P.

In conclusion, we show that sub-chronic exposure to B(a)P up-regulates expression of the *Grin2a* and *Gria1* genes, which may contribute to reduced cognitive function as evidenced by impairments of short-term and spatial memory.

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