### Integrated epigenetics, transcriptomics, and metabolomics to analyze the mechanisms of Benzo[a]pyrene neurotoxicity in the hippocampus

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#### Abstract

Benzo[a]pyrene (B[a]P) is a common environmental pollutant that is neurotoxic to mammals, which can cause changes to hippocampal function and result in cognitive disorders. The mechanisms of B[a]P-induced impairments are complex .To date there have been no studies on the association of epigenetic, transcriptomic and metabolomic changes with neurotoxicity after B[a]P exposure. In the present study, we investigated

the global effect of B[a]P on DNA methylation patterns, non-coding RNAs (ncRNAs) expression, coding RNAs expression, and metabolites in the rat hippocampus. Male Sprague-Dawley rats (SD rats) received daily gavage of B[a]P (2.0 mg/kg body weight [BW]) or corn oil for seven weeks. Learning and memory ability was analysed using the Morris Water Maze (MWM) test and change to cellular ultrastructure in the hippocampus was analysed using electron microscope observation. Integrated analysis of epigenetics, transcriptomics, and metabolomics was conducted to investigate the effect of B[a]P exposure on the signaling and metabolic pathways. Our results suggest that B[a]P could lead to learning and memory deficits, likely as a result of epigenetic and transcriptomic changes that further affected the expression of CACNA1C, Tpo, etc. The changes in expression ultimately affecting LTP, tyrosine metabolism and other important metabolic pathways.

**Keywords** Benzo[a]pyrene; hippocampus; neurotoxicity; epigenetics; transcriptomics; metabolomics

#### Introduction

Benzo[a]pyrene (B[a]P), a typical polycyclic aromatic hydrocarbon (PAH), is ubiquitous in the environmental surroundings. It is produced by incomplete combustion of organic materials, such as those produced by volcanoes, forest fires, and human-made sources like industrial processing and vehicle exhaust. Consumption via contaminated food, water, and air, makes B[a]P exposure almost inevitable (Chepelev, Moffat et al. 2015). The potential point of departure (POD) for neurotoxicity in rodents, based on a standard behavioural test (Morris Water Maze [MWM]), was as low as 0.025 mg B[a]P/kg-body weight (BW)-day compared to 0.54 mg B[a]P/kg-BW-day for rodent forestomach carcinogenicity, suggesting that neurotoxic endpoints are more sensitive to B[a]P exposure than cancer endpoints (Chepelev, Moffat et al. 2015). Therefore, neurotoxicity is worth to be paid much attention. Because of its lipophilicity, B[a]P and its metabolites can cross the blood-brain barrier (BBB), easily reach the brain tissue and cause impairments of the central nervous system (CNS) (Chepelev, Moffat et al. 2015, Zhang, Tian et al. 2016). The "brain growth spurt" (BGS) is the term used to refer to critical period of brain development. This period is characterized by axonal and dendritic outgrowth, establishment of neuronal connections, synaptogenesis, and proliferation of glia cells with accompanying myelinization (Eriksson 1997, Gale, Walton et al. 2003, Viberg, Mundy et al. 2008). Early postnatal exposure to B[a]P, especially in the brain rapid development period (postnatal days [PNDs] 5-11), can cause brain damage which might persist into adolescence and adulthood (Chen, Tang et al. 2012). The hippocampus is part of the brain associated with formation of learning and memory ability and emotional responses. Learning and memory disorders have been recognized as an important manifestation of B[a]P-induced hippocampal injury. Recent studies reported that B[a]P could induce learning and memory impairments, which were mainly related to N-methyl-D-aspartate receptor (NMDAR), cytochrome P450, and DNA damage (Verma, Pink et al. 2012, Einaudi, Courbiere et al. 2014, Chepelev, Moffat et al. 2015, Krais, Speksnijder et al. 2016). B[a]P-induced neurotoxicity is a complex process involving multiple genes, multiple signaling pathways, and multiple regulatory mechanisms. However, current studies of the mechanisms underlying B[a]P neurotoxicity are limited. The mechanisms of B[a]P-induced impairments might include alterations of DNA methylation, ncRNAs, mRNAs, proteins and metabolites, yet very few studies on aberrant epigenetic regulation patterns in DNA methylation and ncRNAs at a global scale have been reported.

The transcriptome refers to the total number of genes expressed by a specific cell in a functional state, representing the identity and expression level of each gene (Velculescu, Zhang et al. 1997). Epigenetics refers to the changes in the gene expression in the absence of DNA sequence differences and this is mainly regulated via DNA methylation, histone modification, and ncRNA interference (Qureshi and Mehler 2011). Methylation is one of the most important mechanisms of epigenetic

regulation found in recent years (Chen and Riggs 2011), which plays an important role in inhibiting gene expression (Fuks, Burgers et al. 2000). Studies have shown that DNA methylation is related to neuronal development and synapse formation (Liu, van Groen et al. 2009, Yu, Baek et al. 2011, Morris and Monteggia 2014), but no studies to date have explored the effect on DNA methylation after B[a]P exposure. The ncRNA is a type of RNA that lacks a protein-coding sequence but has enzymatic, structural, or regulatory function. The ncRNAs can be divided into two major classes: the small ncRNAs which include microRNAs (miRNAs) and other noncoding transcripts of less than 200 nucleotides, and long noncoding RNAs (lncRNAs) which contain from 200 to more than 100000 nucleotides (Costa 2010). The miRNAs are a group of 22nt length endogenous ncRNAs that act as post-transcriptional regulators of protein-coding genes by binding mainly to their 3' untranslated region (UTRs), leading to mRNA degradation or inhibited translation (Cullen 2004, Wang, Niu et al. 2011). The mode of target mRNAs regulation depends on the sequence complementarity between the miRNAs and the mRNAs (Wang, Niu et al. 2011). Hundreds of miRNAs have been identified in the mammalian CNS, some of which have been shown to play a key role in the development of neurodegenerative diseases (Madathil, Nelson et al. 2011, Bhalala, Srikanth et al. 2013, Drusco, Bottoni et al. 2015). lncRNAs, one of the most abundant classes of ncRNAs, are encoded within the genome and are highly expressed in the brain (Ponjavic, Oliver et al. 2009). In contrast to miRNAs, lncRNAs have roles in diverse cellular processes and biological function. For example, lncRNAs can serve as mediators of mRNA decay, as structural scaffolds for nuclear substructures, as host genes for miRNAs, and as regulators of chromatin remodeling (Wilusz, Sunwoo et al. 2009). Although studies have shown that lncRNAs play an important role in neurological development, stress responses and plasticity, little is known about the involvement of lncRNAs in the physiology and pathology of the nervous system (Qureshi, Mattick et al. 2010). In addition, the roles of miRNAs and lncRNAs in B[a]P-induced learning and memory dysfunction are poorly understood.

Metabolomics aims to characterize and quantify all the small molecules in a complex multicellular system to understand systematic changes through time (Nicholson and Lindon 2008). Metabolomics has previously been applied to elucidate metabolite changes in the brain of animals which were exposed to neurotoxins, and for the identification of early differential markers for neurological diseases (Xu, Sun et al. 2015, Zaitsu, Hayashi et al. 2015, Wen, Zhang et al. 2016). In recent years, some metabolomics researchers have discovered adverse effects of B[a]P by studying Manila clam, Pinctada martensii, Hepalclc7 cells, human cells and serum (Zhang, Liu et al. 2011, Pink, Verma et al. 2014, Kalkhof, Dautel et al. 2015, Wang, Zhang et al. 2015, Ba, Huang et al. 2016, Chen, Song et al. 2016), but only a few studies have analyzed the metabolome to study the neurotoxicity of B[a]P and to identify potential biomarkers for its effects on the brain.

In this study, DNA methylation, ncRNAs, mRNAs and metabolite changes are investigated in the rat hippocampus following B[a]P treatment. The results of this study will be helpful to gain comprehensive insights into the mechanisms underlying B[a]P-induced neurotoxicity. This is the first report to describe transcriptome mRNAs, methylated ncRNAs, and metabolite-wide changes in B[a]P-treated rats.

#### Materials and methods

#### Materials

Benzo[a]pyrene (> 96% pure), the derivatization reagent methyl chloroformate (MCF), the internal standard D4-L-alanine, and pyridine were purchased from Sigma-Aldrich (St. Louis, USA). Methanol, chloroform, sodium hydroxide, and sodium bicarbonate were obtained from Merck (Darmastadt, Germany). Anhydrous sodium sulfate was purchased from Fluka (Steinheim, Germany). High-throughput sequencing reagent HiSeq Rapid SBS Kit V2 (200 cycle), HiSeq Rapid PE Cluster Kit V2, HiSeq 3000/4000 SBS Kit (300 Cycles), HiSeq 3000/4000 PE Cluster Kit, HiSeq Rapid SBS Kit V2 (50 cycle) HiSeq, Rapid SR Cluster Kit V2, and EpicentreRibo-Zero rRNA Removal Kit were purchased from Illumina (San Diego, USA). High Sensitivity D1000 Reagent and High Sensitivity D1000 ScreenTape were obtained from Agilent

Technologies (Paro Ato, USA). NEBNext<sup>®</sup> Multiplex Small RNA Library Prep Set for Illumina<sup>®</sup>, NEBNext<sup>®</sup> Ultra<sup>™</sup> RNA Library Prep Kit for Illumina, NEBNext<sup>®</sup> Ultra<sup>™</sup> DNA Library Prep Kit for Illumina and MspI were purchased from NEB (Texas, USA). Qubit<sup>®</sup> dsDNA HS Assay Kit and TRIzol reagent were purchased from Life Technologies (Carlsbad, USA). RNA ScreenTape, RNA Reagent, D1000 ScreenTape, and D1000 Reagent were obtained from Agilent Technologies (El Paso, USA). QIA quick PCR Purification kit and QIAquickMinElute Gel Extraction Kit were obtained from QIAGEN (Nordrhein-Westfalen, Germany). EZ DNA Methylation-God kit was purchased from Zymo Research (Orange, USA). All the reagents in this research were of analytical grade.

#### **Animals and Treatment**

A total of 40 four-day-old neonatal male Sprague-Dawley rats (SD rats) were obtained from Chongqing Medical University Laboratory Animal Center, China [certificate: SCXK (YU) 2007-0001]. The biological age and treatment period of the rats was selected according to the biological age of the mammalian brain development period (Spear 2007, McCallister, Li et al. 2015). Rats were housed in an environment controlled room (temperature  $23 \pm 1 \Box$ ; 12 h light/12 h dark cycle) with free access to food and water. After one day of acclimatization to the surroundings, the rats were randomly divided into two groups: control group (n = 20) and B[a]P-treated group (n = 20).

Other than the B[a]P treatment, both groups were treated indiscriminatingly. B[a]P was dissolved in corn oil due to its lipophilicity and insolubility in water, and the daily equivalent volume of corn oil was given to the control group. The B[a]P-treated group received oral gavage of B[a]P solution (1.0 mL/kg body weight) at a concentration of 2.0 mg B[a]P/kg, once daily, for seven consecutive weeks. This dose was selected based on the level of human environmental B[a]P exposure and is a dose that has been used in a previously published study (Chengzhi, Yan et al. 2011). Oral gavage was chosen for administration because of its resemblance with the most common form of human exposure (McCallister, Li et al. 2015). The BGS that occurs

in neonatal rats reaches its peak around PND 10, and throughout the first 3-4 weeks of life (Eriksson 1997, Gale, Walton et al. 2003, Viberg, Mundy et al. 2008). PND 5-10 is considered the rapid brain development period (Chen, Tang et al. 2012), therefore, our treatment began at PND 5 and lasted seven weeks, to simulate the persistent exposure to B[a]P of infants and children before adulthood. Pups of both groups were weaned on PND 21 and redistributed to their corresponding cages.

The study was conducted in compliance with standard animal use practices and approved by the Ethics Committee of the Chongqing Medical University.

#### Morris Water Maze (MWM) test

After completing the B[a]P treatment for seven weeks (49 days), spatial learning and memory abilities of both groups (n = 8 in each group) were assessed by the MWM, as previously described (Chengzhi, Yan et al. 2011). A circular pool (diameter 130 cm) was filled with water which was made opaque with milk powder ( $22^{\circ}C \pm 1^{\circ}C$ ) and rats were trained to pinpoint a submerged platform. Rats were first trained to pinpoint a clearly marked platform (visible platform, Day 1), which was subsequently changed to a hidden platform beneath the surface of the opaque water (hidden platform, Days 2-4). There were four daily trials for both visible and hidden sessions. The rats were placed into the water facing the edge of the pool in one of four randomized locations. The beginning location was changed for each trial. A trial ended when the rats located the platform. Rats that failed to find the platform within 60 s were led to the platform by placing a finger in front of their swim path. The rats were taken out of the pool after they physically stayed on the platform for a minimum of 3 s.

During visible platform training, the platform was moved to a different quadrant of the pool for each session. For the hidden platform training, the platform location was kept constant. Training during the hidden platform sessions required the rats to learn to find the location of the hidden platform based on extramaze cues. The platform was removed from the pool at Day 5. The rats were then randomly released at any one of the edges and allowed to swim for 120 s in order to test memory retention. The number of times that the rats crossed the target quadrant which the platform had previously been placed and the amount of time that the rats spent in the target quadrant were recorded. The swimming patterns of the rats were recorded by a video tracking software.

#### **Sample Preparation for Electron Microscopy**

Rats from both groups (n = 3 from each group) were randomly chosen and used for transmission electron microscopy, as previously described (Chengzhi, Yan et al. 2011). The rats were perfused with phosphate-buffered saline, followed by a combined fixative solution of phosphate-buffered 2.5% glutaraldehvde and 4% paraformaldehyde, after which a fixed hippocampus was acquired. Subsequently, the hippocampus was sliced into sections of approximately 1 mm<sup>2</sup>, fixed in 2.5% glutaraldehyde for 12 h, and then fixed in 1% osmium tetroxide for 2 h. The fixed specimen was dehydrated in a series of increasing concentrations of alcohol solutions, immersed in propylene oxide, and embedded in 618. Each section was placed on a glass slide and was stained with uranyl acetate and lead citrate, ready for observation by transmission electron microscope.

Synapses of each group were used for the average thickness of postsynaptic density (PSD) analysis via ImageJ software, as previously described (Dosemeci, Tao-Cheng et al. 2001). To calculate the average PSD thickness for each synapse, the outline of the PSD was traced and divided by length of the PSD.

#### High-throughput sequencing and data analysis

Hippocampus tissues from both groups (n = 3 from each group) were randomly selected for high-throughput sequencing, as previously described (Lu, Qin et al. 2016). Hippocampus tissues were disrupted and homogeneously mixed to make two pools representing the two groups (Zhang, Zhao et al. 2014). For each pooled sample, approximately 25 mg and 40 mg of tissue was used for preparation of desoxyribouncleic acid (DNA) and ribouncleic acid (RNA), respectively. The quantity and quality of total DNA and RNA were measured by ND-1000 Nanodrop (Thermo Fisher, USA) and Agilent 2200 TapeStation (Agilent, USA). Subsequently, the reduced representation bisulfite sequencing (RRBS) libraries, RNA-seq libraries, and miRNA-Seq libraries were constructed in this study. The quality of the libraries was assessed on the Agilent 2200 TapeStation (Agilent, USA). After cluster generation, the libraries were sequenced on Hiseq 2500 platform (Illumina, USA). High-throughput sequencing was performed by Ruibo Biotechnology Ltd. Co, Guangzhou, China.

Annotation files of mRNAs and lncRNAs were downloaded from GENCODE rn6, miRNAs annotation data was retrieved from miRBase21, and DNA annotation data was retrieved from GENCODE rn5.

#### Whole genome methylation level and ncRNAs methylation profiles

The DNA, lncRNA, and primary miRNA annotation files of rats were downloaded, as mentioned above. The Bismark software was used to compare the sequence acquired by RRBS with the reference genomes of these regions, to obtain methylation sites and methylation regions. Each C-base methylation site was annotated by ANNOVAR software for genomic elements. Finally, two R language packages, MetylKit and eDMR, were combined for inter-organizational differential methylation regions (DMR) analysis.

The lncRNA and miRNA promoters were defined as 10 kb upstream from the transcriptional start site (TSS) of each lncRNA or pri-miRNA (Xiao, Cao et al. 2017). For each sample, lncRNA or miRNA methylation profiles were contructed using the methylation levels of the promoter region of the lncRNAs or miRNAs.

#### Genes targeted by the differentially expressed methylated ncRNAs

Three online databases were searched to find the predicted miRNAs target genes: miRanda (http://www.microrna.org/microrna/getGeneForm.do), miRDB (http://www.mirdb.org/miRDB/index.html), and TargetScan 7.1 (http://www.targetscan.org/vert\_71/). The miRNAs target genes are the result co-predicted by the three databases. The role of lncRNAs and target genes can be grouped into two major classes: Cis and Trans. The mechanism of Cis is to search all the encoded genes in the 10K up and down range of lncRNAs, and to overlap the genes that have significant functional expression with the lncRNA. These genes, which are near and expressed on the genome, are likely to be regulated by the lncRNA. The Trans effect is to first extract the sequence of lncRNAs and mRNAs, Blast (e < 1e-5) screening, and then use Rnaplex (g < -20) software for filtration, to identify possible target genes for lncRNA.

### PANTHER pathway and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis of mRNA and target genes for methylated miRNAs and lncRNAs

KOBAS (Database for Annotation, Visualization and Integrated Discovery, http://kobas.cbi.pku.edu.cn/) was used to analyze differentially expressed genes (DEG) and differentially expressed methylated ncRNAs. PANTHER pathway and KEGG pathway analysis was performed to determine the involvement of DEG and differentially expressed methylated ncRNAs in various biological pathways. PANTHER pathways and KEGG pathways with P < 0.05 were selected.

# Sample preparation for gas chromatography/mass spectrometry (GC-MS) analysis

The hippocampus (mass = 50 mg ± 0.5 mg) of two groups (n = 6 from each group) were homogenized with 1.5 mL of prechilled methanol/water (1: 1, V: V) and 20  $\mu L$  internal standard in a 2 mL centrifuge tube. The samples were centrifuged (16000 g, 10 min, 4°C) and the supernatant from each sample was transferred into a new 2 mL Eppendorf tube. Solid precipitates were homogenized with 1.6 mL of prechilled dichloromethane/methanol (3: 1, V: V), and centrifuged (16000 g, 10 min, 4°C) to obtain the supernatant. The two supernatants were collected and dried in a vacuum concentrator. To each dried sample 200  $\mu L$  sodium hydroxide was added and the sample was resuspended. The resuspension was then transferred to a silanised glass tube where 167  $\mu$ L methanol and 34  $\mu$ L pyridine was added, followed by 20  $\mu$ L MCF, The sample was mixed for 30 s, followed by another addition of 20  $\mu L$  MCF,

which was again mixed for 30 s. To separate the MCF derivatives 400  $\mu$ L chloroform and 400  $\mu$ L of 50 mM sodium bicarbonate was added, and mixed for 10 s after each addition. After being centrifuged (1500 rpm, 10 min), the aqueous layer was discarded and the samples were dehydrated with anhydrous sodium sulphate before being transferred to GC vials ready for analysis (Dai, Xia et al. 2016, Li, Wang et al. 2017).

#### **GC-MS** Analysis

GC-MS analysis was performed on an Agilent 7890A gas chromatography system coupled with an Agilent 5975C mass spectrometer (Agilent, USA). The system utilized a fused silica Zebron ZB-1701 capillary column (30 m  $\times$  250  $\mu$ m  $\times$  0.15  $\mu$ m) coated with 86% dimethylpolysiloxane and 14% cyanopropylphenyl (Phenomenex, Torrance, CA, USA). A 1.0 *u*L aliquot of sample was injected under split-less mode with the inlet temperature being set to 290°C. The carrier gas was instrument grade helium (99.99%, BOC), and the front inlet purge flow was set to 25 mL/min a minute after injection. The initial temperature was kept at  $45^{\circ}$  for 2 min, raised to  $180^{\circ}$  c at a rate of 9°C/min, held for 5 min; increased to 220°C at a rate of 40°C, held for 5 min; increased to 240°C at a rate of 40°C, held for 11.5 min; finally increased to 280°C at a rate of 40°C, held for 2 min. The transfer line, source, and quadrupole temperature were 250°C, 230°C, and 150°C, respectively. The detector was run in positive ionization mode with electron energy of 70 eV and was turned on 5.5 min into the run. The mass spectrometry data was acquired in scan mode with the range from 38 m/z to 550 m/z with scan time 0.1 s, scan speed 1 562 u/s, and 100 ion counts as the detection threshold. A solvent blank was run after every six samples to check for instrument carryover (Li, Wang et al. 2017).

#### **Metabolomics Date Analysis**

Raw data obtained via GC-MS was converted into a common data format (CDF) by the Automated Mass Deconvolution and Identification System (AMDIS-Http://www.amdis.net/). Metabolites in the hippocampus were identified using an in-house mass spectral library and the NIST 0.5 library. The results included sample information, retention time, and peak intensities. Compounds with a match factor > 70% to one of the libraries were included in the analysis. Relative quantification of peak intensities was used to determine levels of the metabolites. The data was normalized using the internal standard and brain tissue mass. Partial least squares discriminate analysis (PLS-DA) was performed using SIMCA-P 11.5 (Umetrics, Umea, Sweden). Potential biomarkers were selected based on the variable important in the projection (VIP) values (VIP > 1) and statistical significance (P < 0.05) following a Student's *t*-test, which was conducted in SPSS 22.0 (SPSS, Inc, Chicago, IL) (Li, Wang et al. 2017).

#### Metabolic pathway analysis and visualization

Enrichment analysis and pathway topology analysis were performed in metaboanalyst (http://www.metaboanalyst.ca/) to identify specific metabolic pathways that were associated with B[a]P toxicity. Based on the pathway information from KEGG (http://www.kegg.jp/kegg/pathway.html), metabolic and enzyme gene networks were generated by Metscape (a plug-in of Cytoscape).

#### Statistical analysis

Data is presented as mean  $\pm$  standard deviation (*SD*). Statistical analysis was performed using SPSS 22.0 (SPSS, Inc, Chicago, IL). All data from the MWM was analyzed using an unpaired Student's *t*-test, with the exception of a one-way ANOVA followed by an SNK test, which was used for the comparisons among the four training days. P < 0.05 was considered to be statistically significant. Enrichment analysis was performed using a Fisher's exact test. Genes with P < 0.05 were considered DEG, and PANTHER pathways and KEGG pathways with P < 0.05 were considered enriched.

#### Results

#### **Body weight (BW)**

The weights of rats are showed (Table 1). The B[a]P-treated group weighed

significantly less than the control group (P < 0.05).

#### MWM

Data acquired from the place navigation task for control rats and B[a]P-treated rats, showed no significant difference of the swimming speed on different days (Fig. 1A). B[a]P-treated rats showed a significant increase in the escape latency compared to control rats on each trial day (Fig. 1B) (P < 0.05). Compared with the control rats, the number of platform crossings (Fig. 1C, E) and the time in the target area (Fig. 1D, E) was significantly decreased in the B[a]P-treated rats (P < 0.05). Results of the MWM showed that B[a]P could cause spatial learning disabilities.

#### Ultrastructural feature in the hippocampus

The transmission electron microscope was used to capture any ultrastructural alterations of the hippocampus after the seven weeks of B[a]P exposure. Swelled mitochondria and stratified and collapsed myelin sheaths were found in B[a]P-treated rats compared with the control group. In addition, observably thickened PSD were also found in B[a]P-treated rats (P = 0.039) (Fig. 2).

Expression profiles of miRNAs, mRNAs and lncRNAs in hippocampus of SD rats In total, 765 miRNAs, 50,615 mRNAs, and 16,113 lncRNAs were detected and the whole expression profile was presented (Fig. 3A, B, C). Compared with the control group, 45 miRNAs, 1566 mRNAs, and 819 lncRNAs were differentially expressed (log2 (Fold change) > 1.0, P < 0.05) in the B[a]P-treated group, of which 14 miRNAs, 586 mRNAs, and 508 lncRNAs were up-regulated, and 31 miRNAs, 980 mRNAs, and 311 lncRNAs were down-regulated. In addition, 925 DEGs were obtained based on the differentially expressed mRNAs, and B[a]P-treated rats showed an up-regulated expression of CaMKII $\gamma$  (Fold change = 2.43).

#### The whole genome methylation level in the hippocampus of SD rats

There were 1,974,648 C base methylation sites in the control group, and 2,081,484 in

the B[a]P-treated group. Whereas, the B[a]P-treated group had less methylation sites than the control group at the CpG island (CGI) and promoter regions (Fig. 4A). Furthermore, it was found via ANNOVAR software that intergenic was the most prone to methylation, followed by intronic, exonic, upstream, UTR5, downstream, UTR3, ncRNA, and splicing (Fig. 4B). A total of 137 DMRs (P < 0.05) were obtained using R language analysis. The distribution of DMR and differential methylation sites (DMC) on the chromosome are shown in Figure 4C. These DMRs consisted of 166 differential methylation genes (DMG), with seven miRNAs and 86 lncRNAs promoter regions being methylated.

#### Genes targeted by differentially expressed methylated ncRNAs

In comparison to the control group, six miRNAs and two lncRNAs were differentially expressed (log2 (Fold change) > 1.0, P < 0.05) among these promoter methylated miRNAs and lncRNAs in the B[a]P-treated group, including mir-293, mir-483, mir-298, mir-3553, mir-191, mir-133b, LOC100910802, and LOC100911370. 320 target genes were acquired from the miRNAs target gene prediction. Only one target gene was obtained from the lncRNAs target gene prediction.

# Pathway analysis of DEG and differentially expressed methylated ncRNAs predicted target genes

KEGG and PANTHER pathway annotation was applied to DEG and differentially expressed methylated ncRNAs predicted target genes. 54 KEGG pathways and four PANTHER pathways were significantly enriched (P < 0.05) in the DEG analysis, most of which were linked to learning and memory, including the "Alzheimer disease-presenilin pathway" (P00004), and "Tyrosine metabolism" pathways (rno00350). KEGG and PANTHER pathways that related to learning and memory were listed in Table 2. Similarly, 26 KEGG pathways and 17 PANTHER pathways were significantly enriched (P < 0.05) in the analysis of differentially expressed methylated ncRNAs predicted target genes. KEGG and PANTHER pathways that related to learning and memory were listed in Table 3.

#### **Analysis of Brain Metabolomics**

The B[a]P-treated group could be distinguished from the control group in the PLS-DA score plot, which suggested that treatment with B[a]P induces distinct changes in the brain metabolites (Fig. 5).

GC-MS metabolomic profiling of the hippocampus found a total of 120 compounds in both groups, which were used in subsequent multivariate analysis. Results of the PLS-DA showed an obvious distinction between the two groups (R2X = 0.743, R2Y = 0.993, Q2 = 0.596). Based on PLS-DA (VIP > 1) and *t*-test (P < 0.05), 21 significant metabolites were identified (Table 4) and were displayed in a heat map (Fig. 6) to show the difference in brain metabolite levels between the two groups. Pathway analysis was performed using Metaboanalyst (http://www.metaboanalyst.ca/), which suggested that B[a]P significantly impacted a number of metabolic pathways (Table 5, Fig. 7). Enzyme gene compounds reaction network diagram of the significant metabolites was constructed using Metscape. The genes involved in the analysis of enzymes gene compounds reaction of significant metabolites were compared with the genes derived from high-throughput sequencing. The genes (Fold change > 1, P <0.05) that were associated with enzymes gene compounds reaction of significant metabolites are shown (Fig. 8, Table 6).

#### Discussion

B[a]P is potential neurotoxin, and previous studies have observed its neurotoxicity in hippocampus slices, neurosecretory PC12 cells, and primary cortical and striatal neurons (McCallister, Maguire et al. 2008, Slotkin and Seidler 2009, Cheng, Xia et al. 2013). Furthermore, learning and memory deficits have also been observed in animal studies after B[a]P exposure (Qiu, Cheng et al. 2011, Xia, Cheng et al. 2011, Cheng, Xia et al. 2013). In this study, high-throughput sequencing was used to assess hippocampal DNA methylation, ncRNA expression, and gene expression. The DNA methylation level, ncRNAs profile, and gene expression profile were significantly

altered in SD rats following B[a]P exposure. By a series of bioinformatics analysis, DEG and methylated ncRNAs target genes were shown to be significantly correlated with learning and memory deficits. These RNAs could affect not only the expression of learning and memory-related proteins that directly influence learning and memory, but also the expression of enzymes which participate in the metabolic pathways that indirectly influence study memory. Learning and memory deficits caused by B[a]P neurotoxicity involved a complex biological process that involved changes in transcriptomic, epigenetic, and metabolomic profiles of the hippocampus. And coding RNAs and ncRNAs work together, which led to pathway disorders in neurotoxic induced by B[a]P.

The hippocampus is a key brain structure which plays a pivotal role in learning and memory (Maciel, Biasibetti et al. 2014, He, Ji et al. 2016). Fetuses and children are more susceptible than adults to the adverse effects of environment pollutants. Studies have observed neurotoxic effects and behavioral changes when the developing brain has been exposed to repeated oral doses of toxicants (Tchekalarova, Kubova et al. 2005, Karpova, Lindholm et al. 2009, Gassowska, Baranowska-Bosiacka et al. 2016). Nerve injury was found to be irreversible, therefore, early postnatal exposure to B[a]P could cause neurodevelopmental abnormalities and influence adolescenct and adulthood neurobehavior (Bouayed, Desor et al. 2009, Chen, Tang et al. 2012). In this study, after the rats were administered B[a]P orally at a dose of 2.0mg/kg BW, they experienced significant weight loss which was consistent with previous studies (Chengzhi, Yan et al. 2011). MWM is a model to detect long-term memory in animal space learning (Chen, Zhao et al. 1999). MWM performance was significantly different between the B[a]P-treated group and control group, suggesting spatial learning and memory deficits in the B[a]P-treated group. Results of the present study were consistent with previous researches that B[a]P had an adverse effect in neurobehavioral function. In addition, electron micrographs also showed swelled mitochondria, stratified and collapsed myelin sheaths, and thickened PSD in the hippocampal neurons following B[a]P exposure. Mitochondrial swelling is an early manifestation of mitochondrial damage due to inflammation, and the disappearance of mitochondrial cristae with the development of inflammation are characteristics of apoptosis (Zhang, Xu et al. 2013). It has previously been reported that ultrastructural changes such as mitochondrial swelling occurred in the hippocampus of epileptic macaque, and exposure to organic dioxins could lead to inflammatory reactions such as mitochondrial swelling, and degenerative lesions such as demyelination in the hippocampus. (Chen, Liu et al. 2013, Rosinczuk, Dymarek et al. 2015). Additionally, a previous study has linked B[a]P exposure to oxidative stress in the hippocampus (Yang, Chen et al. 2017). Excessive oxygen reactive species (ROS) in the brain has been found to cause neuronal oxidative stress, and to induce and enhance neurobehavioral impairment, mainly through neuroinflammation and neuronal signal alteration (Popa-Wagner, Mitran et al. 2013). Glutathione (GSH) is an important component in the intracellular antioxidant system and plays an essential role in cell defense against ROS, because it can balance the production of ROS that is generated as part of normal cellular metabolism in the brain (Li, Wang et al. 2017). In this study, abnormal glutathione metabolism was found in the hippocampus, indicating dysregulation of the antioxidant system. In analysis of both differentially expressed methylated ncRNA predicted target genes pathways and abnormal metabolite pathways, "Glutathione metabolism" (Table 3, 5) was found simultaneously. Hippocampal ultrastructural changes induced by B[a]P exposure may be attributed to differentially expressed methylated ncRNAs that abnormally regulate the expression of anti-oxidative stress pathway-related genes, eventually resulting in oxidative stress-induced hippocampal injury.

The PSD consists of many different specialized proteins (eg, NMDAR and Ca<sup>2+</sup>/calmodulin kinase II [CaMKII]) apposed to the postsynaptic membrane for scaffolding and signaling at the synapse (Dosemeci, Tao-Cheng et al. 2001, Sheng and Hoogenraad 2007). A preliminary mode of action (MOA) that aryl hydrocarbon receptor (AHR)-dependent modulation of the transcription of NMDAR subunits and NMDAR-mediated loss of neuronal activity and decreased Long-term potentiation (LTP) explained B[a]P-induced neurotoxicity in rodents (Chepelev, Moffat et al. 2015). LTP of the hippocampus is a molecular mechanism for information storage and

memory, and its induction must involve the NMDAR and CaMKII (Shonesy, Jalan-Sakrikar et al. 2014). The NMDAR subunits NR1, NR2A, and NR2B in PSD are binding sites for CaMKII, and thickening of PSDs can be accounted for by an accumulation of CaMKII on their cytoplasmic faces (Shen and Meyer 1999, Dosemeci, Tao-Cheng et al. 2001). Previous research has pointed out that B[a]P exposure can downregulate NMDAR, which is critical for synaptic plasticity (Cheng, Xia et al. 2013). In this study, B[a]P-treated rats showed the up-regulated expression of CaMKII $\gamma$ . Four genes encode isoforms of CaMKII ( $\alpha$ ,  $\beta$ ,  $\gamma$ ,  $\delta$ ), with CaMKII $\alpha$ , CaMKII $\beta$ , and CaMKII $\gamma$  being related to cognitive impairment (Coyle 2009, Bachstetter, Webster et al. 2014). It was speculated that B[a]P exposure influenced the normal binding of CaMKII $\gamma$  and NMDAR, resulting in deposition of CaMKII $\gamma$  on the postsynaptic membrane, leading to PSD thickening, and ultimately affecting the formation of LTP and learning and memory. Therefore, further studies are warranted to confirm the relationship between CaMKII $\gamma$  and cognitive impairment induced by B[a]P exposure.

Obvious changes in pathological lesions found in the study were consistent with changes in learning and memory function, Moreover, ultrastructure changes of to the hippocampal neurons were the structural basis of hippocampal dysfunction.

In recent years, the study of the molecular mechanisms of learning and memory has made great progress, demonstrating that learning and memory are closely related to various physiological processes, such as the release of transmitters, the calcium/kinase phosphatase system, synaptic plasticity, gene transcription and translation, DNA methylation, and so forth (Bradshaw, Kubota et al. 2003, Xia, Cheng et al. 2011, Cheng, Xia et al. 2013, Moore, Le et al. 2013, Pooters, Van der Jeugd et al. 2015). In this study, it was found that there were significantly more DNA methylation sites in the B[a]P-treated rat hippocampus when compared with the control group. B[a]P exposure induced abnormal methylation patterns that might influence the differentiation and maturation of the mammalian CNS, and regulate long-term synaptic plasticity by changing gene expression (Moore, Le et al. 2013, Senut, Sen et al. 2014). The present study also found that compared with the control group,

expression of mRNAs and miRNAs in the B[a]P-treated group was generally down-regulated, while expression of lncRNAs was up-regulated. Both miRNAs and lncRNAs affected the synaptic plasticity by regulating the translation of proteins or proteases that were related to learning and memory (Siegel, Obernosterer et al. 2009, Gao, Wang et al. 2010, Earls, Westmoreland et al. 2014).

According to the gene biological functional analysis, B[a]P-induced impairments in learning and memory were associated with signal transduction and metabolism. The key pathways included eleven "signaling pathways", five "metabolic pathways" and other learning and memory-related pathways, such as "Cell adhesion molecules (CAMs)", "Neuroactive ligand-receptor interaction", "Cytokine-cytokine receptor interaction", and "ECM-receptor interaction" (Table 2). CAMs played an important role in axonal development, synaptic formation, and learning cognition (Jurgenson, Aonurm-Helm et al. 2010, Seyedabadi, Fakhfouri et al. 2014, Thalhammer and Cingolani 2014). Neuroactive ligand-receptor interaction was necessary in signal transduction (Cheng, Xia et al. 2013), as well as cytokine-cytokine receptor interaction and ECM-receptor interaction. Therefore, the neurotoxic lesions caused by B[a]P exposure are partly attributed to disruptions in signal transduction. It was also found that aberrant methylation of ncRNAs could perturb transduction and metabolism. Eighteen "signaling pathways", two "metabolic pathways", "Cell adhesion molecules (CAMs)", "Apoptosis-multiple species", and "Long-term potentiation" were obtained in differentially expressed methylated ncRNAs predicted target genes biological functional analysis (Table 3), indicating that B[a]P mediated the epigenetic expression pattern (Fig. 9). Consistent with previous studies, B[a]P exposure impaired LTP in the hippocampus (Qiu, Cheng et al. 2011) and induced neuronal apoptosis which was related to learning and memory dysfunction in rats (Li, Peng et al. 2012). As a consequence of epigenetic modification, protein expression changes occurred due to transcriptome alterations, which might serve as one of the probable factors leading to learning and memory dysfunction (Mattick and Makunin 2006, Amaral and Mattick 2008, Chengzhi, Yan et al. 2011).

In brief, epigenetic changes appear to be a reasonable mechanism linking B[a]P exposure to learning and memory impairment.

Metabolomic information, the ultimate result of biological function, can directly indicate abnormal physiological status as it is "downstream" from those initial changes that occur at the level of genome, transcriptome, and proteome (Huang, Luo et al. 2016). After bioinformatic analysis, metabolites in the hippocampus were investigated to further understand how B[a]P exposure could lead to learning and memory deficits. Pathway analysis of the 21 significant metabolites revealed the two pathways of "Glycolysis or Gluconeogenesis" and "Tyrosine metabolism" among the 15 metabolic pathways, to be consistent with the DEG pathway and methylated ncRNAs target gene. It was shown that these two pathways were mainly affected by transcriptome changes.

Glucose homeostasis is a crucial physiological function and any abnormalities in glycolysis or gluconeogenesis can directly affect the energy supply of cells and have dramatic consequences for brain cells (Ram'ırez, Vargas et al. 2012). Hippocampus pyramidal neurons have the highest energy requirements in the brain, when their energy requirements are insufficient, the risk of cellular dysfunction and injury, and neurodegenerative disease can increase (LaMANNA and ttARIK 1985, Kapogiannis and Mattson 2011, Bauernfeind and Babbitt 2014, Liu, Gu et al. 2015). Neurotransmitters are important for brain development and the function of the nervous system and cognition (Seyedabadi, Fakhfouri et al. 2014). Tyrosine participates in the synthesis of biogenic amine neurotransmitters such as dopamine (DA), norepinephrine (NE) and epinephrine (A), which are essential for learning, memory, and cognition (Welch, Meyer et al. 1975). The dysregulation of tyrosine metabolism induced by B[a]P exposure might lead to a disturbance in neurotransmitter synthesis, and disruption of neurotransmitter homeostasis in the CNS.

KEGG pathway analysis revealed that differentially expressed protease genes were involved in "Tyrosine metabolism", including Tpo, RGD1308564, Aox2, Aox3,

ALDH3A1, and Adh4 (Supplementary table 1). PANTHER pathway analysis indicated that methylated ncRNAs target genes participated in "Glycolysis", including Hk2 and Eno2 (Supplementary table 2). The abnormal expression of protease genes could be associated with the abnormal metabolism of tyrosine and glycolysis in the hippocampus, which ultimately affected the energy supply to the hippocampus and the synthesis of neurotransmitters. Taken together, it has been speculated that epigenetic changes after B[a]P exposure might further affect protease enzyme gene expression, thus altering enzyme expression, resulting in metabolite changes, which was confirmed by the results of enzyme-gene complex reaction analysis (Fig. 8, Table 6). Synthesis of glycine was regulated by Dpep2 and Pipox, while Tpo and Cpa2 were involved in the synthesis of phenylalanine and leucine, respectively. Glycine and phenylalanine are involved in the synthesis of neurotransmitters and leucine could participate in pathways related to brain energy supply, both of which have been proposed to be closely related to brain dysfunction (Tabatabaie, Klomp et al. 2010, Dai, Xia et al. 2016, Zhang, Wang et al. 2016). Therefore, these four significant metabolite-related enzymes, Dpep2, Pipox, Tpo and Cpa2 should be the focus of future research.

In conclusion, the present study found that B[a]P exposure in rats not only influenced the levels of DNA methylation and expression profiles of ncRNAs and mRNAs, but also resulted in changes to the hippocampal metabolites. This work is the first to demonstrate the potential neurotoxicity of B[a]P from an epigenetics, transcriptomics and metabolomics perspective. These results suggest that B[a]P exposure could lead to learning and memory deficits, likely as a result of epigenetic and transcriptomic changes that further affected the expression of CACNA1C, Tpo, etc. The changes in expression ultimately affecting LTP, tyrosine metabolism, and other important metabolic pathways.

#### Availability of supporting data

The data supporting the results of this article are available in the GEO repository [GEO: GSE109973].

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	Week									
Group	0	1	2	3	4	5	6	7		
Control group	8 58+0 93	14 88+3 23	32 80+10 53	49 68+12 88	74 75+14 39	101 40+17 29	121 03+13 67	142 48+21 41		
weights (g)	0.50±0.75	14.00±3.23	52.00-10.05	19:00=12:00	/+./5±1+.57	101.40±17.27	121.05±15.07	172.70±21.71		
B[a]P group	7 68+1 98	16 19+1 93	25 36+2 75 *	41 62+5 46 *	64 65+9 27 *	81 32+15 35 *	96 28+12 23 *	118 13+11 01 *		
weights (g)	7.00±1.90	10.17-1.75	23.30-2.13	11.02-0.40	01.00-9.27	01.52=10.50	y0.20-12.25	110.15-11.01		

 Table 1
 Variation of weights of the rats throughout the study

\*P < 0.05, when compared with the control group.

Term	Database	ID	P-Value	No. of Genes
Cell adhesion molecules (CAMs)	KEGG PATHWAY	rno04514	7.36277E-10	25
Neuroactive ligand-receptor interaction	KEGG PATHWAY	rno04080	1.91779E-06	26
Cytokine-cytokine receptor interaction	KEGG PATHWAY	rno04060	6.91005E-05	19
ECM-receptor interaction	KEGG PATHWAY	rno04512	0.001	9
Tyrosine metabolism	KEGG PATHWAY	rno00350	0.002	6
Tryptophan metabolism	KEGG PATHWAY	rno00380	0.004	6
Retinol metabolism	KEGG PATHWAY	rno00830	0.005	8
PI3K-Akt signaling pathway	KEGG PATHWAY	rno04151	0.007	19
Linoleic acid metabolism	KEGG PATHWAY	rno00591	0.010	5
Jak-STAT signaling pathway	KEGG PATHWAY	rno04630	0.018	10
TNF signaling pathway	KEGG PATHWAY	rno04668	0.020	8
Chemokine signaling pathway	KEGG PATHWAY	rno04062	0.021	11
Hippo signaling pathway	KEGG PATHWAY	rno04390	0.022	10
GnRH signaling pathway	KEGG PATHWAY	rno04912	0.024	7
Toll-like receptor signaling pathway	KEGG PATHWAY	rno04620	0.030	7
NOD-like receptor signaling pathway	KEGG PATHWAY	rno04621	0.034	5
Arachidonic acid metabolism	KEGG PATHWAY	rno00590	0.039	6
Vitamin B6 metabolism	KEGG PATHWAY	rno00750	0.045	2
Alzheimer disease-presenilin pathway	PANTHER	P00004	0.016	7
Cadherin signaling pathway	PANTHER	P00012	0.024	6
TGF-beta signaling pathway	PANTHER	P00052	0.034	5

 Table 2
 KEGG and PANTHER pathways that related to learning and memory enriched for DEG

\* The DEG involved in the pathways are shown in supplementary table 1.

 Table 3 KEGG and PANTHER pathways that related to learning and memory enriched for differentially expressed

 methylated ncRNAs predicted target genes

Term	Database	ID	P-Value	No. of Genes
MAPK signaling pathway	KEGG PATHWAY	rno04010	9.3126E-06	9
Hippo signaling pathway	KEGG PATHWAY	rno04390	0.001	5
Rap1 signaling pathway	KEGG PATHWAY	rno04015	0.005	5
Phospholipase D signaling pathway	KEGG PATHWAY	rno04072	0.007	4
Cell adhesion molecules (CAMs)	KEGG PATHWAY	rno04514	0.013	4
Apoptosis - multiple species	KEGG PATHWAY	rno04215	0.013	2
TNF signaling pathway	KEGG PATHWAY	rno04668	0.020	3
Neurotrophin signaling pathway	KEGG PATHWAY	rno04722	0.028	3
Glutathione metabolism	KEGG PATHWAY	rno00480	0.038	2
Long-term potentiation	KEGG PATHWAY	rno04720	0.045	2
Wnt signaling pathway	PANTHER	P00057	0.000	6
EGF receptor signaling pathway	PANTHER	P00018	0.001	4
Glycolysis	PANTHER	P00024	0.003	2
FGF signaling pathway	PANTHER	P00021	0.008	3
Beta2 adrenergic receptor signaling pathway	PANTHER	P04378	0.009	2
Beta1 adrenergic receptor signaling pathway	PANTHER	P04377	0.010	2
Toll receptor signaling pathway	PANTHER	P00054	0.011	2
Integrin signalling pathway	PANTHER	P00034	0.017	3
Oxytocin receptor mediated signaling pathway	PANTHER	P04391	0.018	2
VEGF signaling pathway	PANTHER	P00056	0.022	2
5HT2 type receptor mediated signaling pathway	PANTHER	P04374	0.026	2
p53 pathway	PANTHER	P00059	0.027	2
Parkinson disease	PANTHER	P00049	0.044	2

\* The differentially expressed methylated ncRNAs predicted target genes involved in the pathways are shown in

supplementary table 2.

Name	P-Value	VIP*	RT (min)*	Match (%)	FC*
N-Acetylglutamic acid	0.0493321	1.20956	19.507	74	1.0231955
Glycine	0.0477313	1.24744	11.665	96	1.0211368
Glyoxylic acid	0.0330821	1.25821	11.414	74	1.074236
Isoleucine	0.0114773	1.35729	14.297	94	1.0359669
Leucine	0.0085528	1.38005	14.242	98	1.0353758
Benzoic acid	0.0324072	1.38424	9.861	72	-1.112152
2-Hydroxyisobutyric acid	0.0399133	1.40399	9.359	74	1.0588779
Hexanoic acid	0.008316	1.407	6.514	82	1.0524409
Phenylalanine	0.0068899	1.41187	20.305	96	1.0252458
Proline	0.0045741	1.4237	15.242	98	1.0212806
Aspartic acid	0.0246484	1.43881	16.578	98	1.0061667
Phosphoenolpyruvic acid	0.0166334	1.48326	14.765	63	-1.17109874
Valine	0.0009839	1.50823	12.992	92	1.0275114
4-Hydroxyphenylpyruvate	0.0257091	1.51697	16.066	70	-1.031166
Dehydroascorbic acid	0.0011967	1.53076	15.784	86	1.0298389
Cysteine	0.0400343	1.62554	20.466	90	-1.056389
Threonine	0.0132601	1.73652	15.857	97	1.0175589
Caprylic acid	0.0111353	1.79551	9.694	88	-1.017844
Histidine	0.0081575	1.38474	27.545	88	-1.03299
Fumaric acid	0.0314558	1.45179	9.419	84	-1.024587
cis-Aconitic acid	0.0472475	1.53995	15.711	90	-1.017213

 Table 4
 Metabolites significantly changed in hippocampus

\*Fold change (FC) between B[a]P-treated rats and control rats. Fold change with a positive value indicates a higher concentration in B[a]P-treated rats, while a negative value means a relatively lower concentration as compared to the control rats.

\*RT retention time.

\*VIP variable importance in the projection obtained from PLS-DA model with a threshold of 1.0.

Name	Total*	Hit*	P value*	-log( <i>P</i> )	Holm P	FDR	Impact*
Ubiquinone and other terpenoid-quinone biosynthesis	3	1	0.042218	3.1649	1	0.27561	1
Valine, leucine and isoleucine biosynthesis	11	3	0.00038144	7.8716	0.030515	0.015448	0.99999
Phenylalanine, tyrosine and tryptophan biosynthesis	4	2	0.001141	6.7759	0.088996	0.023105	0.5
Phenylalanine metabolism	9	1	0.12163	2.1068	1	0.4926	0.40741
Glycine, serine and threonine metabolism	32	4	0.00083954	7.0827	0.066323	0.022667	0.29197
Histidine metabolism	15	2	0.01817	4.008	1	0.14718	0.24194
Alanine, aspartate and glutamate metabolism	24	2	0.044234	3.1183	1	0.27561	0.1962
Glyoxylate and dicarboxylate metabolism	16	2	0.020589	3.883	1	0.15161	0.14815
Cysteine and methionine metabolism	28	1	0.33386	1.097	1	1	0.12829
Glycolysis or Gluconeogenesis	26	1	0.31406	1.1582	1	1	0.10389
Tyrosine metabolism	42	2	0.11853	2.1326	1	0.4926	0.07225
Citrate cycle (TCA cycle)	20	3	0.0024271	6.0211	0.18688	0.038287	0.06496
Arginine and proline metabolism	44	4	0.0028361	5.8653	0.21554	0.038287	0.064
Primary bile acid biosynthesis	46	1	0.48924	0.7149	1	1	0.02976
Glutathione metabolism	26	2	0.05121	2.9718	1	0.29629	0.00955

#### Table 5 Metabolic pathways in the hippocampus which were altered by B[a]P exposure

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\*Total is the total number compounds in the pathway;

\*Hit is the matched number from our uploaded metabolites;

\*P value and FDR calculated from the enrichment analysis;

\*Impact value calculated from the pathway topology analysis.

Gene	P-value	Fold change	Gene	P-value	Fold change	Gene	P-value	Fold change
Eno1	0.010	1.047	Aga	0.009	-1.129	Ass1	0.002	1.267
Adss	0.011	1.060	Got1	1.57E-23	1.135	Sardh	1.46E-08	1.276
Bcat1	1.54E-34	1.079	Cbs	6.24E-11	1.145	Adssl1	0.050	1.345
Sdhb	3.16E-05	1.083	Nans	2.00E-08	1.153	Cpa2	0.007	-1.567
Sdhc	2.73E-07	1.086	Asns	5.60E-11	1.162	Pipox	0.015	1.722
Got2	7.45E-20	1.090	Enol	2.94E-91	1.166	Тро	0.029	-3.775
Fh	0.001	1.092	Gatm	2.80E-15	1.167	Dpep2	0.000	-8.966
Sdha	7.82E-24	1.109	Adsl	4.58E-13	1.177			
Pck2	0.000	1.129	Gad1	1.98E-39	1.195			

Table 6 Differential genes that can regulate the expression of significant metabolites by regulating enzymes

\*Fold change between B[a]P-treated rats and control rats. Fold change with a positive value indicates a higher concentration, while a negative value indicates a relatively lower concentration present in B[a]P-treated rats as compared to the control rats.

#### **Figure Legends**

**Fig. 1** Performance of the Morris Water Maze: (A) Swim speed, data was expressed as Mean  $\pm SD$  (n = 8) of average swim speed made on different days (F = 0.520, P = 0.483); (B) Escape latency, in seconds, to reach the platform during each trial. Data was expressed as Mean  $\pm SD$  (n = 8) of average escape latency made on different days (F = 6.555, P = 0.023); (C) The number of platform crossings, data sows the average frequency of platform crossings made on day 5. The height of each bar represents the Mean  $\pm SD$  (n = 5) (F = 6.216, P = 0.000); (D) Time spent in target area, data shows the average time spent in the target quadrant on day 5. The height of each bar represents the Mean  $\pm SD$  (n = 5) (F = 3.494, P = 0.014); (E) Representative photographs of rats' swimming tracks. \* P < 0.05, compared with the control group.

Fig. 2 Representative electron micrographs of sections of rat hippocampal neurons. (A) Control group; (B) B[a]P-treated group; (C) PSD thickness, data was expressed as Mean  $\pm SD$  (n = 50); (D) Normal myelinated fibers, with plasma membrane rings of regular heights and dense structure; (E) Damaged myelin sheaths, with stratification and collapse; (F) Normal mitochondria; (G) Swelled mitochondria. The arrows show the synaptic linkages. \* P < 0.05, compared with the control group. Magnification  $\times 20,000$ .

Fig. 3 Expression profiles of miRNAs, mRNAs, and lncRNAs. (A) Volcano plots of all detected miRNAs in both the control group and B[a]P-treated group. The vertical dotted line delimits up-regulation and down-regulation. Red plots represent significant mRNAs with fold change > 2.0, P < 0.05; (B) Volcano plots of all detected mRNAs in both control group and B[a]P-treated group; (C) Volcano plots of all detected lncRNAs in both control group and B[a]P-treated group.

**Fig. 4** B[a]P induced DNA methylation. (A) DNA methylation of the CGI and promoter regions. (B) DNA methylation signal genomic element annotation. (C) Distribution of DMRs and DMCs on the chromosome. The inner ring and the outer ring exhibits DMRs and DMCs, respectively.

Fig. 5 Partial least squares discriminate analysis (PLS-DA) score plot representing the metabolomic profiles of the hippocampus in the B[a]P-treated and control groups (R2X = 0.743, R2Y = 0.993, Q2 = 0.596).

**Fig. 6** Heat map of metabolites that were significantly different between the control group and B[a]P-treated group. Red indicates upregulation, green indicates downregulation.

**Fig. 7** All match pathway *P* values from pathway enrichment analysis and pathway impact value from pathway topology analysis by Metaboanalyst. Enriched metabolic pathways were ranked by false discovery rate (FDR) values. Significant pathways were represented by bigger/red dots and Log *P* value. Pathway impact was calculated as the sum of the importance measures of the matched metabolites normalized by the sum of the importance measures of all metabolites in each pathway. B[a]P altered metabolic pathways of the hippocampus were shown in Table 5.

Fig. 8 Enzyme gene compounds reaction network of the significant metabolites. Figures in red represent significant metabolites, those in pink represent intermediate metabolites; green squares represent enzymes, and violet circles represent genes.

**Fig. 9** A model illustrating the possible regulated processes intertwined by DNA methylation, ncRNAs, and coding genes, after exposure to B[a]P. lncRNAs and miRNAs cooperatively mediate the pathway dysregulation which may be implicated in B[a]P-induced learning and memory dysfunction.



Fig. 1 Performance of the Morris Water Maze: (A) Swim speed, data was expressed as Mean  $\pm$  SD (n = 8) of average swim speed made on different days (F = 0.520, P = 0.483); (B) Escape latency, in seconds, to reach the platform during each trial. Data was expressed as Mean  $\pm$  SD (n = 8) of average escape latency made on different days (F = 6.555, P = 0.023); (C) The number of platform crossings, data sows the average frequency of platform crossings made on day 5. The height of each bar represents the Mean  $\pm$  SD (n = 5) (F = 6.216, P = 0.000); (D) Time spent in target area, data shows the average time spent in the target quadrant on day 5. The height of each bar represents the Mean  $\pm$  SD (n = 5) (F = 3.494, P = 0.014); (E) Representative photographs of rats' swimming tracks. \* P < 0.05, compared with the control group.

259x191mm (300 x 300 DPI)



Fig. 2 Representative electron micrographs of sections of rat hippocampal neurons. (A) Control group; (B) B[a]P-treated group; (C) PSD thickness, data was expressed as Mean ± SD (n = 50); (D) Normal myelinated fibers, with plasma membrane rings of regular heights and dense structure; (E) Damaged myelin sheaths, with stratification and collapse; (F) Normal mitochondria; (G) Swelled mitochondria. The arrows show the synaptic linkages. \* P < 0.05, compared with the control group. Magnification ×20,000.</li>

399x205mm (300 x 300 DPI)



Fig. 3 Expression profiles of miRNAs, mRNAs, and IncRNAs. (A) Volcano plots of all detected miRNAs in both the control group and B[a]P-treated group. The vertical dotted line delimits up-regulation and downregulation. Red plots represent significant mRNAs with fold change > 2.0, P < 0.05; (B) Volcano plots of all detected mRNAs in both control group and B[a]P-treated group; (C) Volcano plots of all detected IncRNAs in both control group and B[a]P-treated group.

394x358mm (300 x 300 DPI)



Fig. 4 B[a]P induced DNA methylation. (A) DNA methylation of the CGI and promoter regions. (B) DNA methylation signal genomic element annotation. (C) Distribution of DMRs and DMCs on the chromosome. The inner ring and the outer ring exhibits DMRs and DMCs, respectively.

251x149mm (300 x 300 DPI)



Fig. 5 Partial least squares discriminate analysis (PLS-DA) score plot representing the metabolomic profiles of the hippocampus in the B[a]P-treated and control groups (R2X = 0.743, R2Y = 0.993, Q2 = 0.596).

20x11mm (300 x 300 DPI)



Fig. 6 Heat map of metabolites that were significantly different between the control group and B[a]P-treated group. Red indicates upregulation, green indicates downregulation.

161x228mm (300 x 300 DPI)



Fig. 7 All match pathway P values from pathway enrichment analysis and pathway impact value from pathway topology analysis by Metaboanalyst. Enriched metabolic pathways were ranked by false discovery rate (FDR) values. Significant pathways were represented by bigger/red dots and Log P value. Pathway impact was calculated as the sum of the importance measures of the matched metabolites normalized by the sum of the importance measures of all metabolites in each pathway. B[a]P altered metabolic pathways of the hippocampus were shown in Table 5.

177x177mm (300 x 300 DPI)



Fig. 9 A model illustrating the possible regulated processes intertwined by DNA methylation, ncRNAs, and coding genes, after exposure to B[a]P. IncRNAs and miRNAs cooperatively mediate the pathway dysregulation which may be implicated in B[a]P-induced learning and memory dysfunction.

383x427mm (300 x 300 DPI)