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ORIGINAL ARTICLE

Postnatal Subacute Benzo(a)Pyrene Exposure Caused Neurobehavioral Impairment and Metabolomic Changes of Cerebellum in the Early Adulthood Period of Sprague-Dawley Rats

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Abstract Benzo(a)pyrene (BaP) is a widespread environmental contaminant that has been associated with neurotoxicity in mammals. It has strong toxic effects on the developing central nervous system. Cerebellum is associated with locomotor activity and anxiety behavior, but there is very little research about the toxic effects of BaP in cerebellum. The present study aims to investigate the global influence of BaP subacute exposure on the metabolome of rat cerebellum. Male neonatal rats (postnatal day 5) were divided into two groups: control group and BaP-treated group (2 mg/kg/day for 7 weeks). Open field test and transmission electron microscopy were performed to analyze neurobehavior and ultramicrostructure alteration. Gas chromatography-mass spectrometry (GC-MS) was used to analyze metabolites of the cerebellum in both groups. The results revealed that postnatal exposure to BaP promoted pathological changes in the cerebellum and increased locomotor and anxiety activities in early adulthood. Twenty differential significant metabolites were identified by multivariate statistical analysis. Further metabolic pathway impact analysis and network analysis suggested that the primary metabolic pathways affected included pathway involved in energy metabolism, methionine and cysteine metabolism, and glutathione metabolism. These findings suggest that BaP-induced cerebellum injury may be correlated with metabolic changes and provide an area to target to reduce the negative effects of BaP.

Keywords Benzo(a)pyrene · Neonatal rat · Cerebellum · Metabolomics · Gas chromatography-mass spectrometry

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Introduction

Benzo(a)pyrene (BaP) is a typical polycyclic aromatic hydrocarbon (PAH): uptake occurs mostly by inhalation and ingestion of contaminated food and water and polluted air (Knuckles et al. 2001; Archibong et al. 2002). BaP and its metabolites can cause central nervous system (CNS) impairments (Chepelev et al. 2015). It has also been reported that the fetus in utero and young children is more susceptible to the toxicant and that postnatal BaP exposure may result in neurobehavioral impairment in adulthood (Brown et al. 2007; Chen et al. 2012). In fact, the developing CNS is much more sensitive to injury from toxicants than the adult CNS and can interfere with any of the processes involved in its development (Rodier 1995).

Every development of an organism has a critical period for its normal maturation. In the development of the brain, it is referred to as the "brain growth spurt" (Eriksson 1997). During this period, the glial cells start to develop, axons grow, dendrites branch, and synapses are formed (Gale et al. 2003). Also in this critical period, animals can develop new motor and sensory abilities and spontaneous motor peaks (Viberg et al. 2008). This is the key period that the brain's physiological structure and function mature from the infant period. Studies have shown that early postnatal toxicant exposure has profound effects on adult neural behavior (Tchekalarova et al. 2005; Viberg et al. 2008; Gassowska et al. 2016). It is worth noting that infants and children are unavoidably exposed to BaP, which is ubiquitous and consistently present in the environment, and is a possible causal factor for abnormal adult neurobehavioral (Chen et al. 2012).

The cerebellum has an important role in gait, reflex adaptation, and motor function and learning (Glickstein and Doron 2008). Recent work has revealed that the cerebellum intervenes in the regulation of some neocortical cognitive function and emotional activities (Habas 2001; Gillig and Sanders 2010). BaP exposure can adversely affect both adult and children's neurobehavior related to the function of the cerebellum, such as mood state, digital span and symbol, visual retention, manual dexterity, anxiety, depression, and attention (Niu et al. 2010; Perera et al. 2012; Qiu et al. 2013; Zhang et al. 2013). A handful of studies of BaP neurotoxicity have mentioned that BaP could disturb gene expression in cerebellum and may correlate with anxiety behavior and motor activity change (Saunders et al. 2002; Grova et al. 2008; Chepelev et al. 2016), but the underlying mechanisms are not clear.

In recent years, metabolomics has been used to study the molecular mechanisms of chemical toxicity (Liu et al. 2015; Xu et al. 2015; Ba et al. 2016) and some diseases (Constantinou et al. 2011; Kimball et al. 2016; Zhou et al. 2016). Metabolomics depicts the metabolic profile in complex systems (Naz et al. 2014), through the systematic identification and quantification of metabolites (Jager et al. 2016). Metabolism is the endpoint of the enzyme (protein) action and the final consequence of biological function, so it can directly indicate aberrant physiological status (Huang et al. 2016). A few reports have shown that BaP disrupts the metabolism of the Manila clam, oyster pinctada, rat serum, murine hepatocytes, and human endothelia (Zhang et al. 2011; Kalkhof et al. 2015; Wang et al. 2015; Chen et al. 2016). An untargeted metabolomic approach has not yet been applied to analyze rat cerebellum following BaP treatment (Naz et al. 2014). In the present study, untargeted metabolomics was employed using GC-MS to explore the effect of postnatal exposure to BaP on rat cerebellum.

Materials and Methods

Chemical

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). All the reagents in this research were of analytical grade.

Animals and Treatment

Thirty-four healthy male neonatal Sprague-Dawley (SD) rats aged postnatal day (PND) 4 (the day of parturition was considered PND 0) and their maternal rats were purchased from the animal center of Chongqing Medical University (Chongqing, China) [certificate SCXK (YU) 2007-0001]. All animals were kept under standard laboratory conditions: 18–28 °C temperature and 40–80% humidity with a light/dark cycle of 12 h. All animals had free access to water and food. Each neonatal rat was marked an individual number on their feet and body by a nontoxic ink. All neonatal rats were randomly divided into two groups: control group and BaP-treated group. Neonatal rats were weaned on PND 21 and housed in the same treatment group. All animal care and experimental procedures were in accordance with the National Institutes of Health Guidelines for Animal Research (Clark et al. 1997). This study was approved by the Ethics Committee of Chongqing Medical University.

Owing to its lipophilicity, BaP was dissolved in corn oil. The BaP-treated group of rats was administered via oral gavage each day with a dose of 2 mg/kg. This dose corresponded to human environmental exposure levels (ATSDR 1995). This dose has also been studied in our previous studies (Chengzhi et al. 2011; Chen et al. 2012; Yang et al. 2017). The control group rats received an equivalent volume of corn oil.

In rats, the brain growth spurt period corresponds to the third human gestational trimester and continues throughout the first years of life, spanning the first 3–4 weeks of neonatal life and reaching its peak around PND 10 (Viberg et al. 2008). In this study, the treatment began at PND 5. To simulate the infant and children continuously being exposed to BaP before maturation, the treatment lasted for 7 weeks.

After 7-week treatment, 18 rats (n = 9 each group) were directly sacrificed by rapid decapitation; the cerebellum was quickly acquired for metabolomic analysis (n = 6 each group) and electron microscopy (n = 3 each group). Sixteen rats (n = 8 each group) performed behavioral tests using the open field test.

Open Field Test

Rats were placed in the testing room for a 30-min period to acclimatize. The open field test apparatus consists of a black square cage (100 × 100 × 40 cm) with a floor arena divided into 25 × 25 cm^2 equal squares. Each rat was placed in the center of the cage. After 30 s of adapting, all behaviors were recorded for 5 min using a camera positioned 200 cm above the arena. The cage was thoroughly cleaned with ethanol swabs between each test. The rats were then returned to their home cage after the test, and then to a hold room once all animals were tested. The locomotor activity (total distance and time) and the number of central activity and rearing behaviors (defined as an upright posture sustained on hind paws) were clearly visible on the video. The test took place in a soundproof room between 9:00 a.m. and 17:00 p.m.

Sample Preparation for Electron Microscopy

The rats were perfused with normal saline solution followed by 4% paraformaldehyde, and the brain was quickly acquired and stored at 4% paraformaldehyde for 12 h. The sample was then sliced into sections of approximately 1 mm^2 and kept in the 2.5% glutaraldehyde for 24 h at 4 °C. Samples were post-fixed in 1% osmium tetroxide for 2 h, dehydrated in a series of alcohol solutions, and embedded in epoxy resin. Ultra-thin (1 μm) sections were placed on grids and stained with uranyl acetate and lead citrate before examination with a transmission electron microscope.

Sample Preparation for GC-MS Analysis

The cerebellum (m = 50 ± 0.5 mg) was homogenized after adding 20 μL internal standard and 1.5 mL of prechilled methanol/water (1:1, v/v) in a 2-mL centrifuge tube. The mixture was centrifuged at 16,000×g for 10 min at 4 °C, and the supernatant was transferred into a new 2-mL Eppendorf tube. Then, 1.6 mL of prechilled dichloromethane/methanol (3:1, v/v) was added to precipitate. The mixture was again centrifuged under the same conditions, to obtain the supernatant. The two supernatants were combined and dried in a vacuum concentrator. Subsequently, 200 μL of sodium hydroxide was added to each of the dried samples before they were transferred to a silanized glass tube. One-hundred sixty-seven microliters of methanol and 34 μL of pyridine were added to the solution, followed by the addition of 20 μL of MCF with vigorous mixing for 30s, and then, a further 20 μL of MCF was added, accompanied by vigorous mixing for 30 s. Four hundred microliters of chloroform and 400 μL of 50 mM sodium bicarbonate were added, followed by 10 s of vigorous mixing to separate the MCF derivatives. After centrifuging at 1500 rpm for 10 min, the aqueous layer was discarded and the remaining sample was dehydrated with anhydrous sodium sulfate before being transferred to GC vials for analysis (Smart et al. 2010; Dai et al. 2016).

GC-MS Analysis

GC-MS analysis was performed on an Agilent 7890A gas chromatography system coupled with an Agilent 5975C mass spectrometer (Agilent, Santa Clara, CA, USA). The system utilized a fused silica Zebron ZB-1701 capillary column (30 × 250 × 0.15 μm) coated with 86% dimethylpolysiloxane and 14% cyanopropylphenyl (Phenomenex, Torrance, CA, USA). The GC-MS parameters were based on Thomas et al. (2015). A 1.0 μL amount of the sample was injected into the GC-MS under pulsed splitless mode. The inlet temperature was set to 290 °C. Carrier gas was instrument grade helium (99.99%, BOC); the front inlet purge flow was set to 25 mL/min 1 min after injection. The GC oven initial temperature was kept at 45 °C for 2 min, raised to 180 °C at rate of 9 °C/min, held 5 min; increased 220 °C at rate of 40 °C/min, held 5 min; increased 240 °C at rate of 40 °C/min, held 11.5 min; finally increased 280 °C at rate of 40 °C/min, held 2 min. The transfer line, source, and quadrupole mass analyzer temperatures were 250, 230, and 150 °C, respectively. The detector was run in positive ionization mode with electron energy of 70 eV and 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 1562 μ/s, and 100 ion counts as the detection threshold. A solvent blank was run after every six sample injections to check for instrument carryover.

Metabolomic Data Analysis

The raw data obtained from the GC-MS was converted into common data format (CDF) by the Automated Mass Deconvolution and Identification System (AMDIS—http://www.amdis.net/). Different metabolites of the cerebellum were identified by an in-house mass spectral library and the NIST0.5 library (Thomas et al. 2015). The results include

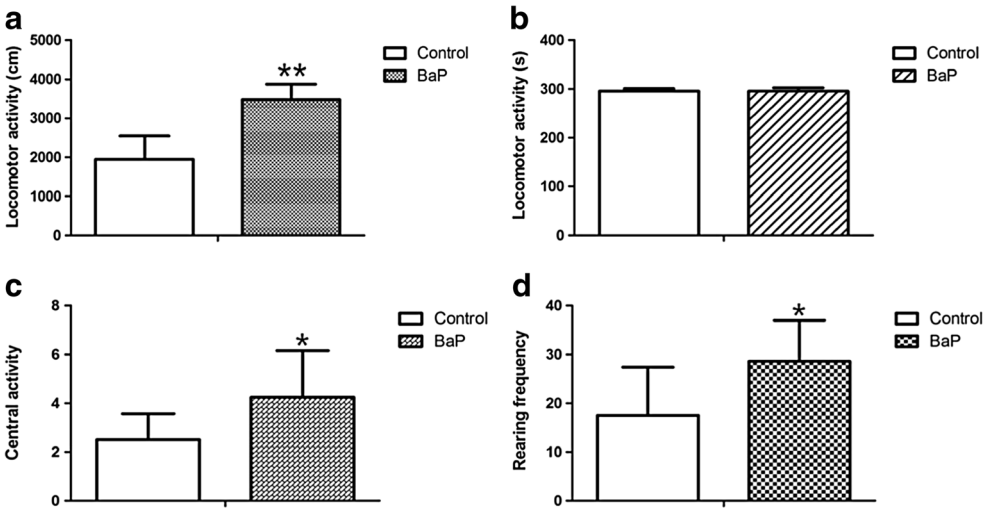

Fig. 1 Results of the behavioral tests between control and BaP group. **a** The locomotor activity (the total distance). **b** The locomotor activity (time. **c** The number of central activity. **d** The number of rearing activity. $^{**}P < 0.01$; $^{*}P < 0.05$

sample information, retention time, and peak intensities. Compounds with a match factor > 70% to one of the libraries were included in the analysis (Thomas et al. 2015). We used the relative quantification of peak intensities to estimate the level of the metabolites. The data was normalized using an internal standard and weight of the brain tissue. Principal

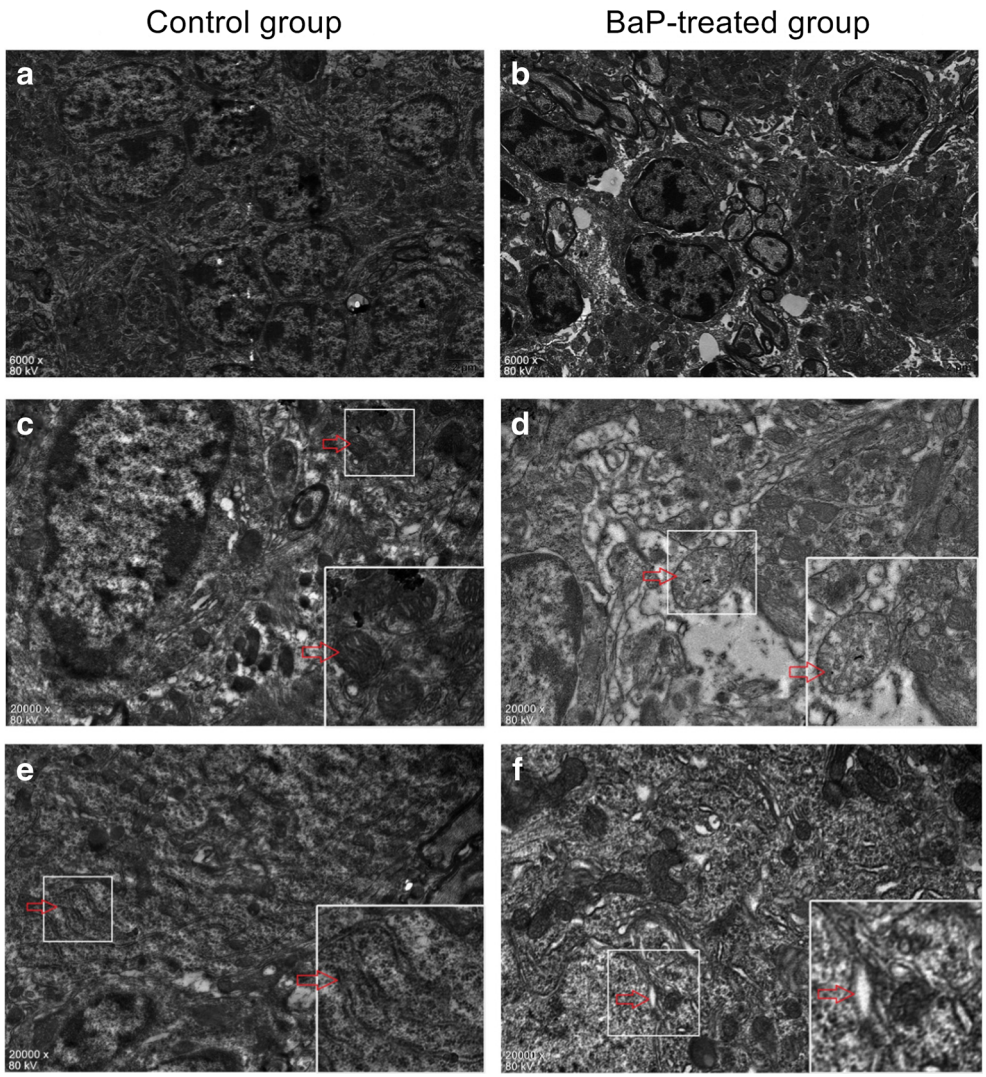

Fig. 2 Ultrastructural changes in cerebellum of control and BaP-treated rats. **a** Unaltered structure of neural cell and neuropil. **b** Neural cells and neuropil swelling. **c** Ultrastructurally unchanged mitochondria. **d** Mitochondria swollen and deformation. **e** Ultrastructurally unchanged endoplasmic reticulum. **f** Endoplasmic reticulum expansion. Pictures are representative for each of three control and BaP-treated group rats

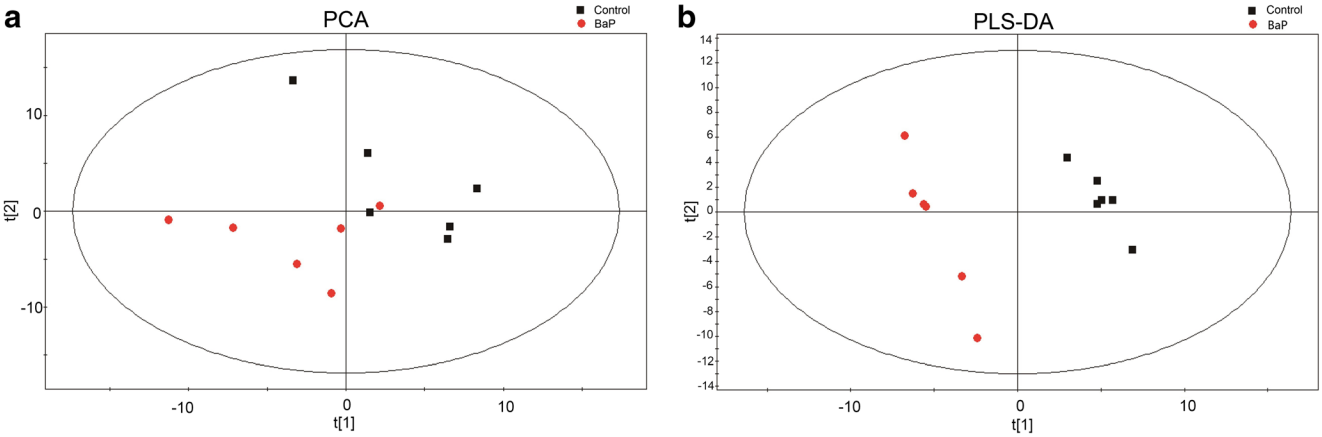

Fig. 3 Metabolomic analysis of the cerebellum in the control group and BaP-treated group. **a** Principal component analysis (PCA) score plot ($R^2X = 0.68$, $Q^2 = 0.32$). **b** Partial least squares-discriminate analysis (PLS-DA) score plot ($R^2X = 0.416$, $R^2Y = 0.992$, $Q^2 = 0.915$)

component analysis (PCA) was used to observe the distribution of all samples, and partial least-squares discriminate analysis (PLS-DA) was used to differentiate the control group from the BaP-treated group by SIMCA-P 11.5 (Umetrics, Umea, Sweden). Significantly changed compounds were selected based on their variable of importance in the project (VIP) value. To ensure that the selected metabolites significantly changed among groups, a Student's *t* test was conducted in SPSS 17.0 (SPSS, Inc., Chicago, IL) with the significance level set at VIP > 1 and $P < 0.05$. In addition, metabolic pathway analysis was performed in Metaboanalyst (http://www.metaboanalyst.ca/), followed by network analysis performed in Metscape (a plug-in of Cytoscape 3.5.0) to select the main affected pathway.

Statistical Analysis

The data obtained in the open field test was analyzed using a Student's *t* test. Data was reported as mean ± SD, and $P < 0.05$ was considered to be statistically significant in the open field test. All statistical analyses were performed in SPSS v. 17.0.

Table 1 Metabolites significantly changed in cerebellum after treatment with BaP

Name	RT (min)	Match (%)	*P* value	VIP value	Fold change
Leucine	14.242	98	0.000	2.008	− 1.167
Cystathionine	35.531	78	0.000	1.961	1.252
Lysine	26.672	82	0.000	1.844	1.041
Creatinine	16.935	86	0.000	1.801	− 1.040
Proline	15.242	98	0.000	1.755	− 1.025
S-Adenosylhomocysteine	17.31	79	0.000	1.763	1.1283
2-Aminoadipic acid	20.38	80	0.000	1.755	1.060
Glutaric acid	10.947	73	0.000	1.747	− 1.048
Serine	17.63	96	0.000	1.734	− 1.023
Ornithine	25.065	71	0.001	1.711	1.038
4-Hydroxyphenylpyruvate	16.066	70	0.003	1.578	− 1.026
NADP_NADPH	11.049	81	0.004	1.553	1.040
Nervonic acid	33.804	70	0.005	1.534	1.062
Glutathione	19.206	92	0.012	1.475	− 1.014
Phosphoenolpyruvic acid	14.748	73	0.018	1.350	1.095
Hexanoic acid	6.514	82	0.025	1.307	− 1.027
2-Pyrrolidinone	11.76	74	0.028	1.282	− 1.025
Valine	12.992	92	0.029	1.281	− 1.020
Asparagine	16.796	88	0.035	1.239	− 1.011
Dehydroascorbic acid	15.784	86	0.043	1.214	− 1.017

Fold change is the ratio of BaP-treated group and control group. *P* value from Student's *t* test, $P < 0.05$

RT retention time, *VIP* variable important in the project (obtained from PLS-DA with a threshold of 1.0)

Results

Open Field Test

Locomotor activity and mood changes were evaluated in the open field test. Compared with the control group, the rats in the BaP-treated group had significantly higher total distance locomotor activity (Fig. 1a, $t = 6.039$, $P < 0.000$), but there was no statistically significant difference in the time of the locomotor activity (Fig. 1b, $t = 0.077$, $P = 0.940$). Moreover, the number of central activities (Fig. 1c, $t = 2.263$, $P = 0.040$) and rearing activities performed by the BaP-treated group was significantly increased (Fig. 1d, $t = 2.426$, $P = 0.029$).

Ultrastructural Feature in Cerebellum

Compared with the control group (Fig. 2a, c, e), the BaP-treated group showed ultrastructural features of typical cellular swelling. The characterization of cellular swelling in the BaP-treated group was local swelling which was recognized by a pale and watery cytoplasm under electron microscopic analysis (Fig. 2b) and swollen organelles, including mitochondria deformation (Fig. 2d) and endoplasmic reticulum expansion (Fig. 2f), which were obvious in the cell body and the neuropil.

Analysis of Brain Metabolomics

The GC-MS metabolomic profiling of the cerebellum samples produced a total of 120 compounds across the two groups, which were used in the subsequent multivariate analysis. The PCA plot showed that the BaP-treated group could be distinguished from the control group ($R^2X = 0.68$, $Q^2 = 0.32$; Fig. 3a), and the PLS-DA plot showed an obvious distinction between the two groups ($R^2X = 0.416$, $R^2Y = 0.992$, $Q^2 = 0.915$, Fig. 3b). Based on the PLS-DA (VIP > 1) and t test ($P < 0.05$), 20 differential metabolites were identified in the cerebellum between the control group and BaP-treated group (Table 1). In order to visualize the metabolomic results, a heat map (Fig. 4) was generated to show the level of the 20 significant metabolites. Pathway analysis, performed by inputting metabolites into Metaboanalyst (http://www.metaboanalyst.ca/), suggested that BaP significantly impacted a number of metabolic pathways (Fig. 5, Table 2). Further analysis was conducted based on the 20 significant metabolites, to further study the associations and interaction patterns between metabolites. There were only six metabolites that were highly linked and were considered as a hub of a network (Fig. 6). This network hub included pathways related to energy metabolism, methionine cycle, and glutathione metabolism. Figure 7 visually summarizes the potential mechanisms of BaP in rat cerebellum through the metabolomic changes observed.

Discussion

The open field test was used to comprehensively assess locomotor activity and anxiety-like and exploratory behaviors (Prut and Belzung 2003; Tatem et al. 2014). In this study, compared with the control group, the rats in the BaP-treated group had significantly increased locomotor activity, rearing activity, and central activity. These results suggest that early life exposure to BaP can affect locomotor activity and anxiety-like behavior in adulthood. These findings are in accordance with previous research (Chen et al. 2012; Perera et al. 2012; Patel et al. 2016). Moreover, electron microscope analysis revealed that BaP-treated rats had obvious neural cell and neuropil swelling; mitochondria deformation including elongation, swelling, or shrinking; and endoplasmic reticulum expansion. Therefore, subacute postnatal BaP oral exposure may have subsequent effects on the development of rat cerebellum.

The primary metabolism of the SD rat cerebellum exposed to BaP after 7 weeks by oral gavage was studied, using GC-MS metabolomics for the acquisition of the metabolic profiles. Metabolomic analysis enabled the identification of 20 metabolites that were significantly different between the two groups, and they were involved in major metabolic pathways and functions. Our findings demonstrated that the rat cerebellum is metabolically responsive to BaP. Thus, the metabolic changes observed in mammalian cerebellum indicate that BaP may affect brain metabolism.

The metabolomic observations indicate that insufficient energy was being supplied in the cerebellum. There was no significant change in some metabolites that represent glycolysis and the tricarboxylic acid (TCA) cycle, but the level of phosphoenolpyruvic (PEP) increased, and the levels of some amino acids, including asparagine, proline, valine, leucine, and serine, decreased. The creatine/phosphocreatine/creatine kinase (Cr/PCr/CK) system was interrupted, indicating that there was shortage of energy supply in cerebellum. PEP is a high-energy intermediate product of glycolysis, which can be converted into pyruvate (Saiki et al. 1997). This step is essentially irreversible in the whole process of glycolytic and oxidative phosphorylation (Saiki et al. 1997). The accumulation of PEP suggests that glycolysis was abnormal. While the TCA cycle is the main pathway of oxidative phosphorylation in mitochondria (Fernie et al. 2004), and is the major common pathway for oxidation, some amino acids are metabolized to obtain energy needed to maintain CNS function (Rodrigues and Cerdán 2007). The levels of asparagine, proline, valine, and leucine were decreased which may have indicated that amino acid oxidation was occurring to compensate for the shortage of energy (Reitzer 2004; Fernstrom 2005; Wyse and Netto 2011). The Cr/PCr/CK system, a high-energy phosphate shuttle from mitochondria to cytoplasmic sites and an intracellular buffer for ATP, is another high-energy reservoir for CNS (Beard and Braissant 2010; Lage et al. 2013). CK

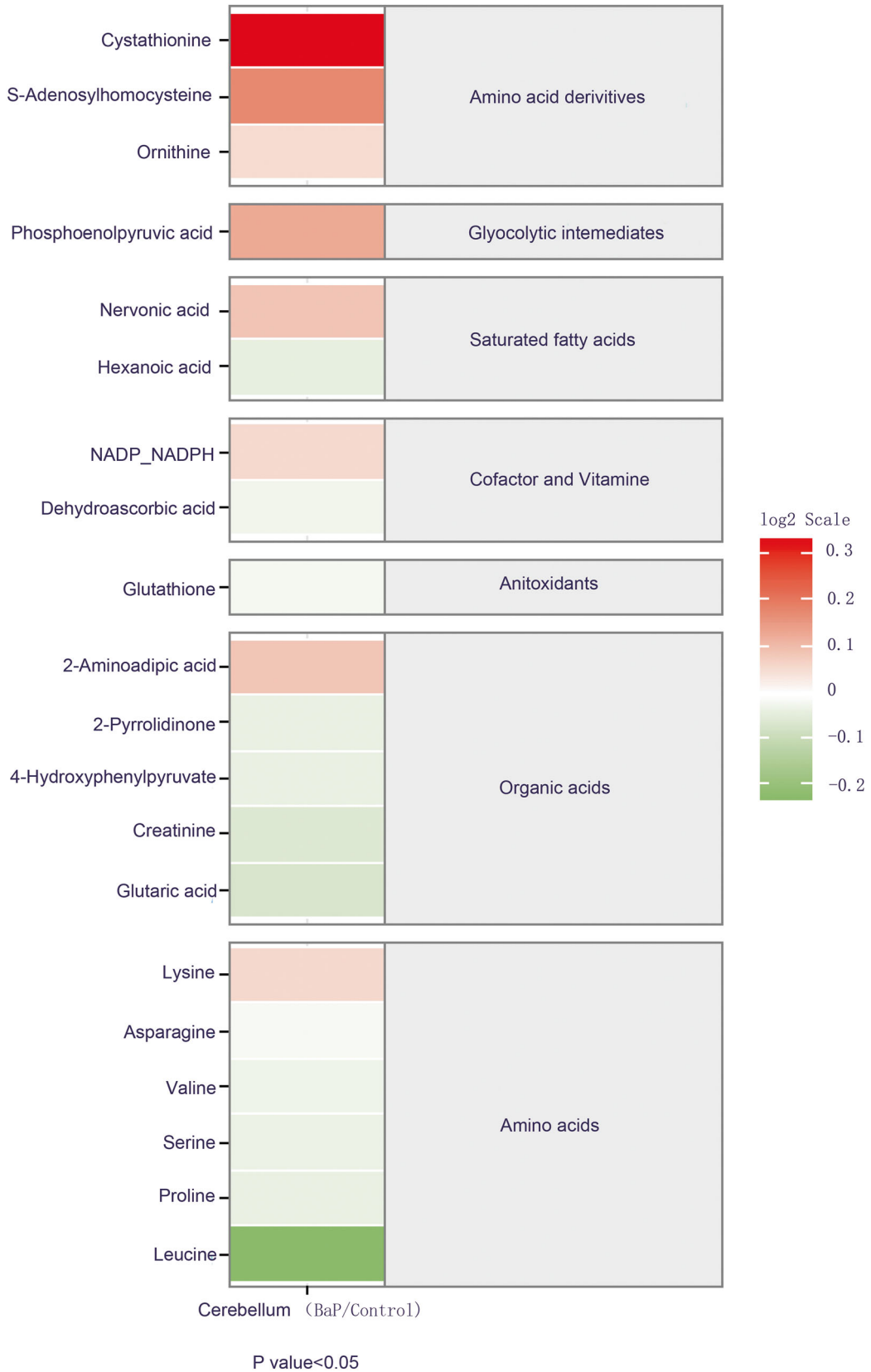

Fig. 4 The heat map of the levels of the 20 metabolites. Red indicates that the concentration of the metabolite is greater than average, while green indicates that the concentration of the metabolite is less than average

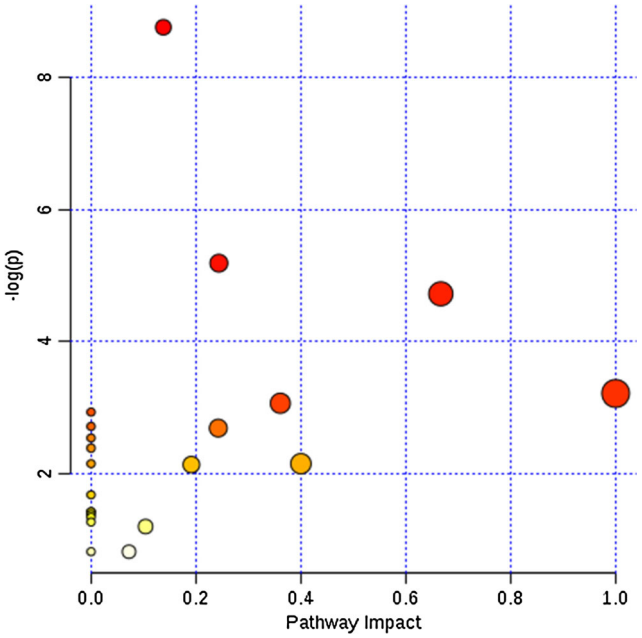

Fig. 5 The pathway impact of BaP on rat cerebellum metabolites. All matched pathway according to *P* values from pathway enrichment analysis and pathway impact value from pathway topology analysis by Metaboanalyst. Enrich metabolic pathways were ranked by FDR values. The significant pathways for BaP-treated group compared to control group were represented by bigger/red dots and by those dots with higher log *P* value. And the pathway impact is 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. Specifics are shown in Table 2

activity is important for brain energy metabolism homeostasis and especially ATP transfer and buffering and could be inhibited because of the increased levels of lysine (Tonin et al. 2009). The decrease of creatinine, which is final product of creatine, was further evidence that the Cr/PCr/CK system was disrupted. The shortage of energy can impair the main functions of CNS, including generation, processing, and transmission of impulses. Because action potentials generated and transmitted rely on Na^+, K^+, and Ca^+ to maintain electrochemical disequilibrium across the neuronal plasma membrane, the CNS is in need of a constant supply of energy (Erecinska and Silver 1989). Sodium-potassium adenosine triphosphatase (Na^+-K^+-ATPase), a highly expressed membrane protein that hydrolyzes ATP to transduce Na^+ and K^+, can modulate the resting transmembrane potential, some postsynaptic activities, and neurotransmitter turnover (Villa et al. 2013; Kurauchi et al. 2016). Calcium adenosine triphosphatase (Ca^+-ATPase) can hydrolyze ATP to maintain the homeostasis of intracellular Ca^+ (Kurauchi et al. 2016). The rates of ion transport and ATPase activity require a large supply of energy (Erecinska and Silver 1989). If this equilibria was broken, it could lead to nerve ending depolarization with Ca^+ entry into the cell, followed by neurotransmitter release and neuronal swelling (de Lores Arnaiz and Ordieres 2014). Our previous studies found that BaP exposure can lead to decreased ATPase activity as well as Ca^+ overload (Duan et al. 2013; Dong et al. 2015; Yang et al. 2017). Furthermore, the upregulation of locomotor activity and anxiety aggravated the consumption of energy and accelerated amino acid metabolism—particularly valine and leucine metabolism.

The upregulation of NADP/NADPH and the downregulation of glutathione (GSH) and dehydroascorbic acid (DRA) suggest that the antioxidant system is disordered in the cerebellum. NADP/NADPH and GSH are known as classic molecules involved in energy metabolism, reductive biosynthesis, and antioxidation (Wu et al. 2004; Ying 2008). They are important components in the intracellular antioxidant system and play an essential role in cell defense against reactive oxygen species (ROS) (Wu et al. 2004; Ying 2008). NADPH mediates cellular antioxidation mainly through its effects on GSH regeneration: the regeneration of GSH from GSSH, through the action of glutathione reductase, requires NADPH (Hayes and McLellan 1999; Ying 2008). DRA, an oxidized form of ascorbic acid (AA), could inhibit mitochondrial damage and cell

Table 2 The detail pathway impact results in cerebellum from pathway analysis

Name	Total	Hit	*P* value	−Log (*P*)	Holm *P*	FDR	Impact
Ubiquinone and other terpenoid-quinone biosynthesis	3	1	0.040136	3.2155	1	0.68647	1
Valine, leucine, and isoleucine biosynthesis	11	2	0.008902	4.7215	0.70327	0.24036	0.66666
Methane metabolism	9	1	0.11588	2.1552	1	0.73223	0.4
Glutathione metabolism	26	2	0.046613	3.0659	1	0.68647	0.36069
Cysteine and methionine metabolism	28	3	0.005585	5.1877	0.44677	0.22618	0.24395
Glycine, serine, and threonine metabolism	32	2	0.067799	2.6912	1	0.68647	0.2428
Arginine and proline metabolism	44	2	0.11752	2.1412	1	0.73223	0.19136
Aminoacyl-tRNA biosynthesis	67	6	0.000158	8.7525	0.012804	0.012804	0.13793
Glycolysis or gluconeogenesis	26	1	0.30092	1.2009	1	1	0.10389
Tyrosine metabolism	42	1	0.44105	0.81861	1	1	0.07225

Total is the total number of 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

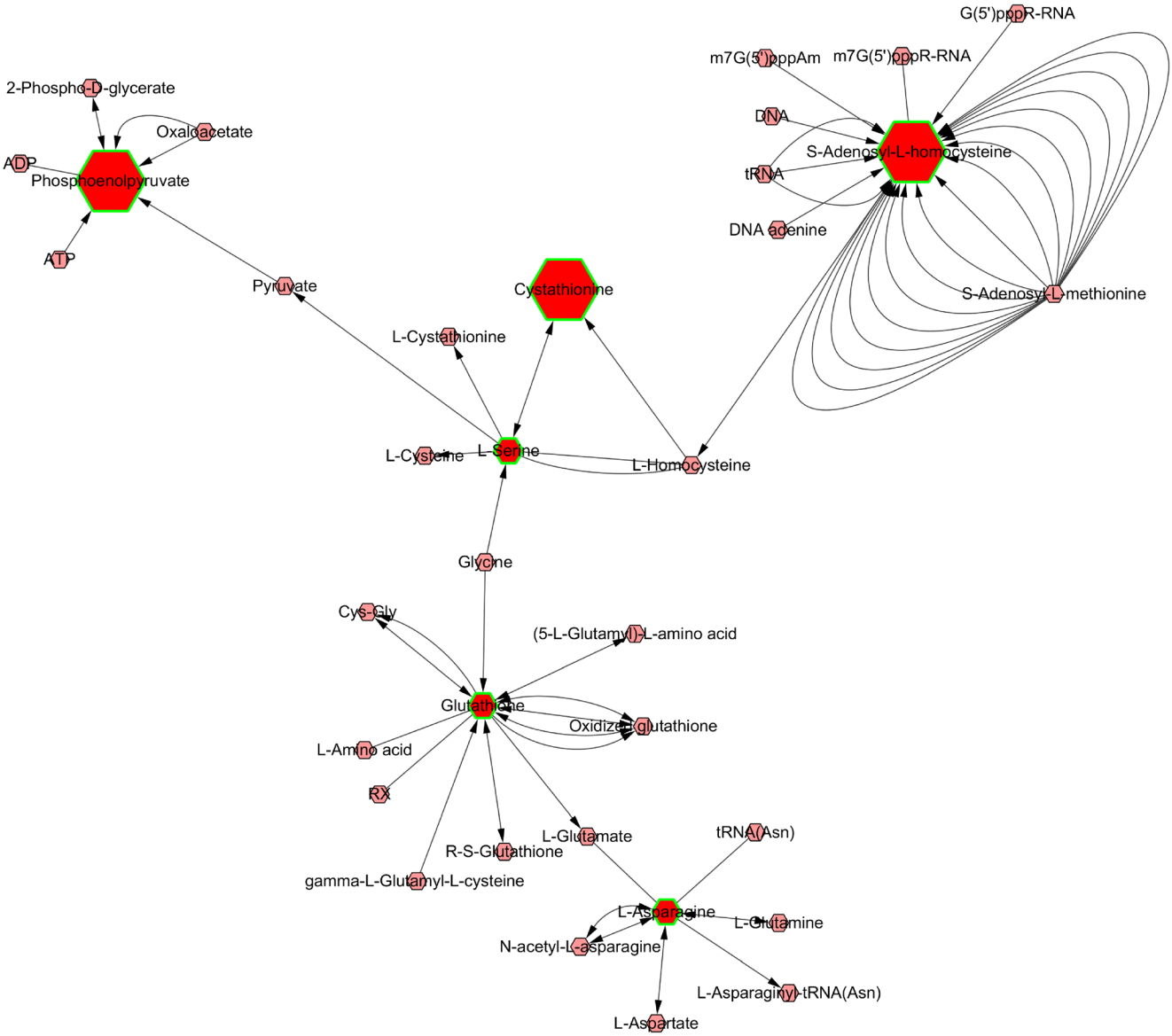

Fig. 6 Integrated network combining the major signaling pathway associated with the differential metabolites between control group and BaP-treated group. The network analysis of differential metabolites was constructed using Metscape (a plug-in Cytoscape 3.5.0). Nodes in red represent the hub of network. The scale of red node represents the size of impact. There are six metabolites highly linked that were considered as a hub of network and involved three metabolisms including energy metabolism, methionine cycle, and glutathione metabolism

death following oxidative injury and can also complement the lack of AA at the expense of GSH (Kim et al. 2008). The antioxidant system is responsible for balancing the production of ROS that is generated as part of normal cellular metabolism in the brain (Liu et al. 2015). The antioxidant system breakdown following BaP treatment suggests that there was a ROS generation overload, resulting in neuronal oxidative stress. Also, the upregulated level of intracellular Ca^{+} can trigger the mitochondria to produce more ROS (Bakthavachalam and Shanmugam 2017). These findings are in accordance with our previous studies which found that BaP could induce oxidative stress in the rat brain (Duan et al. 2013; Yang et al. 2017). Excessive ROS induced and enhanced neurobehavioral impairment and neural cell damage, mainly through altered neuronal signaling, neuroinflammation, and activation of cell death mechanisms (Popa-Wagner et al. 2013).

S-adenosylhomocysteine (AdoHcy), an important intermediate product in the methionine cycle, can influence cellular methylation function through regulation of the levels of S-adenosylmethionine (AdoHmet) and homocysteine (HCys) (Miller 2003). AdoHcy is generated from AdoHmet after donation of a methyl group (Miller 2003). The level of AdoHcy and AdoHmet can modulate cellular methylation; thus, the ratio of AdoHcy/AdoHmet is often used as an index of

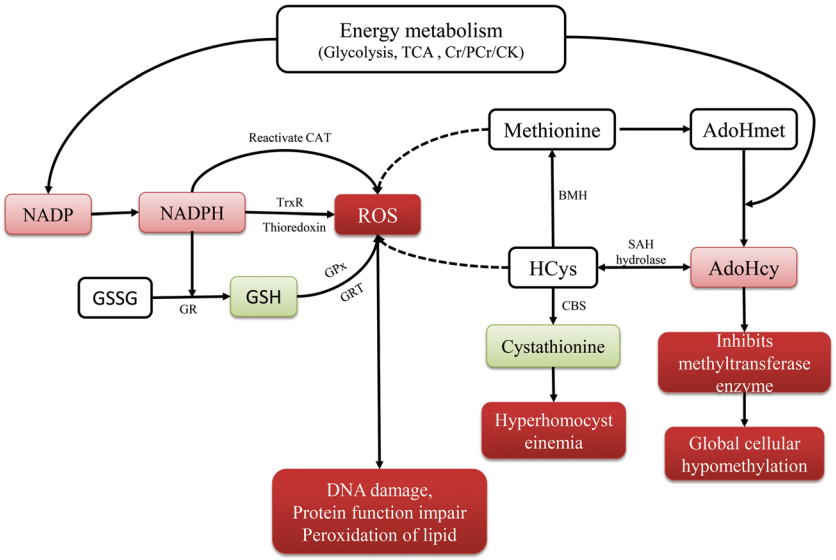

Fig. 7 The summary overview of the metabolite pathway of BaP affected neural function in rat cerebellum. Molecular upregulation (pink). Molecular downregulation (green). Potential mechanism of toxic effects (green). Cr/PCr/CK creatine/phosphocreatine/creatine kinase system, TCA tricarboxylic acid cycle, GSSG oxidized glutathione, GSH glutathione, GPx glutathione peroxidase, GST glutathione-S-transferases, CAT reactivating H_2O_2-inactiated catalase, TrxR thioredoxin reductase, HCys homocysteine, AdoHcy S-adenosylhomocysteine, AdoHmet S-adenosylmethionine, SAH hydrolase S-adenosylhomocysteine hydrolase, BHM betaine-homocysteine methyltransferase, CBS cystathionine-beta-synthase, TCA tricarboxylic acid

methylation capacity (Choumenkovitch et al. 2002). AdoHcy acts as a potent methyltransferase inhibitor that could cause global cellular hypomethylation (Esse et al. 2014). AdoHcy hydrolase cleaves AdoHcy to adenosine and Hcys in the methionine cycle (Miller 2003). The increase of AdoHcy could induce the pathophysiology of cystathionine-beta-synthase (CBS) deficiency (Choumenkovitch et al. 2002; Esse et al. 2014). HCys is converted to cystathionine by CBS and is associated with the incidence of hyperhomocysteinemia that could cause or promote CNS dysfunction (Gerritsen and Waisman 1964; Robert et al. 2003; Troen 2005). In this study, we found that cystathionine downregulated HCys metabolism which suggests that CBS deficiency plays a critical role in metabolism. In addition, CBS deficiency could also induce the increase of HCys and AdoHcy (Choumenkovitch et al. 2002; Esse et al. 2014). The accumulation of HCys in neurons could not act on the extracellular transport of HCys. High brain concentrations of homocysteine or its oxidized derivatives might indirectly alter neurotransmission and induce excitotoxicity in neurons, or induce AdoHcy metabolism and cerebrovascular function damage, leading to secondary neuronal dysfunction and degeneration (Troen 2005). Methionine and HCys may also modulate ROS generated (Gomez et al. 2011), which suggests that the concentration of AdoHcy may be associated with ROS generation.

This is the first study to apply a metabolomic approach to investigate the effect of BaP on rat cerebellum metabolism. Administration of BaP caused anxiety behavior and increased locomotor activity and neural cell swelling. Our findings suggest that postnatal exposure to BaP induced toxic effects on the brain. Untargeted GC-MS-based metabolomics revealed that the cerebellum neurodevelopment was predominantly affected in three key areas: energy metabolism, the antioxidant system, and the methionine cycle.

1. Energy metabolism plays an important role in maintaining CNS function. Insufficient energy supply is linked with neurobehavioral impairment and cell swelling and could also promote ROS production and disrupt the methionine cycle.
2. ROS were modulated by GSH (glutathione) and NADP-NADPH by glutathione peroxidase (GPx), glutathione-S-transferases (GST), reactivating H_2O_2-inactiated catalase (CAT), and thioredoxin reductase (TrxR)-mediated regeneration of thioredoxin. Methionine and homocysteine (HCys) may modulate ROS generation in the brain.
3. S-adenosylhomocysteine (AdoHcy), a potent methyltransferase inhibitor, could induce global cellular hypomethylation. The accumulation of AdoHcy and the absence of cystathionine may lead to BHM enzyme inactivity and CBS deficiency, which are correlated with hyperhomocysteinemia that can cause CNS dysfunction.

In this study, the untargeted GC-MS metabolomic approach was used to investigate the effects of BaP on cerebellar development. More detailed mechanisms should be explored in future studies.

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Compliance with Ethical Standards

Conflict of Interest The authors declare that they have no conflict of interest in the submission of this manuscript.

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