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Maternal di-(2-ethylhexyl) phthalate exposure inhibits cerebellar granule precursor cell proliferation via down-regulating the Shh signaling pathway in male offspring

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ABSTRACT

Di-(2-ethylhexyl) phthalate (DEHP) is an endocrine disrupting chemical (EDC) widely used as a plasticizer in many materials. Epidemiological investigations have shown that DEHP exposure during early development is related to cerebellar-related adverse neurodevelopmental outcomes. However, animal studies involving the effect of DEHP exposure on cerebellar development have rarely been reported and the potential mechanisms are unclear. The aim of this study was to investigate the effect of maternal DEHP exposure on the proliferation of cerebellar granule cell precursor cells (GCPs) and the mechanisms involved. Wistar rats were randomly assigned to four exposure groups and given 0, 30, 300, or 750 mg/kg/d DEHP by intragastric administration from gestational day (GD) 0 to postnatal day (PN) 21. Exposure to 300 and 750 mg/kg/d DEHP restrained GCPs proliferation and impaired neurodevelopment for males. Furthermore, exposure to 300 and 750 mg/kg/d DEHP decreased male pups protein expressions and mRNA levels of molecules related to proliferation, including Shh, Gli1, N-Myc, CyclinD1. In addition, the estrogen level and aromatase expression also reduced in male pups after maternal exposure to DEHP. However, effects on females were not obvious. These results suggested that 300 and 750 mg/kg/d DEHP exposure inhibit the proliferation of GCPs in male offspring and ultimately contribute to the impairment of neuromotor development. This, may be caused by the down-regulation of Shh signaling. And the susceptibility of male offspring to DEHP exposure may be attributed to the decreased estrogen level and aromatase expression in male pup's cerebellum.

1. Introduction

Di-(2-ethylhexyl) phthalate (DEHP) is widely used as a plasticizer as it renders softness and elasticity to normally tough plastic (Kay et al., 2013). It has a wide spectrum of commercial and industrial applications, such as food and beverage packaging, children's toys, personal care products, building materials and medical devices (Bernard et al., 2015; Sakhi et al., 2017; Tickner et al., 2001). It is generally believed that DEHP is not covalently bound to the polymer matrix, which makes it susceptible to leaching into the environment (Chiellini et al., 2011; Erythropel et al., 2014). Consequently, people can be daily exposed to it through the ingestion and inhalation as well as by dermal contact (Martinez-Arguelles and Papadopoulos, 2016). The gastrointestinal tract is usually considered the main absorption pathway for DEHP (Howdeshell et al., 2008; Martino-Andrade and Chahoud, 2010; Wittassek et al., 2011). When entering the body by an oral approach, most DEHP is quickly hydrolyzed to mono-(2-ethylhexyl) phthalate (MEHP), mono-2-ethyl-5-hydroxyhexyl phthalate (MEHHP), mono-2-ethyl-5-oxohexyl phthalate (MEOHP), mono-2-ethyl-5-carboxypentyl phthalate (MECPP) and 2-carboxymethylhexyl phthalate (MCMHP) (Wittassek and Angerer, 2008). Urine is the major route of excretion for such metabolites (Chang-Liao et al., 2013; Koch et al., 2010).

As a representative of environmental endocrine disrupting chemicals (EDCs), the toxicity of DEHP has been widely investigated, mainly regarding reproductive development (Hannon et al., 2015; Pocar et al., 2017; Wang et al., 2016). Recently, the focus on DEHP of neurodevelopment is emerging. It is worth noting that DEHP and its metabolites can pass through the placental barrier or exist in breast milk to be taken up by the fetus or nursing infant, and then affected the growth and development of the progeny (Fromme et al., 2011; Koch et al., 2010; Shin et al., 2014). Many prior studies have indicated that prenatal exposure to phthalates may be inversely associated with the mental and psychomotor developmental and indices of infants' and school age children's IQ (Cho et al., 2010; Kim et al., 2011; Téllezrojo et al., 2013; Whyatt et al., 2012). In addition, there is a positive correlation between DEHP metabolite concentration in urine and symptoms of attention deficit hyperactivity disorder (ADHD) (Cho et al., 2010; Chopra et al., 2014). ADHD is a multiple etiological disorder that involve in many encephalic regions which include cerebellum (Vieira et al., 2018). People with ADHD are often reported to have cerebellar abnormalities (Valera et al., 2007). Further-

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more, it was considered that DEHP exposure during pregnancy and lactation affected neurodevelopment of offspring in a sex-specific manner (Luu et al., 2017; Rebuli and Patisaul, 2016; You et al., 2018). This might involve in the level of estrogen and the aromatase activity, a key enzyme for estrogen synthesis, in the brain of male pups (Dai et al., 2015; Smith and Holahan, 2014; Smith et al., 2011). Although many epidemiological investigations have shown a relationship between maternal DEHP exposure and cerebellar-related neurodevelopment in offspring, few animal investigations exist concerning about the effects of DEHP exposure on the development of the cerebellum and its motor function.

The cerebellum is thought to be mainly related to motor function, coordination and motor learning. Once the function of the cerebellum is impaired, alterations in body balance, motor coordination, muscle strength and even neurodevelopmental disorders occur (Deuschl et al., 2001; Koziol et al., 2012; Thach et al., 1992; Timmann et al., 2010). These alterations can be detected by behavioral tests including surface righting reflex, grip strength and negative geotaxis reflex and so on, which are related to the normal development of cerebellar granule cells (GCs) (Gallegos et al., 2016; Souza et al., 2015; Zhao et al., 2015). GCs make up the largest number of cells in the cerebellum, and a reduction in number can result in the formation of a smaller cerebellum and abnormal foliation (Cairns et al., 2016). It is generally considered that the number of cerebellar GCs relied on normal cellular proliferation (Miyazawa et al., 2000). Therefore, the normal proliferation of GCs during the proliferative phase will eventually affect the morphology and function of the cerebellum. GCs arise from cells in the outer granular layer (EGL), known as cerebellar granule cell precursor cells (GCPs). GCPs proliferation mainly occurs two weeks postnatally (Ceccarelli et al., 2015; Klein et al., 2001), the Sonic hedgehog (Shh) signal pathway plays an important role during the proliferation of GCPs (Fernandez et al., 2010; Subkhankulova et al., 2010; Vaillant and Monard, 2009).

Shh is a morphogen that is primarily involved in cerebellar formation and maturation. More importantly, Shh is required to regulate the proliferation of GCPs (Manto and Patrice, 2012). As the transcriptional effector of Shh signaling, Gli1 can activate target genes including N-Myc and CyclinD1 (Wallace, 1999), which directly promote precursor to entry into the cell cycle and DNA duplication (Kenney et al., 2003; Knoepfler et al., 2002; Oliver et al., 2003).

Although a great deal of study has provided evidence for the bad consequences of DEHP on animal and human health, such as damage to the reproductive system (Kay et al., 2013), disruption of the endocrine system (Boas et al., 2010; Ghisari and Bonefeld-Jorgensen, 2009), and dysregulation of the immune system (Wang et al., 2014, 2017), its hazardous effect and specific mechanisms in the nervous system, especially in the cerebellum, are unclear. Consequently, in our study, female rats were exposed to DEHP by gavage during pregnancy and lactation to study the effects of maternal DEHP exposure on the proliferation of cerebellar GCPs and behavioral development in pups. We also, investigated the underlying mechanisms involved.

2. Materials and methods

2.1. Animals

Female Wistar rats (230–250 g) were obtained from the Center for Experimental Animals at China Medical University (Shenyang, China) with a National Animal Use License number of SCXK-LN2013-0007. All experiments and surgical procedures were approved by the Animal Use and Care Committee at China Medical University, which complies with the National Institutes of Health Guide for the Care and Use of Laboratory Animals. All efforts were made to minimize the number of animals used and their suffering. Rats were housed at a temperature of 24 ± 1 °C with 12 h light/12 h dark cycles. DEHP-free food and water were provided ad libitum.

2.2. DEHP administration

Female Wistar rats were randomly assigned into four groups (n = 20 per group), the control group and three DEHP treatment groups. Female rats were adaptive fed for 1 week and then mated with the normal male rats (♀/♂ = 2:1). The day of the vaginal plug was taken as gestational day (GD) 0. Then pregnant rats were administered via oral gavage 0,30 mg/kg/d, 300 mg/kg/d, 750 mg/kg/d DEHP (Sigma-Aldrich, purity >99.5%, Germany) in 0.1 ml corn oil (Sigma-Aldrich, Germany)/20 g body weight from GD 0 to postnatal day (PN) 21. This dose range was selected with reference to the effects measured in neurodevelopment in previous studies (Moore et al., 2001; Smith et al., 2011). According to the conversion coefficient between human and rat (Reagan-shaw et al., 2008), 30 mg/kg/d represents the general population exposure (Campioli et al., 2014), Furthermore, the European Union and the United States determined that DEHP did not affect human health with a limit of 48 mg/kg/d, which converted to rat equivalent dose based on the body surface area was approximately 300 mg/kg/d (Koch et al., 2010; Virtanen et al., 2005). Moreover, 750 mg/kg/d is 1/40 half lethal dose of DEHP. Each litter was culled to eight to nine pups at PN 4 (same number of males and females in each group, if possible).

2.3. Measurement of urine DEHP metabolites

The urine of six pregnant rats per group were collected at GD 19 to measure the concentration of MEHP, MEHHP, MEOHP, MECPP and MCMHP with gas chromatography-mass spectrometry (GC-MS) (Agilent 7890B-7000C, Agilent Technologies, Palo Alto, CA, USA). The isotopic label of the five metabolites MEHP-^{13}C4, MEHHP-^{13}C4, MEOHP-^{13}C4, MECPP-^{13}C4, MCMHP-^{13}C4 (Cambridge, Isotope Laboratories, American) were mixed equally as internal standard substance mixtures. Then urine was taken enzymatic hydrolysis in 37 °C water baths for 120min. Followed by the HLB column (Waters corporation, Milford Massachusegtts USA) was eluted with 1 ml mixture of urine samples incubated. Finally, the eluate was dried with high purity nitrogen and then were added 20 μl of BSTFA (Sigma-Aldrich, USA), 1 ml of TMCS (Sigma-Aldrich, USA) and 80 μl of pyridine (Sigma-Aldrich, USA) to derive at 30 °C for 30 min. The concentrations were measured by GC-MS. The limit of detection (LOD) for MEHP, MECPP, MEOHP, MEMHP, MEHHP is 3.8 ng/ml, 11.6 ng/ml, 25.6 ng/ml, 0.6 ng/ml, 2.6 ng/ml, respectively.

2.4. Immunofluorescence

Pups from each group were perfused transcardially with 4% paraformaldehyde at PN 7 and PN 14. Then the fixed cerebellum was embedded in paraffin and sectioned into 4 μm thick sagittal sections. For immunofluorescence, sections were incubated overnight at 4 °C with the rabbit anti-pax 6 antibody (Millipore Corporation, MA, USA; 1:400 dilution) and the mouse anti-proliferating cell nuclear antigen (PCNA) antibody (Abcam, MA, USA. 1:400 dilution). Tissue sections were then washed three times in PBS followed by incubation with secondary antibody conjugated to the fluorescent markers FITC and TRITC (Zhongshan Biotechnology, Beijing, China. 1:100 dilution) at room temperature for 2 h. The pictures were obtained from

fluorescence microscopy (BX61+DP-71, Olympus/IPP, JAPAN/USA) at a magnification of 200× (objective 20× and ocular 10×). The proliferative and no proliferative GCs in the cerebellar EGL were counted by a blinding method using Image-Pro Plus 6.0 software (Media Cybernetics, Inc, Silver Spring, MD, USA). Three different fields were selected from lobule 4–5 regions per section, and three sections per animal were evaluated to obtain a mean value. Six male rats and six female rats from each group were used to obtain an overall value for subsequent statistical analysis for each time point. Results were expressed as proliferation rate which was the ratio of the number of proliferative GCs versus the number of total GCs.

2.5. Behavioral tests

Surface righting reflex was performed at PN 3, PN 5 and PN 7. Pups were individually placed in a supine position on a flat surface and the time that pups returned to prone posture with all four paws on the surface was recorded. The righting reflex from a supine to a prone position was present on the first day after birth (Sekulić et al., 2009). There was no significant change in the amount of time which the rats overturned from supine to prone position seven days after birth (Jing et al., 2014). So, we choose PN 3, PN 5 and PN 7 as representatives to do surface righting reflex. Furthermore, the time we chose in surface righting reflex coincided with other literature (Zhao et al., 2015). Negative geotaxis reflex was carried at PN 7, PN 8 and PN 9, pup was oriented toward the top on the rough surface with a slope of 45° and the time to rotate 180° was recorded. The negative geotaxis reflex develops in the second week of life in normal pups (Hobbs et al., 2008). Pups were tested at PN 7 to ensure the reflex had developed, and that the eyelids remained closed. The experiment was conducted on PN 7, 8, 9 which were in consistent with other reports (Brys et al., 2014; Omer et al., 1991). On PN 14, PN 15 and PN16, forelimb grip strength was conducted to evaluate the forelimb strength of pups, each pup was suspended by its forelimb from a fixed wire a few centimeters above the floor, and the time it held on to the rod before falling was recorded. Rats can use their front claw to catch the iron bar on PN 10, but the ability of grip suspension was stable after 14 days of the birth. (Zhao et al., 2015). Therefore, the test was carried out on PN 14, 15 and 16 which is in accordance with the following literature (Brys et al., 2014). Twelve pups (gender in half) from each group were collected to conduct in the above three experiments.

2.6. Real-time PCR

Total RNA was isolated from cerebellar homogenates using the TRIzol Reagent (Invitrogen, Carlsbad, CA, USA) and reverse transcribed into cDNA with Prime Script RT kit as previously described (DRR047A, Takara, Japan) (Wang et al., 2017). Primers were designed with Primer 3 (http://bioinfo.ut.ee/primer3-0.4.0/primer3/), and the sequences were checked using a BLAST search (http://blast.ncbi.nlm.nih.gov/Blast.cgi). The sequences of specific primer pairs are described as Table 1. GAPDH was set as internal control for determining ΔCT values. Fold increases in expression were normalized to the control group by determining $2^{-\Delta\Delta CT}$ values.

2.7. Western blotting

The proteins of pups cerebellum from each group were prepared as previously described at PN 7, PN 14 (Wang et al., 2017). Concentrations of tissue lysates were estimated by Pierce BCA Protein Assay Kit (Thermo Scientific, USA) and diluted to 3 μg/μl. Then, sample was separated on 10% SDS-acrylamide gels. Proteins were separated by applying a constant voltage of 100 V for 1.5 h and were then transferred onto PVDF membranes at a constant voltage of 100 V for 60min. After blocking nonspecific sites with TBS containing 0.1% Tween 20 (TBST) and 5% defatted dried milk, membranes were washed and incubated with goat primary antibody *anti*-Shh (Santa Cruz Biotechnology, Inc. 1:200 dilution), rabbit *anti*-N-Myc (Santa Cruz Biotechnology, Inc. 1:200 dilution), mouse *anti*-Cyclin D1 (Santa Cruz Biotechnology, Inc. 1:200 dilution), rabbit *anti*-Gli1 (Abcam, MA, USA. 1:500 dilution), rabbit *anti*-aromatase (Abcam, MA, USA. 1:500 dilution) and rabbit *anti*-β-actin (Santa Cruz Biotechnology, Inc. 1:200 dilution) overnight at 4 °C. Membranes were then incubated with goat anti-rabbit, or mouse anti-goat horseradish peroxidase-conjugated secondary antibody (Zhongshan Biotechnology, Beijing, China, 1:2500 dilution). Finally, blots were visualized using the Bioanalytical Imaging System (Azure Biosystems, Inc). The relative density of the blot was quantified via Image-Pro Plus 6.0 software (Media Cybernetics, Inc, Silver Spring, MD, USA).

2.8. ELISA measurement of estradiol in cerebellum tissue

The tissue sample of each rat was homogenized in Pierce RIPA Buffer (Thermo Scientific, USA) containing protease and phosphatase inhibitors. The sample was sonicated and incubated on ice for 40 min and then centrifuged at 13,000 g for 10 min at 4 °C. The resulting supernatant was re-centrifuged and saved. The protein was estimated by Pierce BCA Protein Assay Kit (Thermo Scientific, USA), and the concentration of tissue lysates was diluted to 1 μg/μl. For determination of estrogen level, a commercially available ELISA kit (MEIMIAN, China) was used according to the manufacturer's instruction. Photometric analysis was done with the Varioskan Flash (Thermo Fisher, USA).

2.9. Statistics

All analyses were carried out using SPSS software, version 21.0 (SPSS Inc., Chicago, IL, USA), and all experiments were performed in at least triplicate. The data of net optical density of bands were presented as means ± standard deviations (SD), and a one-way analysis of variance followed by the Student-Newman-Keuls test was used to compare the treated groups with the control group. Several independent sample nonparametric tests were used to compare the proliferation rate and DEHP metabolites concentration in the four different

Table 1
Primer sequences for real-time PCR.

Gene	Forward Primer (5′-3′)	Reverse Primer (5′-3′)
Shh	AGGCTGGATTCGACTGGGTCTA	AACTTGGTGCCACCCTGCTC
Gli1	AACATGGCAGTCGGTAACATGAG	CCGCGTGTGTGTAGCCATTTAG
CyclinD1	CATCACAGCAGTCAGGGCAAC	TGATGCACCAGAGACTCAGAACAA
NMYC	GTGGAAGTTCGGGACACTTAGGAG	GGAATGACTTGTTTGGAAACTTGGA
GAPDH	GGCACAGTCAAGGCTGAGAATG	ATGGTGGTGAAGACGCCAGTA

groups, and the Kruskal-Wallis was used to compare the treated groups with the control group. A p value of <0.05 was considered statistically significant.

3. Results

3.1. Concentration of DEHP metabolites

MEHP, MECPP, MEOHP, MEMHP and MEHHP are valuable biomarkers of DEHP exposure, that, represent the major share of DEHP metabolites excreted in urine (Koch et al., 2006). The concentrations of the five DEHP metabolites were measured by GC-MS in the four groups. The concentrations of the five DEHP metabolites in the control group were below LOD. In addition, significantly higher concentrations of the five DEHP metabolites MEHP (Fig. 1. H=21.934, *df*==3, $p<0.05$), MECPP (Fig. 1. H=21.943, *df*=3, $p<0.05$), MEOHP (Fig. 1. H=21.934, *df*=3, $p<0.05$), MEMHP (Fig. 1. H=21.934, *df*=3, $p<0.05$) and MEHHP (Fig. 1. H=21.943, *df*=3, $p<0.05$) in the rats of 30, 300 and 750 mg/kg/d exposure groups were observed compared to untreated control rats. Concentrations of metabolites increased as the dose of DEHP contamination rose. These results demonstrated that we were successful in establishing an animal model for studying DEHP exposed.

3.2. Effect of maternal DEHP exposure on cerebellar granule cell proliferation in pups

The transcription factor Pax6 is predominantly expressed throughout the life cycle of GCs and has been considered as an appropriate marker for newly formed neurons (Chung et al., 2010; Engelkamp et al., 1999; Swanson et al., 2005). In addition, it can regulate the transition of GCs from proliferation to differentiation and is critical for cerebellar GC development (Yamasaki et al., 2001). PCNA was identified as a proliferation marker that is expressed in the nuclei of cells during the DNA synthesis phase of the cell cycle (Leonardi et al., 1992; Scheuer et al., 2017). Therefore, $Pax6^+$ and $PCNA^+$ were labelled in proliferating GCs. To explore the effects of DEHP exposure on the proliferation of GCs, the number of proliferating ($Pax6^+PCNA^+$) and non-proliferating ($Pax6^+$ $PCNA^-$) cell was observed in the EGL. The proliferation rate was found to be significantly decreased in rats belonging to the 300 and 750 mg/kg/d groups compared to the untreated control at PN 7 (Fig. 2A. H=17.473, *df*=3, $p<0.05$) and PN 14 (Fig. 2B. H=17.393, *df*=3, $p<0.05$) in male offspring. However, a significant difference was not found between male offspring of rats of the 30 mg/kg/d and control groups. In addition, for female offspring, a significant change was also not observed at PN 7 and PN 14 (Fig. 2C and D). These findings, therefore, suggested that DEHP exposure resulted in the inhibition of proliferation in GCPs for male offspring.

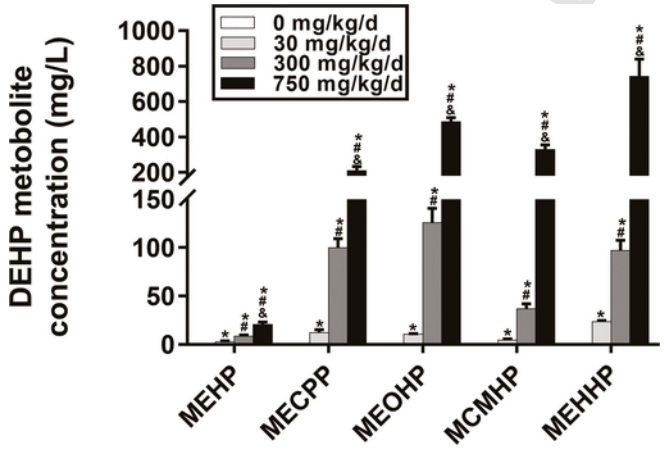

Fig. 1. The concentration of DEHP metabolites in the urine of pregnant rats on GD19. $^*p<0.05$ vs untreated control; $^\#p<0.05$ vs 30 mg/kg/d contamination group; $^\&$ $p<0.05$ vs 300 mg/kg/d contamination group; NO, not observed.

3.3. Effect of maternal DEHP exposure on the neuromotor development of pups

The neuromotor development of the offspring was assessed via behavioral tests, such as measuring a surface righting reflex, negative geotaxis reflex, as well as forelimb grip strength (Gallegos et al., 2016; Souza et al., 2015; Zhao et al., 2015). For the righting reflex, the latency times in male pups from the 300 and 750 mg/kg/d exposure groups were significantly increased compared with those of the control group at PN 3 (Fig. 3A. PN 3, $F_{(3,20)}=12.950$, $p<0.05$), PN 5, (Fig. 3A; $F_{(3,20)}=8.263$, $p<0.05$) and PN 7 (Fig. 3A; $F_{(3,20)}=22.039$, $p<0.05$). Subsequently, forelimb grip strength was measured from PN14 to PN16, and the hanging times in male offspring of the 300 and 750 mg/kg/d exposure groups were significantly reduced relative to those of the control group at PN 14 (Fig. 3C; $F_{(3,20)}=5.187$, $p<0.05$), PN 15 (Fig. 3C; $F_{(3,20)}=6.002$, $p<0.05$) and PN 16 (Fig. 3C; $F_{(3,20)}=5.296$, $p<0.05$). However, a difference in these four groups for female offspring was not evident (Fig. 3B and D). In addition, the negative geotaxis reflex test was conducted at

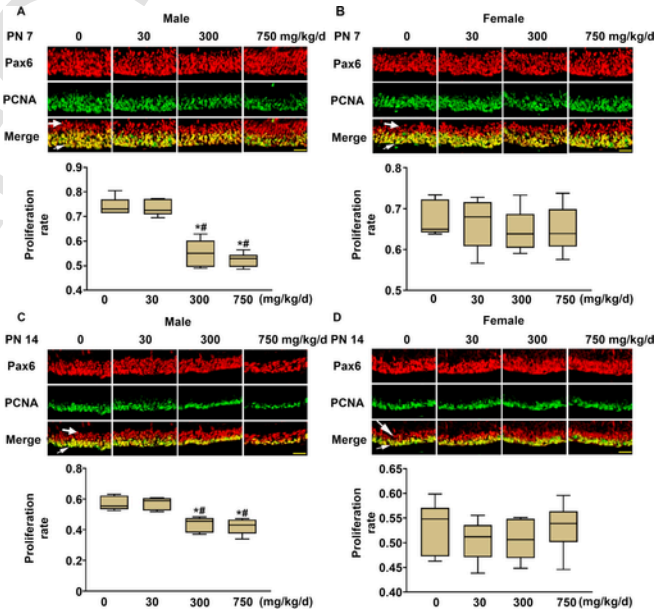

Fig. 2. Effect of maternal DEHP exposure on cerebellar granule cell proliferation in pups. Representative photomicrographs show fluorescence staining of anti-Pax6 (red) and *anti*-PCNA(green) antibodies in GCs on (A) PN 7 for males, (B) PN 7 for females, (C) PN 14 for males, and (D) PN14 for females Merged images show overlapping localization of these two proteins. Pax6+ and PCNA− (thick arrows) indicate the non-proliferating GCs, Pax6+ and PCNA+ (thin arrows) labeling in proliferating GCs. Scale bar = 25 μm. The scale bar is the same for all images in the figure. Corresponding bar graphs shows the proliferation rate of GCs in the four different groups. $^*p<0.05$ vs control; $^\#p<0.05$ vs 30 mg/kg/d contamination group (n=6).

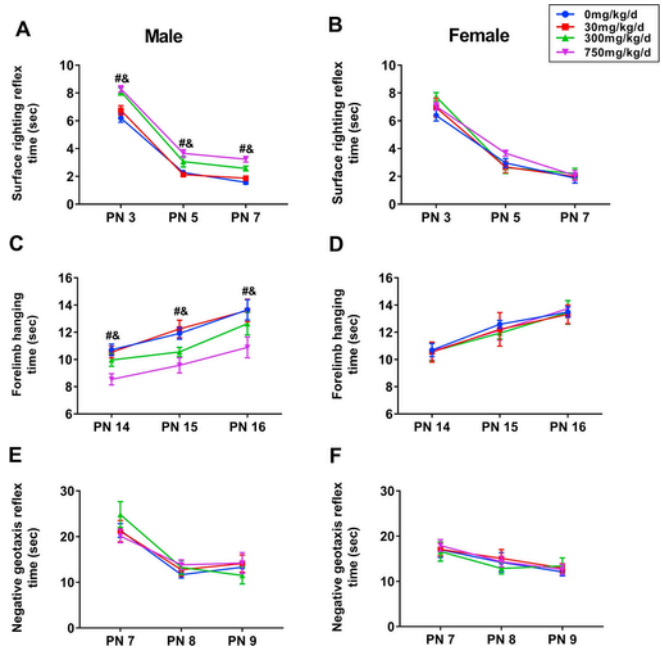

Fig. 3. Effect of maternal DEHP exposure on cerebellar behavioral changes in pups. (A) Male offspring surface righting reflex, (B) female offspring surface righting reflex, (C) forelimb grip strength of male offspring, (D) forelimb grip strength of female offspring, (E) male offspring negative geotaxis, and (F) female offspring negative geotaxis. $^{\#}p<0.05$ between control and 300 mg/kg/d contamination group; $^{\&}p<0.05$ between control and 750 mg/kg/d contamination group (n=6).

PN 7, PN 8 and PN 9, but significant variations were not found in male (Fig. 3E) and female (Fig. 3F) offspring of the four groups.

3.4. Effect of maternal DEHP exposure on the Shh signaling pathway

The Shh signaling pathway is responsible for rapid GCPs proliferation in the EGL (Kenney and Rowitch, 2000; Luca et al., 2016). And the loss of Shh leads to reduced proliferation and cerebellar defects (Vaillant and Monard, 2009). To quantify any alterations in the Shh pathway induced by DEHP exposure, RT–PCR was performed to assess mRNA levels including Shh, Gli1, N-Myc, cyclin D1. Compared with the control group, a significantly lower expression of Shh (Fig. 4A. PN 7: $F_{(3,20)}=7.636$, $p<0.05$; PN 14: $F_{(3,20)}=6.581$, $p<0.05$), Gli1 (Fig. 5A. PN 7: $F_{(3,20)}=4.955$, $p<0.05$; PN 14: $F_{(3,20)}=16.183$, $p<0.05$), N-Myc (Fig. 6A. PN 7: $F_{(3,20)}=18.214$, $p<0.05$; PN 14: $F_{(3,20)}=10.537$, $p<0.05$), and cyclin D1 mRNAs (Fig. 7A. PN 7: $F_{(3,20)}=8.903$, $p<0.05$; PN 14: $F_{(3,20)}=16.447$, $p<0.05$) was noted in male pups from the 300 and 750 mg/kg/d groups on PN 7 and PN 14. However, no evident differences were found between the control and 30 mg/kg/d group in the level of Shh, Gli1, N-Myc and cyclin D1 mRNAs. To confirm this, western blotting was conducted to measure Shh, Gli1, N-Myc, cyclinD1 protein levels. Consistently, a significant down-regulation of Shh (Fig. 4B. PN 7: $F_{(3,20)}=21.763$, $p<0.05$; PN 14: $F_{(3,20)}=20.409$, $p<0.05$), Gli1 (Fig. 5B. PN 7: $F_{(3,20)}=23.944$, $p<0.05$; PN 14: $F_{(3,20)}=13.554$, $p<0.05$), N-Myc (Fig. 6B. PN 7: $F_{(3,20)}=23.422$, $p<0.05$; PN 14: $F_{(3,20)}=21.473$, $p<0.05$) and cyclinD1 (Fig. 7B. PN 7: $F_{(3,20)}=28.053$, $p<0.05$; PN 14: $F_{(3,20)}=35.733$, $p<0.05$) was observed in pups from 300 to 750 mg/kg/d groups when compared with those of the control group on PN 7 and PN 14. In addition, a statistical difference between control and 30 mg/kg/d groups was

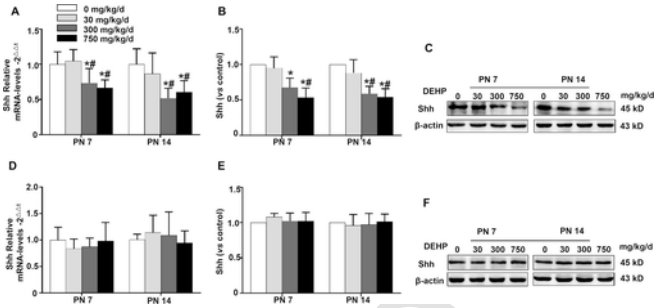

Fig. 4. Effect of maternal DEHP exposure on Shh mRNA and protein levels. (A) Relative expression of Shh mRNA was assayed by real-time PCR for male offspring on PN 7 and PN 14. (B) Semi-quantitative measurements of Shh for male offspring. (C) Western blots showing representative protein findings for male offspring. (D) The relative expression of Shh mRNA was assayed by real-time PCR for female offspring on PN 7 and PN 14. (E) Semi-quantitative measurements of Shh for female offspring. (F) Western blots showing representative protein findings for female offspring. Mean expression in offspring from 0, 30, 300, and 750 mg/kg/d exposure group is shown as fold-change compared to mean expression in the 0 mg/kg/d group that has been ascribed an arbitrary value of 1. With each time point, $^{*}p<0.05$ vs control; $^{\#}p<0.05$ vs 30 mg/kg/d contamination group (n=6).

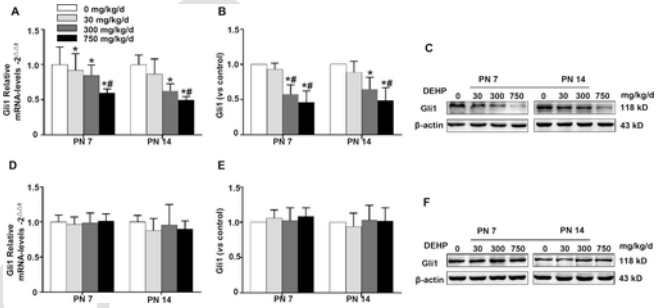

Fig. 5. Effect of maternal DEHP exposure on Gli1 mRNA and protein levels. (A) Relative expression of Gli1 mRNA was assayed by real-time PCR for male offspring on PN7 and PN14. (B) Semi-quantitative measurements of Gli1 for male offspring. (C) Western blots showing representative protein findings for male offspring. (D) The relative expression of Gli1 mRNA was assayed by real-time PCR for female offspring on PN7 and PN14. (E) Semi-quantitative measurements of Gli1 for female offspring. (F) Western blots showing representative protein findings for female offspring. With each time point, $^{*}p<0.05$ vs control, $^{\#}p<0.05$ vs 30 mg/kg/d contamination group (n=6).

not evident for these proteins, though their expression was slightly reduced in male pups. Of interest, a marked difference was not seen in levels of the four mRNA (Figs. 4D, 5D and 6D, 7D) and proteins (Figs. 4E, 5E and 6E, 7E) in female pups.

3.5. Effect of maternal DEHP exposure on the estrogen levels and aromatase expressions of offspring

Estrogen plays an important role in the central nervous system, which can affect the development, structure, function of brain tissue (Cersosimo and Benarroch, 2015). Aromatase is a key rate-limiting enzyme in the synthesis of estrogen and its activity is highly correlated with the concentration of estrogen in the brain (Wang et al., 2003). In order to investigate whether the gender difference in DEHP is related to the level of estrogen in the brain, the aromatase expression and estrogen level were measured in the four groups at PN 7 and PN 14. The results demonstrated that the aromatase expression was

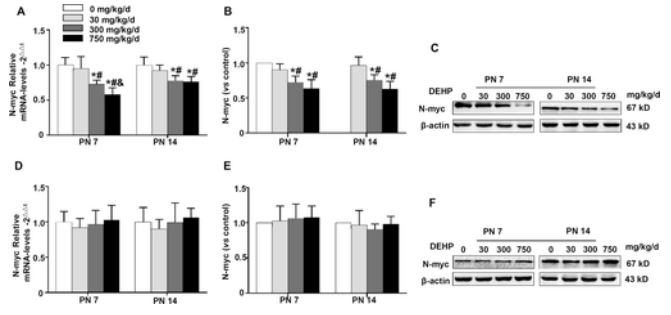

Fig. 6. Effect of maternal DEHP exposure on N-Myc mRNA and protein levels. (A) Relative expression of N-Myc mRNA was assayed by real-time PCR for male offspring on PN7 and PN14. (B) Semi-quantitative measurements of N-Myc for male offspring. (C) Western blots showing representative protein findings for male offspring. (D) The relative expression of N-Myc mRNA was assayed by real-time PCR for female offspring on PN 7 and PN 14. (E) Semi-quantitative measurements of N-Myc for female offspring. (F) Western blots showing representative protein findings for female offspring. With each time point, $^{*}p<0.05$ vs control, $^{\#}p<0.05$ vs 30 mg/kg/d contamination group (n = 6).

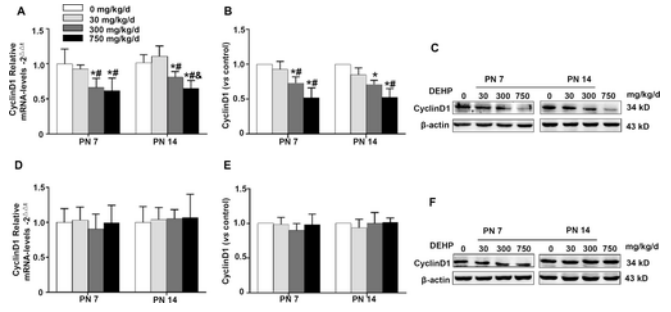

Fig. 7. Effect of maternal DEHP exposure on cyclin D1 mRNA and protein levels. (A) Relative expression of cyclin D1 mRNA was assayed by real-time PCR for male offspring on PN 7 and PN 14. (B) Semi-quantitative measurements of cyclin D1 for male offspring. (C) Western blots showing representative protein findings for male offspring. (D) The relative expression of cyclin D1 mRNA was assayed by real-time PCR for female offspring on PN 7 and PN 14. (E) Semi-quantitative measurements of cyclin D1 for female offspring. (F) Western blots showing representative protein findings for female offspring. With each time point, $^{*}p<0.05$ vs control, $^{\#}p<0.05$ vs 30 mg/kg/d exposure group (n = 6).

found to be significantly decreased in the 300 and 750 mg/kg/d groups in comparison with the control at PN 7 (Fig. 8B. PN 7: $F_{(3,20)}=19.373$, $p<0.05$) and PN 14 (Fig. 8B. PN 14: $F_{(3,20)}=42.721$, $p<0.05$) in male offspring. In addition, the estrogen level decreased in male offspring coming from the 300 and 750 mg/kg/d groups when compared to the untreated control at PN 7 (Fig. 8E. PN 7: $F_{(3,20)}=7.755$, $p<0.05$), and PN 14 (Fig. 8E. PN 14: $F_{(3,20)}=13.196$, $p<0.05$). Furthermore, there was no obvious difference among the four groups of female offspring in aromatase expression (Fig. 8D) and estrogen level (Fig. 8F) at PN 7 and PN 14.

4. Discussion

DEHP is a ubiquitous contaminant of the environment in modern life that causes a number of possible human health risks (Kamrin, 2009; Latini, 2005; Meeker et al., 2009). An increasing concern exists for the influence of DEHP exposure on developing neuronal networks and motor function in the young (Quinnies et al., 2017; Xu et al., 2015; Zarean et al., 2016). Therefore, in the present study, we es-

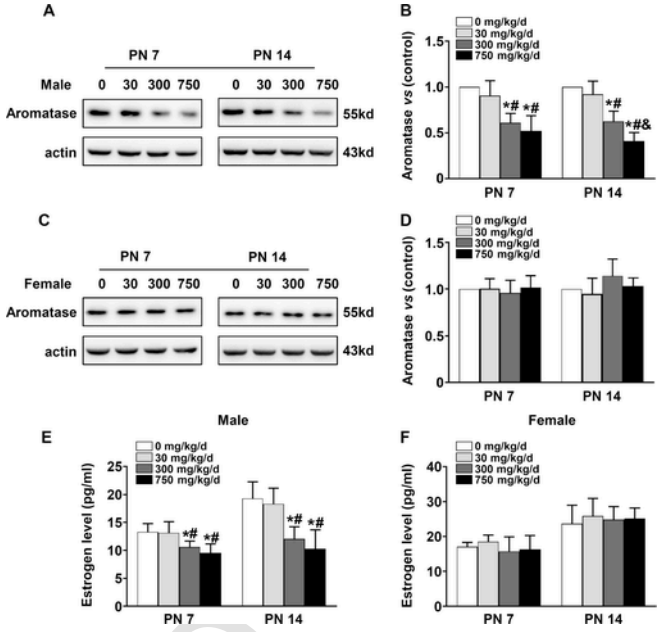

Fig. 8. Effect of maternal DEHP exposure on the estrogen levels and aromatase expressions of offspring. (A) Western blots showing representative protein findings for male offspring at PN 7 and PN 14. (B) Semi-quantitative measurements of aromatase for male offspring. (C) Western blots showing representative protein findings for female offspring at PN 7 and PN 14. (D) Semi-quantitative measurements of aromatase for female offspring. (E) Estrogen levels in male offspring cerebellum at PN 7 and PN14. (F) Estrogen levels in female offspring cerebellum at PN 7 and PN14. $^{*}p<0.05$ vs control, $^{\#}p<0.05$ vs 30 mg/kg/d exposure group, $^{\&}p<0.05$ vs 300 mg/kg/d exposure group (n = 6).

tablished an animal model for DEHP exposure by gavage with DEHP and observed the effects on neurodevelopment function in the cerebellums of offspring. We established an animal model of four groups, consisting of 0 mg/kg/d, 30 mg/kg/d, 300 mg/kg/d, 750 mg/kg/d. The proliferation of GCPs was our focus owing to its prominent role in cerebellar development and function. Moreover, Shh signaling is known to be related to the impairment of proliferation in cerebellar GCPs and the restrained proliferation may contribute to behavioral development disorders.

The development process of GCPs continues from embryonic 12.5 to PN 14, which is the basis for obtaining normal cerebellar size and lobules (Behesti and Marino, 2009). In this investigation, we found that the number of proliferating GCPs was decreased in 300 and 750 mg/kg/d groups at PN 7 and PN 14 compared with the untreated control group for male pups. This indicated that maternal DEHP exposure can impair the proliferation of GCPs in male offspring. Similarly, in an in vitro experiment that MEHP, the active metabolite of DEHP, was shown to inhibit PC12 cell proliferation by interfering with DNA synthesis and causing G2/M cell cycle arrest (Chen et al., 2011). The cell cycle checkpoints, G2/M and G1/S, are essential in maintaining DNA integrity and regulating cells through the cell cycle, damage of such checkpoints involves the disruption of cell proliferation (Pietenpol and Stewart, 2002). In addition, other EDCs can inhibit the proliferation of GCs (Collins et al., 2008). If DEHP exposure interferes with proliferation, subsequent consequences on GCs differentiation and apoptotic programs may occur, which ultimately damage the final formation and function of the cerebellum (Fox et al., 2010).

Behavioral development indicators are mainly used to identify behavioral dysfunction prior to various other clinical and biochemical

indicators. The righting reflex reflects the coordinated development of both sides of the body (Ronca and Alberts, 2000) while grip suspension reacts to the muscle strength and body balance ability of offspring (Zhao et al., 2015). In the present study, DEHP exposure during gestation and breast feeding impaired neuromotor development as reflected in the increased latency of the surface righting reflex and less grip strength in male offspring. Similarly, epidemiological studies have suggested that maternal exposure to DEHP is associated with delayed motor development, higher incidences of hypotonia and increased activity levels (Gascon et al., 2015; Park et al., 2015). Furthermore, animal studies have also shown that other EDCs can affect pup neurodevelopment, which can manifest as a delayed development of the righting reflex and impaired rotarod performance (Gonçalves et al., 2010; Kawasaki et al., 2003). Therefore, it was speculated that DEHP exposure affected the righting reflex and grip strength, which might be partly attributed to the impaired cerebellar development caused by the inhibition of GCPs proliferation.

The Shh signaling pathway is crucial to the regulation of GCPs proliferation (Jiang et al., 2011; Varjosalo and Taipale, 2008), and sustains normal cerebellum foliation in the developing cerebellum (Lewis et al., 2004). Shh is a major mitogenic factor secreted by Purkinje cell, which is thought to primarily and actively regulate the amplification of GCPs proliferation in the EGL (Luca et al., 2016; Smeyne et al., 1995). The addition of Shh to GCPs results in a prolonged proliferation reaction, whereas GCP proliferation is inhibited by the down-regulation of Shh (Dahmane and Ruizialtaba, 1999; Lewis et al., 2004). In this study, it was found that DEHP exposure decreased Shh mRNA levels and protein expression in males at PN 7 and PN 14. Similarly, previous research has shown that *trans*-placental exposure to Bisphenol A, another representative of EDCs, can inhibit neural precursor cell proliferation by down-regulating of Shh (Yang et al., 2014). Therefore, it is speculated that decreased Shh expression in males may contribute to the inhibition of GCPs proliferation.

Gli1 is a transcription factor of the Shh signaling pathway and represents a feedback pathway that acts as a sensor of Shh activity (Cohen et al., 2011; Corrales et al., 2004; Feijóo et al., 2011). Activated Gli1 can induce the expression of pro-proliferative target genes, including N-Myc and cyclin D1, which promote GCPs proliferation. Shh inhibitor treatment of the cultured cell can lead to a reduction in Gli1 expression (He et al., 2014). In this study, we observed that Gli1 protein and mRNA levels decreased in male offspring after maternal DEHP exposure. Therefore, this suggest that maternal DEHP exposure may reduce the Gli1 protein level via the down-regulation of Shh. In turn, this may interfere with cerebellar GCPs proliferation and cause a cerebellar developmental disorder.

N-Myc plays a crucial role as a downstream effector of the Shh signaling pathway in GCP proliferation during the development of the cerebellum (Oliver et al., 2003). N-Myc is delivered by GCPs in the EGL of the cerebellum and activated in response to Shh (Kenney et al., 2003). The present study indicates that maternal DEHP exposure inhibits N-Myc mRNA and protein levels in male offspring at PN 7 and PN 14. Another study has reported that GCPs lacking N-Myc expression after utilizing a Nestin-Cre transgene showed impaired GCPs proliferation and a reduction in GC numbers accompanied by a smaller and disorganized cerebellum (Knoepfler et al., 2002; Ma et al., 2015). Our data suggest that the down-regulation of N-Myc protein expression in the cerebellum of male offspring caused by maternal DEHP exposure may lead to reduced GCPs proliferation.

The mitogenic effects of Shh on GCPs appear to be regulated by downstream genes, such as cyclin D1, whose expression is specifically up-regulated in response to Shh (Zhao et al., 2002). Cyclin D1 is a direct target of the Shh pathway and functions to regulate cell-cycle progression in granule cell precursors by increasing G1 cyclin expression (Kenney and Rowitch, 2000; Pogoriler et al., 2006). It is up-regulated in response to Shh signaling in GC cells and plays an essential role in the postnatal expansion of the GC population (Rowitch and David, 2000). In this study, we observed that maternal DEHP exposure could decrease cyclin D1 mRNA protein level in male offspring. Consistent with our data, conditional mutant mice in which cyclin D1 was deleted in neuronal precursors displayed reduced cell proliferation and caused a delay in acquiring normal cerebellum (Pogoriler et al., 2006). Therefore, we speculate that maternal DEHP exposure may restrain GCPs proliferation by decreasing cyclin D1 expression to ultimately affect the development of GCs.

In our investigation, an interesting phenomenon occurred was that obvious changes were not found in female pups, suggesting that male offspring may be more susceptible to DEHP exposure than female rats. The increased sensitivity to DEHP in males may be attributed to its action as an anti-androgenic compound. Researches have shown that DEHP treatment early in life reduced estrogen levels in the male brain by reducing the production of testosterone and inhibiting aromatase activity (Andrade et al., 2006; Borch et al., 2006). Our results also indicated that DEHP exposure decreased estrogen level of cerebellum in male pups and reduced the aromatase expression at PN 7 and PN 14. Aromatase converts peripheral circulating androgens into estrogen that is the main source of this hormone in the male brain (Wu et al., 2009). The level of estrogen in the brain is associated with the development of GCPs. The decreased estrogen level in male offspring might involve in the phenomenon that male offspring may be more vulnerable to DEHP exposure than female rats. During the development of the rat, brain sexual differentiation has a critical period of about a few days before birth to a dozen days after birth. It was speculated that DEHP affected the pattern of brain differentiation by interfering with the synthesis of estrogen in a critical period of development, making the male brain more susceptible to DEHP.

In conclusion, maternal DEHP exposure of 300 and 750 mg/kg/d inhibited the proliferation of cerebellar GCPs in male offspring of Wistar rats, which may be due to the down-regulation of Shh signaling, to contribute to the impairment of coordinated movement and muscle strength. Furthermore, the gender difference in DEHP may related to the decreased estrogen level and aromatase expression in male pups. This study will contribute to a better understanding of neurodevelopment after DEHP exposure. In addition, thorough investigations are needed to delineate the underlying differential mechanism(s) of DEHP induced changes in cerebellar development.

Conflicts of interest

The authors declare that they have no competing interests.

Acknowledgments

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References

Andrade, A.J., Grande, S.W., Talsness, C.E., Grote, K., Chahoud, I., 2006. A dose-response study following in utero and lactational exposure to di-(2-ethylhexyl)-phthalate (DEHP): non-monotonic dose-response and low dose effects on rat brain aromatase activity. Toxicology 227, 185–192.

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Behesti, H., Marino, S., 2009. Cerebellar granule cells: insights into proliferation, differentiation, and role in medulloblastoma pathogenesis. Int. J. Biochem. Cell Biol. 41, 435–445.
Bernard, L., Cueff, R., Chagnon, M., Abdoulouhab, F., Décaudin, B., Breysse, C., Kauffmann, S., Cosserant, B., Souweine, B., Sautou, V., 2015. Migration of plasticizers from PVC medical devices: development of an infusion model. Int. J. Pharm. 494, 136–145.
Boas, M., Frederiksen, H., Feldtrasmussen, U., Skakkebæk, N.E., Hegedüs, L., Hilsted, L., Juul, A., Main, K.M., 2010. Childhood exposure to phthalates: associations with thyroid function, insulin-like growth factor I, and growth. Environ. Health Perspect. 118, 1458–1464.
Borch, J., Metzdorff, S.B., Vinggaard, A.M., Brokken, L., Dalgaard, M., 2006. Mechanisms underlying the anti-androgenic effects of diethylhexyl phthalate in fetal rat testis. Toxicology 223, 144–155.
Brys, I., Pupe, S., Bizarro, L., 2014. Attention, locomotor activity and developmental milestones in rats prenatally exposed to ethanol. Int. J. Dev. Neurosci. Off. J. Int. Soc. Dev. Neurosci. 38, 161–168.
Cairns, J., Swanson, D., Yeung, J., Sinova, A., Chan, R., Potluri, P., Dickson, P., Mittleman, G., Dan, G., 2016. Abnormalities in the Structure and Function of Cerebellar Neurons and Neuroglia in the Lc/+ Chimeric Mouse Model of Variable Developmental Purkinje Cell Loss. Cerebellum, 1–15.
Campioli, E., Martinezarguelles, D.B., Papadopoulos, V., 2014. In utero exposure to the endocrine disruptor di-(2-ethylhexyl) phthalate promotes local adipose and systemic inflammation in adult male offspring. Nutr. Diabetes 4, e115.
Ceccarelli, M., Micheli, L., D'Andrea, G., De, B.M., Scheijen, B., Ciotti, M., Leonardi, L., Luvisetto, S., Tirone, F., 2015. Altered cerebellum development and impaired motor coordination in mice lacking the Btg1 gene: involvement of cyclin D1. Dev. Biol. 408, 109–125.
Cersosimo, M.G., Benarroch, E.E., 2015. Estrogen actions in the nervous system: complexity and clinical implications. Neurology 85, 263–273.
Chang-Liao, W.L., Hou, M.L., Chang, L.W., Lee, C.J., Tsai, Y.M., Lin, L.C., Tsai, T.H., 2013. Determination and pharmacokinetics of di-(2-ethylhexyl) phthalate in rats by ultra performance liquid chromatography with tandem mass spectrometry. Molecules 18, 11452–11466.
Chen, T., Yang, W., Li, Y., Chen, X., Xu, S., 2011. Mono-(2-ethylhexyl) phthalate impairs neurodevelopment: inhibition of proliferation and promotion of differentiation in PC12 cells. Toxicol. Lett. 201, 34–41.
Chiellini, F., Ferri, M., Latini, G., 2011. Physical-chemical assessment of di-(2-ethylhexyl)-phthalate leakage from poly(vinyl chloride) endotracheal tubes after application in high risk newborns. Int. J. Pharm. 409, 57–61.
Cho, S.C., Bhang, S.Y., Hong, Y.C., Shin, M.S., Kim, B.N., Kim, J.W., Yoo, H.J., Cho, I.H., Kim, H.W., 2010. Relationship between environmental phthalate exposure and the intelligence of school-age children. Environ. Health Perspect. 118, 1027–1032.
Chopra, V., Harley, K., Lahiff, M., Eskenazi, B., 2014. Association between phthalates and attention deficit disorder and learning disability in U.S. children, 6–15 years. Environ. Res. 128, 64–69.
Chung, S.H., Kim, C.T., Jung, Y.H., Lee, N.S., Jeong, Y.G., 2010. Early cerebellar granule cell migration in the mouse embryonic development. Anat. Cell Biol. 43, 86–95.
Cohen, M., Kicheva, A., Ribeiro, A., Blassberg, R., Page, K.M., Barnes, C.P., Briscoe, J., 2011. Ptch1 and Gli regulate Shh signalling dynamics via multiple mechanisms. Nat. Commun. 6, 6709.
Collins, L.L., Williamson, M.A., Thompson, B.D., Dever, D.P., Gasiewicz, T.A., Opanashuk, L.A., 2008. 2,3,7,8-Tetracholorodibenzo-p-dioxin exposure disrupts granule neuron precursor maturation in the developing mouse cerebellum. Toxicol. Sci. Off. J. Soc. Toxicol. 103, 125–136.
Corrales, J.D., Rocco, G.L., Blaess, S., Guo, Q., Joyner, A.L., 2004. Spatial pattern of sonic hedgehog signaling through Gli genes during cerebellum development. Development 131, 5581.
Dahmane, N., Ruizialtaba, A., 1999. Sonic hedgehog regulates the growth and patterning of the cerebellum. Development 126, 3089–3100.
Dai, Y., Yang, Y., Xu, X., Hu, Y., 2015. Effects of uterine and lactational exposure to di-(2-ethylhexyl) phthalate on spatial memory and NMDA receptor of hippocampus in mice. Horm. Behav. 71, 41–48.
Deuschl, G.N., Raethjen, J., Lindemann, M., Krack, P., 2001. The pathophysiology of tremor. A review. Muscle Nerve 24, 716–735.
Engelkamp, D., Rashbass, P., Seawright, A., Van, H.V., 1999. Role of Pax6 in development of the cerebellar system. Development 126, 3585–3596.
Erythropel, H.C., Maric, M., Nicell, J.A., Leask, R.L., Yargeau, V., 2014. Leaching of the plasticizer di (2-ethylhexyl)phthalate (DEHP) from plastic containers and the question of human exposure. Appl. Microbiol. Biotechnol. 98, 9967–9981.
Feijóo, C.G., Oñate, M.G., Milla, L.A., Palma, V.A., 2011. Sonic hedgehog (Shh)-Gli signaling controls neural progenitor cell division in the developing tectum in zebrafish. Eur. J. Neurosci. 33, 589–598.
Fernandez, C., Tatard, V.M., Bertrand, N., Dahmane, N., 2010. Differential modulation of sonic-hedgehog-induced cerebellar granule cell precursor proliferation by the IGF signaling network. Dev. Neurosci. 32, 59–70.
Fox, D.A., Opanashuk, L.A., Weiss, B., 2010. Gene-chemical interactions in the developing mammalian nervous system: effects on proliferation, neurogenesis and differentiation. Neurotoxicology 31, 589–597.
Fromme, H., Gruber, L., Seckin, E., Raab, U., Zimmermann, S., Kiranoglu, M., Schlummer, M., Schwegler, U., Smolic, S., Völkel, W., 2011. Phthalates and their metabolites in breast milk--results from the bavarian monitoring of breast milk (BAMBI). Environ. Int. 37, 715–722.
Gallegos, C.E., Bartos, M., Bras, C., Gumilar, F., Antonelli, M.C., Minetti, A., 2016. Exposure to a glyphosate-based herbicide during pregnancy and lactation induces neurobehavioral alterations in rat offspring. Neurotoxicology 53, 20–28.
Gascon, M., Valvi, D., Forns, J., Casas, M., Martínez, D., Júlvez, J., Monfort, N., Ventura, R., Sunyer, J., Vrijheid, M., 2015. Prenatal exposure to phthalates and neuropsychological development during childhood. Int. J. Hyg Environ. Health 218, 550–558.
Ghisari, M., Bonefeld-Jorgensen, E.C., 2009. Effects of plasticizers and their mixtures on estrogen receptor and thyroid hormone functions. Toxicol. Lett. 189, 67–77.
Gonçalves, C.R., Cunha, R.W., Barros, D.M., Martínez, P.E., 2010. Effects of prenatal and postnatal exposure to a low dose of bisphenol A on behavior and memory in rats. Environ. Toxicol. Pharmacol. 30, 195–201.
Hannon, P.R., Brannick, K.E., Wang, W., Gupta, R.K., Flaws, J.A., 2015. Di(2-ethylhexyl) phthalate inhibits antral follicle growth, induces atresia, and inhibits steroid hormone production in cultured mouse antral follicles. Toxicol. Appl. Pharmacol. 284, 42–53.
He, X.L., Zhang, L.G., Chen, Y., Remke, M., Shih, D., Lu, F., Wang, H., Deng, Y., Yu, Y., Xia, Y., 2014. The g-protein alpha subunit Gsα is a tumor suppressor in sonic hedgehog-driven medulloblastoma. Nat. Med. 20, 1035–1042.
Hobbs, C., Thoresen, M., Tucker, A., Aquilina, K., Chakkarapani, E., Dingley, J., 2008. Xenon and hypothermia combine additively, offering long-term functional and histopathologic neuroprotection after neonatal hypoxia/ischemia. Stroke J. Cerebral Circ. 39, 1307.
Howdeshell, K.L., Wilson, V.S., Furr, J., Lambright, C.R., Rider, C.V., Blystone, C.R., Hotchkiss, A.K., G.L Jr., 2008. A mixture of five phthalate esters inhibits fetal testicular testosterone production in the sprague-dawley rat in a cumulative, dose-additive manner. Toxicol. Sci. 105, 153–165.
Jiang, M., Stanke, J., Lahti, J.M., 2011. The connections between neural crest development and neuroblastoma. Curr. Top. Dev. Biol. 94, 77–127.
Jing, L., Ming, X., Alhashem, H.M., Yong, Z., Tilak, V., Patel, A., Siegel, A., Jiang, H.Y., Bekker, A., 2014. Effects of prenatal propofol exposure on postnatal development in rats. Neurotoxicol. Teratol. 43, 51–58.
Kamrin, M.A., 2009. Phthalate risks, phthalate regulation, and public health: a review. J. Toxicol. Environ. Health B Crit. Rev. 12, 157–174.
Kawasaki, K., Takatori, A., Ishii, Y., 2003. Effects of perinatal exposure to bisphenol A on the behavior of offspring in F344 rats. Environ. Toxicol. Pharmacol. 14, 99.
Kay, V.R., Chambers, C., Foster, G.W., 2013. Reproductive and developmental effects of phthalate diesters in females. Crit. Rev. Toxicol. 43, 200.
Kenney, A.M., Rowitch, D.H., 2000. Sonic hedgehog promotes G1 cyclin expression and sustained cell cycle progression in mammalian neuronal precursors. Mol. Cell. Biol. 20, 9055–9067.

Kenney, A.M., Cole, M.D., Rowitch, D.H., 2003. Nmyc upregulation by sonic hedgehog signaling promotes proliferation in developing cerebellar granule neuron precursors. Development 130, 15–28.
Kim, Y., Ha, E.H., Kim, E.J., Park, H., Ha, M., Kim, J.H., Hong, Y.C., Chang, N., Kim, B.N., 2011. Prenatal exposure to phthalates and infant development at 6 Months: prospective mothers and Children's environmental health (MOCEH) study. Environ. Health Perspect. 119, 1495–1500.
Klein, R.S., Rubin, J.B., Gibson, H.D., Dehaan, E.N., Alvarezhernandez, X., Segal, R.A., Luster, A.D., 2001. SDF-1 alpha induces chemotaxis and enhances Sonic hedgehog-induced proliferation of cerebellar granule cells. Development 128, 1971–1981.
Knoepfler, P.S., Cheng, P.F., Eisenman, R.N., 2002. N-myc is essential during neurogenesis for the rapid expansion of progenitor cell populations and the inhibition of neuronal differentiation. Gene Dev. 16, 2699–2712.
Koch, H.M., Preuss, R., Angerer, J., 2006. Di(2-ethylhexyl)phthalate (DEHP): human metabolism and internal exposure - an update and latest results. Int. J. Androl. 29, 155–165.
Koch, H.M., Preuss, R., Angerer, J., 2010. Di(2-ethylhexyl)phthalate (DEHP): human metabolism and internal exposure an update and latest results 1 . Int. J. Androl. 29, 155–165.
Koziol, L.F., Budding, D.E., Chidekel, D., 2012. From movement to thought: executive function, embodied cognition, and the cerebellum. Cerebellum 11, 505–525.
Latini, G., 2005. Monitoring phthalate exposure in humans. Clin. Chim. Acta 361, 20–29.
Leonardi, E., Girlando, S., Serio, G., Mauri, F.A., Perrone, G., Scampini, S., Palma, P.D., Barbareschi, M., 1992. PCNA and Ki67 expression in breast carcinoma: correlations with clinical and biological variables. J. Clin. Pathol. 45, 416–419.
Lewis, P.M., Gritli-Linde, A., Smeyne, R., Kottmann, A., Mcmahon, A.P., 2004. Sonic hedgehog signaling is required for expansion of granule neuron precursors and patterning of the mouse cerebellum. Dev. Biol. 270, 393–410.
Luca, A.D., Cerrato, V., Fucà, E., Parmigiani, E., Buffo, A., Leto, K., 2016. Sonic hedgehog patterning during cerebellar development. Cell. Mol. Life Sci. 73, 291–303.
Luu, B.E., Green, S.R., Childers, C.L., Holahan, M.R., Storey, K.B., 2017. The roles of hippocampal microRNAs in response to acute postnatal exposure to di(2-ethylhexyl) phthalate in female and male rats. Neurotoxicology 59, 98–104.
Ma, M., Wu, W., Li, Q., Li, J., Sheng, Z., Shi, J., Zhang, M., Yang, H., Wang, Z., Sun, R., 2015. N-myc is a key switch regulating the proliferation cycle of postnatal cerebellar granule cell progenitors. Sci. Rep. 5, 12740.
Manto, M.U., Patrice, J., 2012. Cerebellum: links between development, developmental disorders and motor learning. Front. Neuroanat. 6, 1.
Martinez-Arguelles, D.B., Papadopoulos, V., 2016. Prenatal phthalate exposure: epigenetic changes leading to lifelong impact on steroid formation. Andrology 4, 573–584.
Martino-Andrade, A.J., Chahoud, I., 2010. Reproductive toxicity of phthalate esters. Mol. Nutr. Food Res. 54, 148–157.
Meeker, J.D., Sathyanarayana, S., Swan, S.H., 2009. Phthalates and other additives in plastics: human exposure and associated health outcomes. Phil. Trans. Roy. Soc. Lond. 364, 2097–2113.
Miyazawa, K., Himi, T., Garcia, V., Yamagishi, H., Sato, S., Ishizaki, Y., 2000. A role for p27/Kip 1 in the control of cerebellar granule cell precursor proliferation. J. Neurosci. Off. J. Soc. Neurosci. 20, 5756–5763.
Moore, R.W., Rudy, T.A., Lin, T.M., Ko, K., Peterson, R.E., 2001. Abnormalities of sexual development in male rats with in utero and lactational exposure to the antiandrogenic plasticizer Di(2-ethylhexyl) phthalate. Environ. Health Perspect. 109, 229–237.
Oliver, T.G., Grasfeder, L.L., Carroll, A.L., Kaiser, C., Gillingham, C.L., Lin, S.M., Wickramasinghe, R., Scott, M.P., Wechslerreya, R.J., 2003. Transcriptional profiling of the Sonic hedgehog response: a critical role for N-myc in proliferation of neuronal precursors. Proc. Natl. Acad. Sci. U.S.A. 100, 7331–7336.
Omer, V.E.V.S., Ali, S.F., Holson, R.R., Duhart, H.M., Scalzo, F.M., W.S Jr., 1991. Behavioral and neurochemical effects of prenatal methylenedioxymethamphetamine (MDMA) exposure in rats. Neurotoxicol. Teratol. 13, 13–20.
Park, S., Lee, J.M., Kim, J.W., Cheong, J.H., Yun, H.J., Hong, Y.C., Kim, Y., Han, D.H., Yoo, H.J., Shin, M.S., 2015. Association between phthalates and externalizing behaviors and cortical thickness in children with attention deficit hyperactivity disorder. Psychol. Med. 45, 1601.
Pietenpol, J.A., Stewart, Z.A., 2002. Cell cycle checkpoint signaling: cell cycle arrest versus apoptosis. Toxicology s 181–182, 475–481.
Pocar, P., Fiandanese, N., Berrini, A., Secchi, C., Borromeo, V., 2017. Maternal exposure to di(2-ethylhexyl)phthalate (DEHP) promotes the transgenerational inheritance of adult-onset reproductive dysfunctions through the female germline in mice. Toxicol. Appl. Pharmacol. 322, 113–121.
Pogoriler, J., Millen, K., M, Du, W., 2006. Loss of cyclin D1 impairs cerebellar development and suppresses medulloblastoma formation. Development 133, 3929–3937.
Quinnies, K.M., Harris, E.P., Snyder, R.W., Sumner, S.S., Rissman, E.F., 2017. Direct and transgenerational effects of low doses of perinatal di-(2-ethylhexyl) phthalate (DEHP) on social behaviors in mice. PloS One 12, e0171977.
Reagan-shaw, S., Nihal, M., Ahmad, N., 2008. Dose translation from animal to human studies revisited. Faseb J. Off. Publ. Fed. Am. Soc. Exp. Biol. 22, 659.
Rebuli, M.E., Patisaul, H.B., 2016. Assessment of sex specific endocrine disrupting effects in the prenatal and pre-pubertal rodent brain. J. Steroid Biochem. Mol. Biol. 160, 148–159.
Ronca, A.E., Alberts, J.R., 2000. Effects of prenatal spaceflight on vestibular responses in neonatal rats. J. Appl. Physiol. 89, 2318–2324.
Rowitch, A.M.K., David, H., 2000. Sonic hedgehog promotes G1 cyclin expression and sustained cell cycle progression in mammalian neuronal precursors. Mol. Cell. Biol. 20, 9055.
Sakhi, A.K., Sabaredzovic, A., Cequier, E., Thomsen, C., 2017. Phthalate metabolites in Norwegian mothers and children: levels, diurnal variation and use of personal care products. Sci. Total Environ. 599–600, 1984–1992.
Scheuer, T., Sharkovska, Y., Tarabykin, V., Marggraf, K., Brockmöller, V., Bührer, C., Endesfelder, S., Schmitz, T., 2017. Neonatal hyperoxia perturbs neuronal development in the cerebellum. Mol. Neurobiol. 1–15.
Sekulić, S., Lukac, D., Drabsin, M., Suknjaja, V., Keković, G., Grbić, G., Martać, L., 2009. The righting reflex from a supine to a prone position in the Guinea pig fetus. Gen. Physiol. Biophys. 28, 284–288.
Shin, I.S., Lee, M.Y., Cho, E.S., Choi, E.Y., Son, H.Y., Lee, K.Y., 2014. Effects of maternal exposure to di(2-ethylhexyl)phthalate (DEHP) during pregnancy on susceptibility to neonatal asthma. Toxicol. Appl. Pharmacol. 274, 402–407.
Smeyne, R.J., Chu, T., Lewin, A., Bian, F., Sanlioglu, S., Kunsch, C., Lira, S.A., Oberdick, J., 1995. Local control of granule cell generation by cerebellar Purkinje cells. Mol. Cell. Neurosci. 6, 230–251.
Smith, C.A., Holahan, M.R., 2014. Reduced hippocampal dendritic spine density and BDNF expression following acute postnatal exposure to di(2-ethylhexyl) phthalate in male Long Evans rats. PloS One 9, e109522.
Smith, C.A., Macdonald, A., Holahan, M.R., 2011. Acute postnatal exposure to di(2-ethylhexyl) phthalate adversely impacts hippocampal development in the male rat. Neuroscience 193, 100–108.
Souza, A.C., Souza, A., Medeiros, L.F., De, O.C., Scarabelot, V.L., Da, S.R., Bogo, M.R., Capiotti, K.M., Kist, L.W., Bonan, C.D., 2015. Maternal caffeine exposure alters neuromotor development and hippocampus acetylcholinesterase activity in rat offspring. Brain Res. 1595, 10–18.
Subkhankulova, T., Zhang, X., Leung, C., Marino, S., 2010. Bmi1 directly represses p21Waf1/Cip1 in Shh-induced proliferation of cerebellar granule cell progenitors. Mol. Cell. Neurosci. 45, 151–162.
Swanson, D.J., Tong, Y., Goldowitz, D., 2005. Disruption of cerebellar granule cell development in the Pax6 mutant, Sey mouse. Dev. Brain Res. 160, 176–193.
Téllezrojo, M.M., Cantoral, A., Cantonwine, D.E., Schnaas, L., Peterson, K., Hu, H., Meeker, J.D., 2013. Prenatal urinary phthalate metabolites levels and neurodevelopment in children at two and three years of age. Sci. Total Environ. 462, 386–390.
Thach, W.T., H.P.G, Keating, J.G., 1992. The cerebellum and the adaptive coordination of movement. Annu. Rev. Neurosci. 15, 403–442.
Tickner, J.A., Schettler, T., Guidotti, T., Mccally, M., Rossi, M., 2001. Health risks posed by use of Di-2-ethylhexyl phthalate (DEHP) in PVC medical devices: a critical review. Am. J. Ind. Med. 39, 100–111.
Timmann, D., Drepper, J., Frings, M., Maschke, M., Richter, S., Gerwig, M., Kolb, F.P., 2010. The human cerebellum contributes to

motor, emotional and cognitive associative learning. A review. Cortex 46, 845–857.
Vaillant, C., Monard, D., 2009. SHH pathway and cerebellar development. Cerebellum 8, 291–301.
Valera, E.M., Faraone, S.V., Murray, K.E., Seidman, L.J., 2007. Meta-analysis of structural imaging findings in attention-deficit/hyperactivity disorder. Biol. Psychiatry 61, 1361–1369.
Varjosalo, M., Taipale, J., 2008. Hedgehog: functions and mechanisms. Gene Dev. 22, 2454–2472.
Vieira, D.M.B.B., Trigueiro, M.J., Rodrigues, P.P., 2018. Systematic overview of neuroanatomical differences in ADHD: definitive evidence. Dev. Neuropsychol. 43, 52–68.
Virtanen, H.E., Rajpertde, M.E., Main, K.M., Skakkebaek, N.E., Toppari, J., 2005. Testicular dysgenesis syndrome and the development and occurrence of male reproductive disorders. Toxicol. Appl. Pharmacol. 207, 501–505.
Wallace, V.A., 1999. Purkinje-cell-derived Sonic hedgehog regulates granule neuron precursor cell proliferation in the developing mouse cerebellum. Curr. Biol. 9, 445–448.
Wang, L., Andersson, S., Warner, M., Gustafsson, J.A., 2003. Estrogen receptor (ER)beta knockout mice reveal a role for ERbeta in migration of cortical neurons in the developing brain. Proc. Natl. Acad. Sci. U. S. A. 100, 703–708.
Wang, I.J., Lin, C.C., Lin, Y.J., Hsieh, W.S., Chen, P.C., 2014. Early life phthalate exposure and atopic disorders in children: a prospective birth cohort study. Environ. Int. 62, 48–54.
Wang, Y., Yang, Q., Liu, W., Yu, M., Zhang, Z., Cui, X., 2016. DEHP exposure in utero disturbs sex determination and is potentially linked with precocious puberty in female mice. Toxicol. Appl. Pharmacol. 307, 123.
Wang, B., Liu, F., Dong, J., You, M., Fu, Y., Li, C., Lu, Y., Chen, J., 2017. Maternal exposure to environmental DEHP exacerbated OVA-induced asthmatic responses in rat offspring. Sci. Total Environ. 615, 253–261.
Whyatt, R.M., Liu, X., Rauh, V.A., Calafat, A.M., Just, A.C., Hoepner, L., Diaz, D., Quinn, J., Adibi, J., Perera, F.P., 2012. Maternal prenatal urinary phthalate metabolite concentrations and child mental, psychomotor, and behavioral development at 3 Years of age. Environ. Health Perspect. 120, 290–295.
Wittassek, M., Angerer, J., 2008. Phthalates: metabolism and exposure. Int. J. Androl. 31, 131–138.
Wittassek, M., Koch, H.M., Angerer, J., Brüning, T., 2011. Assessing exposure to phthalates – the human biomonitoring approach. Mol. Nutr. Food Res. 55, 7–31.
Wu, M.V., Manoli, D.S., Fraser, E.J., Coats, J.K., Tollkuhn, J., Honda, S.I., Harada, N., Shah, N.M., 2009. Estrogen masculinizes neural pathways and sex-specific behaviors. Cell 139, 61–72.
Xu, X., Yang, Y., Wang, R., Wang, Y., Ruan, Q., Lu, Y., 2015. Perinatal exposure to di-(2-ethylhexyl) phthalate affects anxiety- and depression-like behaviors in mice. Chemosphere 124, 22.
Yamasaki, T., Kawaji, K., Ono, K., Bito, H., Hirano, T., Osumi, N., Kengaku, M., 2001. Pax6 regulates granule cell polarization during parallel fiber formation in the developing cerebellum. Development 128, 3133.
Yang, C.W., Chou, W.C., Chen, K.H., Cheng, A.L., Mao, I.F., Chao, H.R., Chuang, C.Y., 2014. Visualized Gene Network Reveals the Novel Target Transcripts Sox2 and Pax6 of Neuronal Development in Trans-placental Exposure to Bisphenol A, vol. 9, e100576.
You, M., Dong, J., Fu, Y., Cong, Z., Fu, H., Wei, L., Wang, Y., Wang, Y., Chen, J., 2018. Exposure to di-(2-ethylhexyl) phthalate during perinatal period gender-specifically impairs the dendritic growth of pyramidal neurons in rat offspring. Front. Neurosci. 12, 444.
Zarean, M., Keikha, M., Poursafa, P., Khalighinejad, P., Amin, M., Kelishadi, R., 2016. A systematic review on the adverse health effects of di-2-ethylhexyl phthalate. Environ. Sci. Pollut. Res. Int. 23, 24642–24693.
Zhao, Q., Kho, A., Kenney, A.M., Yuk, D.I., Kohane, I., Rowitch, D.H., 2002. Correction: identification of genes expressed with temporal-spatial restriction to developing cerebellar neuron precursors by a functional Genomic approach. Proc. Natl. Acad. Sci. U.S.A. 99, 5704–5709.
Zhao, X., Cheng, Z., Zhu, Y.I., Li, S., Zhang, L., Luo, Y., 2015. Effects of paternal cadmium exposure on the sperm quality of male rats and the neurobehavioral system of their offspring. Exp. Therap. Med. 10, 2356–2360.