



# Effects of uterine and lactational exposure to di-(2-ethylhexyl) phthalate on spatial memory and NMDA receptor of hippocampus in mice



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## ABSTRACT

Di-(2-ethylhexyl) phthalate (DEHP) is an environmental endocrine disrupter. Currently, little is known about neurodevelopmental toxicity of DEHP in wildlife and humans. The present study investigated the effects of DEHP, focusing on the changes in the behavior of offspring mice at the ages of 6 and 12 w, respectively, following utero and lactational exposure to DEHP (10, 50, and 200 mg/kg/d) from gestation day 7 through postnatal day 21. The results of open field tasks showed that DEHP increased the grooming of males at age 6 w and females at age 12 w but decreased the frequency of rearing of 6-w-old females and the number of grid crossings of 12-w-old females. In the Morris water maze task, 50 and 200 mg/kg/d DEHP significantly prolonged the time of searching the hidden platform in water maze and reduced the time staying in the target quadrant during a probe trial of 6-w-old male mice, but not of 6-w-old females nor 12-w-old mice of both sexes, suggesting an impaired spatial learning and memory among younger males after perinatal exposure to DEHP. Western blot analyses further showed that DEHP at 50 and 200 mg/kg/d decreased the levels of the N-methyl-D-aspartic acid (NMDA) receptor subunits NR1 and NR2B in the hippocampus of 6-w-old males. These results suggest that uterine and lactational exposure to low doses of DEHP sex-specifically impacted behaviors, including locomotion activity and spatial memory, via the concomitant inhibition of the NMDA receptor of the hippocampus in offspring mice.

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## Introduction

Environmental endocrine disruptors (EEDs) are exogenous and synthetic chemicals that mimic or antagonize the actions of endogenous hormones. Exposure to EEDs has been associated with a range of developmental, reproductive, neurological, immune, and metabolic diseases in humans and wildlife. Phthalates, which are EEDs, constitute a family of industrial chemicals used primarily to increase the flexibility and durability of plastic products (Singh and Li, 2012). Di-(2-ethylhexyl) phthalate (DEHP) is one of the most commonly used phthalates with major applications in plastics and many other products, including food storage containers, children's toys, pharmaceuticals, cosmetics, and personal care products. DEHP has been measured in individuals across all developmental stages, with compelling evidence showing that infants and children have a greater exposure to these chemicals than adults (Chopra et al., 2014). DEHP can interfere with the normal behavior of estrogen and androgen, especially during critical periods of organ or system developmental stages (in utero and lactational), thereby affecting the development and long-term function of hormone-sensitive

tissues (Andrade et al., 2006). Currently, the developmental and reproductive toxicity of DEHP in wildlife and humans has raised widespread concern. However, much less is known about the effects of phthalates on brain development.

Gonadal hormones are critical for the development of the brain (McEwen and Alves, 1999), and thus it is likely that exposure to EEDs influences the development of several brain structures related to behavior and cognitive processes. Because maternal DEHP and its metabolites can transfer either to the fetus across the placenta during pregnancy or to the nursing infant through breast milk during lactation and due to the developmental immaturity of the blood–brain barrier in early life, DEHP and its metabolites can easily pass into the brain tissue of offspring (Xu et al., 2007). It is likely that the developmental neurotoxicity of DEHP may begin during gestation and continue through the lactation period. Uterine and lactational exposure to DEHP has been found to sex- or age-specifically change the aromatase activity of the hypothalamic/preoptic area in offspring, indicating that the effects of DEHP exposure on the male and the female brain are distinctly different throughout development (Andrade et al., 2006). Brain aromatase catalyzes the conversion of local androgens to estrogens and is critical for the masculinization of the brain. Therefore, we hypothesize that developmental exposure to DEHP may influence the behavior and sexual differences of the brain. There is growing concern that EEDs may disrupt hormone-dependent events during brain development, thereby affecting behavior (Patisaul and Polston, 2008). Behavioral evaluation on chick pre-hatch exposure

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to DEHP (100 mg/kg) showed an abolishment of imprinting performance from the control preference ratio (Abdul-Ghani et al., 2012). Exposure to DEHP at 30 mg/kg/d from postnatal day (PND) 1 through the day of behavior test at day 45 or 60 significantly increased anxiety-like behaviors in male but not in female rats (Carbone et al., 2013). Epidemiological studies have associated the concentrations of phthalates in maternal urine with attention deficit disorder and learning disabilities in American children aged 6–15 years (Chopra et al., 2014). However, studies on DEHP's effects on non-reproductive behaviors have been limited, and the exact mechanism is not well understood.

The hippocampus is considered to be closely related to learning and memory. It has been reported that acute DEHP exposure during PND16 to PND 22, a critical period of hippocampal development, reduced the axonal markers in the CA3 distal stratum oriens and the cell density of both immature and mature neurons in the dentate gyrus and CA3 of male rats, suggesting that the hippocampus is highly sensitive to DEHP (Smith et al., 2011). Gonadal hormones regulate the synaptic plasticity and physiology of the hippocampus, as well as the memory behaviors mediated by the hippocampus. In addition, the N-methyl-D-aspartic acid (NMDA) receptor, one of the ionotropic glutamate receptors present at excitatory synapses, is critical for the establishment of synaptic connections and normal brain function (Stephenson et al., 2008). NMDA receptors in the hippocampus are involved in long-term potentiation (LTP), a cellular model for learning and memory, which are under the influence of estrogen and other gonadal hormones (Foy et al., 1999). Estrogen is involved in the formation of excitatory synapses in the hippocampus (Jelks et al., 2007) and exerts profound effects on the sprouting of hippocampal dendritic spines and the concomitant up-regulation of NMDA receptors (McEwen, 2002; Woolley and McEwen, 1994). The purpose of the current study was to examine the effects of perinatal exposure to DEHP on neurobehavioral outcomes, especially on learning and memory, in male and female offspring mice. It was hypothesized that exposure to DEHP during this developmental period would negatively impact neurobehaviors and the NMDA receptors in the hippocampus, with the possibility of differential effects between genders.

## Materials and methods

### Animals and treatment

Male (30–35 g) and female (25–30 g) ICR mice (purchased from the Experimental Animal Center, Jinhua Institute for Drug Control) were housed under standard conditions (temperature  $24 \pm 1$  °C; humidity 50–60%; 12:12 h light/dark cycle) with free access to food and water. To minimize background exposure to DEHP beyond the treatment regimen, mice were housed in white poly-propylene cages with ad libitum access to DEHP-free water provided in glass bottles and diet. After acclimatization for 1 week, female mice were housed with male (female:male = 1:1), and vaginal plugs and vaginal smears were checked daily. The presence of sperm-positive smears and vaginal plugs determined gestational day 0 (GD 0); if detected, the pregnant dams were each placed in an individual cage. All of the experiments in the present study were conducted in accordance with the Care and Use Standard of the Laboratory Animal (the National Institute of Health Guide for the Care and Use of Laboratory Animals, Beijing, China).

Dams were randomly assigned to five experimental groups ( $n = 12$  litters for each condition) and were orally administered DEHP at doses of 10, 50, or 200 mg/kg/d (1 g/mL, pure > 99%, AMRESCO, USA) dissolved in 0.56% Tween 80 (AMRESCO LLC, Solon, OH, USA) from GD 7 to weaning (postnatal day 21, PND 21). Tween 80 (<0.56%) was used as the vehicle control and water, as the blank control (Kessler et al., 2004). All of the dams were allowed to feed their offspring until weaning. The doses of DEHP in the present study were below the no-observed-adverse-effect level (NOAEL, 48 mg/kg/d) of DEHP (10, 50, and 200 mg/kg/d, converted to human equivalent dose based on the

body surface area was approximately 0.73, 3.67, 14.70 mg/kg/d) and were previously found to have no reproductive toxicology (Smith et al., 2011).

### Body length, body weight, reproductive organ weights, and serum levels of hormones in pups

Three days after birth, the body lengths of the pups were recorded. The body weights of pups were recorded every week. Pups from the vehicle- and the blank-control and DEHP groups were weaned at PND 21. On PND 35, offspring from each litter were housed on a same-sex basis. On PND 42 (6 w), half of the pups of each sex from each litter submitted to a behavior test ( $n = 12$  pups from 12 litters) or were used for protein analyses ( $n = 4$  pups from 4 litters) and reproductive organ weight measurements. The remaining pups were used for the experiment requiring offspring in adulthood on PND 84 (12 w) (Fig. 1). For the examination of serum hormone (estradiol or testosterone) levels, blood samples ( $n = 8$ ) were collected from the orbitals after the behavior test, and the samples were centrifuged at 3000 rpm for 10 min. The serum was then separated, and the hormone (estradiol in females or testosterone in males) levels were measured using radioimmunoassays (performed by Medical Solutions Diagnostics and Assay Core of Jinhua Central Hospital) (Dorgan et al., 2010).

### Tissue preparation and Western blotting analyses

On PND 42 or PND 84, the protein expressions in the hippocampus were analyzed (4 litters for each group, one male and one female from each litter,  $n = 4$ ). The mice were sacrificed, and the hippocampus was dissected below 4 °C and then stored at  $-80$  °C until use. Tissues were homogenized in ice-cold RIPA buffer (Beyotime) containing 1% Triton X-100, 1% deoxycholate, 0.1% SDS, and 1 mM PMSF (Beyotime) and then centrifuged at 14,000 g for 15 min at 4 °C. The supernatant with an appropriate loading buffer (60 mM Tris, pH 6.8, 2% SDS, 25% glycerol, 14.4 mM 2-mercaptoethanol, 0.1% bromophenol blue) was bathed for 5 min at 100 °C and then used for SDS-PAGE separation and Western blot analysis of the levels of NMDA receptor subunit NR2B and  $\beta$ -actin.

Equivalent amounts of protein from each sample were loaded and run on a 4% acrylamide stacking gel and a 10% acrylamide resolving gel. After electrophoresis, proteins were transferred to nitrocellulose membranes for 2 h at 300 mA. Membranes were incubated in blocking buffer (5% fat-free milk in TBS-Tween 20) for 2 h at 37 °C to block non-specific binding. The blots were reacted with primary antibody, anti-NR1, anti-NR2B (1:500, Santa Cruz Biotechnology, USA), or anti- $\beta$ -actin (1:1000, Cell signaling Technology, USA) in the blocking buffer (1% BSA in TBS-Tween 20) overnight at 4 °C. After washing ( $3 \times 10$  min in TBS-Tween 20), the blots were incubated in secondary antibody, horseradish peroxidase-conjugated goat anti-rabbit IgG heavy and light chains (Jackson ImmunoResearch) with blocking buffer (0.5% BSA) for 1.5 h at 37 °C.  $\beta$ -Actin was utilized as an internal reference. Finally, membranes were washed with TBS-Tween 20 ( $3 \times 10$  min) and then immunolabeled by chemiluminescence (ECL, Beyotime). Immunodetection of the bands was determined using a computer image system (Quantity one, Bio-Rad Laboratories).

### Behavioral test

All sessions of behavior tests were automatically recorded with a computer-based video tracking system (VideoMot2 BWM; TSE System GmbH, Germany). The investigator was not visible to the mice. All behavioral data were collected using software (TSE System GmbH, Germany) by a trained observer who was blind to the experimental groups until the data analysis was completed. To avoid the influence of the estrus cycle in females on various behavioral characteristics, the estrus stage of adult females (12-w-old) was checked by taking vaginal

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