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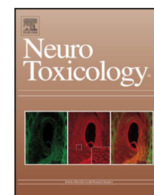

Full Length Article

The roles of hippocampal microRNAs in response to acute postnatal exposure to di(2-ethylhexyl) phthalate in female and male rats

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ABSTRACT

Previous studies have shown that di(2-ethylhexyl) phthalate (DEHP) exposure impairs the normal development of pre- and post-synaptic elements of the male, but not female, rat hippocampus. While males seem to be vulnerable to the neurodevelopmental deficits resulting from DEHP exposure, females appear to show a protective response. The purpose of the present study was to characterize hippocampal microRNAs in female and male rats exposed to DEHP to assess whether any patterns emerged that would be consistent with vulnerability in males and resilience in females. Male and female rats were treated with 0, 1, 10, or 20 mg/kg of DEHP by intraperitoneal injections from postnatal day 16 (PND16) – PND22 and brains were removed and flash frozen on PND78. A group of 85 microRNAs which have been previously shown to play a role in the development and maintenance of hippocampal neurons was assessed with RT-qPCR. In response to DEHP exposure, there were 19 microRNAs that increased in females and 52 that decreased in males. The strongest microRNA response in females occurred in conjunction with the 10 mg/kg of DEHP dose, whereas suppression of microRNAs in males appeared to be dose-dependent. Select hippocampal microRNAs (such as miR-132-3p and miR-191-5p), previously shown to regulate dendrite morphology, were modulated by DEHP exposure in this study. The results suggest that DEHP exposure has the potential to regulate microRNAs in a sex-specific manner which may interfere with proper hippocampal development in males and preserve hippocampal development in females.

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1. Introduction

Phthalates, like the commonly-used Di(2-ethylhexyl) phthalate (DEHP), are synthetic plasticizers used to provide flexibility and malleability to plastics made from polyvinyl chloride (PVC). Phthalates are bound to plastics due to their molecular shape, allowing them to be entangled in a plastic-phthalate complex without covalent bonding and as such, these compounds can leach away from the plastic resulting in human exposure through various routes (Heudorf et al., 2007; Schettler et al., 2006; Wittassek et al., 2011). Due to the ubiquitous use of phthalates, it is important to consider the possible effects they may have on human physiology and health.

Phthalate exposure in children and infants is of particular concern as multiple studies have shown that this population is exposed to higher levels of phthalates than adults (Cirillo et al., 2011; Green et al., 2005; Koch et al., 2011, 2005, 2004; Sathyanarayana et al., 2008; Wittassek et al., 2011). Phthalates have the potential to cause developmental alterations through their endocrine disrupting actions. They have been shown to decrease testosterone production and bind to androgen receptors (AR), impairing endocrine function thereby interfering with normal development (Chen et al., 2014; Desdoits-Lethimonier et al., 2012; Harris et al., 1997; Hong et al., 2005). Investigations have determined maladaptive outcomes on the male reproductive system with phthalate exposure causing a reduction in anogenital distance (an important marker for anti-androgenic activity) and cryptorchidism (Braun et al., 2013; Lyche et al., 2009; Swan, 2008). These effects are likely due to the fact that several phthalates, such as DEHP, have been implicated in inhibition of fetal testosterone production via actions on Leydig cells (Borch et al., 2006; Saillenfait et al., 2013; Swan et al., 2010).

Effects on neurodevelopmental mechanisms of the brain are another concern of phthalate exposure and not well-characterized.

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One brain region that shows connectivity-based changes during sensitive developmental periods is the hippocampus. Between postnatal day (PND) 18 and PND 24 in the rat, there exists a sensitive phase of hippocampal development wherein axonal terminal fields in the CA3 region shift from the stratum lucidum to the stratum oriens resulting in the onset of a more adult-like morphology and improved spatial functioning (Holahan et al., 2007; Keeley et al., 2010). While the enhanced plasticity during this time has favorable functional outcomes (Wartman et al., 2012), the increased neural plasticity during this time may result in the brain being more vulnerable to the potentially damaging effects of toxicants and other environmental insults (Anderson et al., 2011; Arain et al., 2013; Holahan and Smith, 2015). With respect to phthalates, male, but not female, rats injected (i.p.) with 10 mg/kg DEHP from PND16 − 22 showed reduced axonal innervation of the CA3 region within the dorsal hippocampus (Smith et al., 2011) and a reduction in spine density on CA3 pyramidal neuron dendrites associated with a reduction in brain-derived neurotrophic factor (BDNF) mRNA levels (Smith and Holahan, 2014). Lipid composition in nervous system tissue plays an important role in the development of the morphology of the brain by influencing cell survival, myelination, and receptor trafficking among other processes and it is known to be altered in response to DEHP exposure (Ramstedt and Slotte, 2002; Uauy et al., 2001). DEHP treatment from PND16–PND 24 resulted in sex-specific changes in lipid profiles such that levels of multiple sphingomyelins and phosphatidylcholine species were more than doubled in concentration in female hippocampal tissue whereas in male tissue, these remained similar or decreased (Smith et al., 2015). The differential responses in male and female rat hippocampal lipid composition following DEHP exposure is consistent with the observed histological differences and suggest that there may be a neuroprotective effect of elevated lipid species in females.

A question remains as to why these neurodevelopmental effects occur after phthalate exposure. One possible mediating factor might be changes in microRNA expression. MicroRNAs are short non-coding RNAs that play an important role in post-transcriptional regulation by inhibiting translation of RNAs into functional proteins through complementary binding of specific mRNAs. These short RNAs are highly conserved throughout vertebrates and therefore play shared roles between species in regulating metabolism, cell differentiation, and the cell cycle (Biggar and Storey, 2012; Bueno and Malumbres, 2011; Rottiers and Näär, 2012; Shivdasani, 2006). The role of specific microRNAs in regulating the development of hippocampal cells in culture has been explored in relation to axonal outgrowth and also NMDA receptor stimulated plasticity (Van Spronsen et al., 2013). Another study has demonstrated the importance of neuronal activity in altering the expression profile of microRNAs in the hippocampi of mice (Eacker et al., 2011). Altered expression of microRNAs related to the apoptotic pathway in rat hippocampus has also been associated with chronic temporal lobe epilepsy by inducing an anti-apoptotic effect (Li et al., 2014). Lead exposure in male rats was reported to alter levels of microRNAs miR-204, miR-211, miR-448, miR-34c, miR-34b, miR-449a, and mir-494 which are related to important neurophysiological pathways involved in neural damage, synapse and axon regulation, and neural regeneration suggesting that alteration in microRNAs may mediate toxin-induced pathogenic effects in the hippocampus (An et al., 2014). The effects of phthalates on microRNA expression in the hippocampus to date has not been explored however, the impaired development of pre- and post-synaptic elements of the male rat hippocampus following DEHP exposure might result from altered microRNA expression profiles specific to axonal and dendritic outgrowth. Because microRNAs may play an integral role in the progression of neurodevelopment and may contribute to neuropathology, alterations in their expression patterns should be of growing interest in toxicological studies. As such, the present study investigated the expression of 85 hippocampal microRNAs in male and female rats following developmental (PND18–24) exposure to DEHP.

2. Materials and methods

2.1. Animals

Six timed pregnant female Long Evans rats (approximately 13 days gestation) were purchased from Charles River Laboratories (St. Constant, Québec). The pregnant females were singly-housed in polycarbonite $48 \times 26 \times 20\,cm^3$ cages within a temperature controlled environment. The day the pups were born was recorded as postnatal (PND) 0. Pups ($n = 26$ males; $n = 28$ females) were weaned on PND22 and group-housed (2 rats per cage), with males and females in separate cages. All rats were on a 12 h light-dark cycle (lights on at 8:00 a.m.) with ad libitum access to food (Purina rat chow) and tap water. All experiments were conducted at Carleton University and were approved by the Institutional Animal Care Committee as per guidelines established by the Canadian Council on Animal Care.

2.2. DEHP injections

Rats were injected i.p. with either 1, 10 or 20 mg/kg DEHP (Sigma-Aldrich; St. Louis, MO, USA) or vehicle (corn oil) daily from PND16 to PND22 inclusive: 1 mg/kg ($n = 6$ males; $n = 7$ females), 10 mg/kg ($n = 7$ males; $n = 7$ females) and 20 mg/kg ($n = 6$ males; $n = 7$ females).

Each rat was injected in the late morning (between 10:30 and 11:30 AM) and was returned to their home cage following the injection. Rats were randomly assigned to treatment (DEHP) and control (vehicle) groups counterbalanced across all litters. DEHP solutions were prepared using DEHP (1000 mg/kg) and corn oil immediately before each injection. Rats ($n = 7$ males; $n = 7$ females) receiving vehicle injections were injected with corn oil.

2.3. Tissue extraction

A subset of rats (4 males and 4 females for each dose condition selected from different litters to control for litter effects) were euthanized on PND78 by live decapitation and brains were rapidly removed and hemisected. One hemisphere was flash frozen and stored at -80° C while the other hemisphere was postfixed in 4% paraformaldehyde.

2.4. Total RNA extraction

Total RNA extraction was performed using TRIzol reagent (Invitrogen, Carlsbad, CA) as previously described (Luu and Storey, 2015). Briefly, frozen hippocampal samples were homogenized in 1 mL of TRIzol reagent, followed by the addition of 300 μL of chloroform. Samples were vigorously shaken by hand and allowed to incubate at room temperature for 5 min before centrifugation at $10{,}000 \times g$ for 15 min at 4 °C. The aqueous phases were combined with 750 μL isopropanol, shaken vigorously, and rested at room temperature for 15 min to facilitate RNA precipitation. Samples were centrifuged at $12{,}000 \times g$ for 15 min at 4 °C, and RNA pellets washed with 1 mL 70% ethanol. RNA pellets were briefly air dried and suspended in 50 μL RNase-free water. The quality of RNA was assessed by the appearance of two sharp bands representing 28S and 18S ribosomal RNA bands on a 1% agarose gel stained with SybrGreen dye. Purity was further assessed using a 260/280 nm

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