REVIEW

Autistic-Like Traits in Laboratory Rodents Exposed to Phthalic Acid Esters During Early Development – an Animal Model of Autism?

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Received September 2, 2020
Accepted February 25, 2021
Epub Ahead of Print May 12, 2021

Summary

Phthalates are chemical substances that are widely used to provide flexibility and durability to plastic materials. They leach from products in which they are mixed and reach living organisms. Results from experimental studies suggest that exposure to phthalates can have a negative impact on an individual's neuronal system and behavior. In this regard, exposure during early ontogenesis seems to be particularly dangerous due to the extensive growth and development of body structures and functions. Disruption during this critical time can result in alterations of behavior and the emergence of neurodevelopmental disorders, such as autism spectrum disorder (ASD). Various animal models have been used to elucidate the pathogenesis of this disease. They are fundamental for research, and although the translation of results to humans is difficult, new animal models are being developed. The aim of this review is to summarize laboratory rodent studies in which early developmental phthalate exposure resulted in brain alterations and autistic-like behavioral traits. We also discuss the possibility of using early developmental phthalate exposure in rodents to create a new animal model of autism.

Key words

Phthalates • Early-life exposure • Brain development • Behavior • Animal model

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Introduction

The diesters of 1,2-benzenedicarboxylic acid (phthalic acid) are commonly known as phthalates. Phthalates are man-made chemical substances that are presently widely used to provide flexibility and durability to plastic materials and that can also act as solvents. They can be found in a wide range of products used in daily life, such as vinyl flooring, shower curtains, raincoats, bath products, nail polish, perfumes, cosmetics, medications, and food storage containers (Schettler 2006, Diamanti-Kandarakis *et al.* 2009). As there is no covalent bond between phthalates and the plastic, in which they are mixed, phthalates may leach or outgas into their surroundings. They are widespread in the environment and the sources of exposure vary. They can enter our bodies parenterally (while delivering intravenous injections), but also through dermal contact (personal care products), inhalation (indoor air and dust contain phthalates that leach/outgas from various household products), and mostly food consumption (contaminated food) (Schettler 2006, Swan 2008, Mullerová *et al.* 2016).

Phthalates are usually quickly metabolized into monoester metabolites after they enter the body. Low-molecular-weight phthalates are subsequently excreted and high-molecular-weight phthalates are further conjugated, and then excreted (Frederiksen *et al.* 2007). Their half-life is usually less than 24 h (Koch *et al.* 2005, Klein *et al.* 2016). Phthalate monoesters are used as biomarkers of exposure to both low-molecular and high-

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molecular-weight phthalates (Frederiksen *et al.* 2007). Phthalate metabolites are detected in bodily fluids including urine, saliva, amniotic fluid, or breast milk (Frederiksen *et al.* 2007). The concentration of these metabolites is usually estimated from urine samples (Swan 2008). The rate of exposure to phthalates is repetitive and relatively constant over time, and several authors have agreed that exposure assessment can be based on a single urine sample (Hoppin *et al.* 2002, Hauser *et al.* 2004).

Various studies have already demonstrated that phthalates have a negative impact on living organisms (Blystone *et al.* 2010, Boberg *et al.* 2011). These compounds have been studied in terms of their effects on susceptible subjects, including pregnant women, infants, and children (Factor-Litvak *et al.* 2014, Weng *et al.* 2017, Kolatorova *et al.* 2018, Tsai *et al.* 2018, Zhang *et al.* 2018).

Phthalates are endocrine disruptors, which means they can interfere with the function of natural blood-borne hormones (Diamanti-Kandarakis *et al.* 2009). Fetal exposure to phthalates can have negative effects on the function of the reproductive system of humans and animals (Swan *et al.* 2005, Hsieh *et al.* 2008, Repouskou *et al.* 2019). This impact has been studied intensively and has already been reviewed elsewhere (Martino-Andrade and Chahoud 2010, Zamkowska *et al.* 2018).

Phthalates can also have a negative impact on the developing brain and there has been discussion about a possible role of phthalates in the etiology of neurodevelopmental disorders, e.g. autism spectrum disorder (ASD) (Colborn 2004, Grandjean and Landrigan 2006).

Animal models have an important role in the field of science. Large numbers of animal models are currently being used to elucidate the causes behind various human disorders, including ASD (Olexová *et al.* 2012, Ergaz *et al.* 2016, Möhrle *et al.* 2020). ASD is characterized by impaired social interaction and social communication, as well as restricted and/or repetitive behaviors and interests (American Psychiatric Association 2013). Several methods are used to induce an autistic phenotype in animals, including the administration of exogeneous chemicals (Ergaz *et al.* 2016).

In this review, we will address the role of prenatal and early postnatal phthalate exposure in brain development and review rodent studies in which the impact of phthalates on the brain was examined. This aspect draws much attention and is increasingly being studied.

Next, we will discuss the presence of autistic-like traits in rodents that are often seen in studies investigating behavior after prenatal and early postnatal phthalate exposure.

Lastly, since rodents are usually used for the development of animal models of various disorders, we will consider the prospect of using rodents that were prenatally and early postnatally exposed to phthalates as a new potential animal model of ASD.

Phthalates and their impact on brain development

The brain is highly vulnerable and sensitive to exogenous factors at its early developing stage. This susceptibility originates from the fact that, during a short period of prenatal and early postnatal life, the brain develops from a strip of cells into a complex organ consisting of billions of precisely located, highly interconnected, and specialized cells (Grandjean and Landrigan 2006). Exposure to environmental chemicals during the brain growth spurt in the prenatal period has been suggested as a possible causal factor for impaired neurodevelopment and the emergence of neurodevelopmental and behavioral disorders, including ASD (Colborn 2004).

Only a limited number of studies have investigated the effect of phthalates on brain development. Therefore, the underlying mechanism, induced by prenatal and early postnatal phthalate exposure, that could lead to an autistic phenotype is not yet clear. Structural, functional, or molecular abnormalities in the hippocampus, amygdala, and prefrontal cortex are often mentioned. Pathomechanisms leading to these abnormalities were investigated in studies focusing solely on ASD, but also in some studies focusing on the role of phthalate exposure in brain development and ASD-linked changes. Below, we review these studies.

Abnormalities in the hippocampus

The hippocampus has for long been known to have an important role in spatial memory that enables spatial navigation (O'Keefe and Dostrovsky 1971). However, spatial memories are not the only ones supported by the hippocampus. It is also crucial for declarative memory, the ability to learn and remember everyday facts and events (reviewed in Cohen 2015). It was also found that the hippocampus is essential for social memory formation (Hitti and Siegelbaum 2014)

and that it supports decision making and helps us to use memory in order to guide our behavior (Anderson *et al*. 2014, Cohen 2015).

Atypical hippocampal functioning was suggested as playing a role in the etiology of ASD (Ring *et al.* 2017), however, exact pathomechanisms are still to be elucidated. Synaptopathology and neuron loss are often mentioned.

Excitation and inhibition imbalances in synaptic transmission have been implicated in ASD (Lee *et al*. 2017). A decrease in the density of parvalbumin-expressing interneurons in the hippocampus was suggested as a potential mechanism for impaired social cognition in a study by Piskorowski *et al.* (2016). These interneurons provide an inhibitory output onto their target cells (Bartos and Elgueta 2012) and were found to be crucial for social memory (Deng *et al.* 2019). Furthermore, mutations in genes that are essential for synaptic function were found in ASD individuals (reviewed in detail in Guang *et al*. 2018).

Abnormalities in the function of synapses in the hippocampus are often seen in experimental studies on laboratory rodents investigating the role of phthalates in brain development. Li *et al*. (2013) administered di-n-butyl phthalate (DBP) to pregnant rat dams from gestational day six and throughout lactation. Pups of these dams demonstrated signs of synaptopathology in the hippocampus. Expression of synaptophysin (a protein important for the formation, maintenance, and plasticity of synapses) was decreased, and both the slope and amplitude of field excitatory postsynaptic potentials were reduced. Moreover, loss of synapses, decreased number of presynaptic vesicles, reduced postsynaptic density, and a broader synaptic gap were observed. These authors suggest that DBP exposure may induce synaptic loss and synaptic function failure, leading to cognitive impairment (Li *et al.* 2013). Early postnatal exposure of experimental rats to di(2-ethylhexyl)phthalate (DEHP) resulted in a decreased axonal innervation to the hippocampus (Smith *et al*. 2011) and interfered with normal synaptogenesis and connectivity in this region of the brain (Smith and Holahan 2014). Furthermore, hippocampal brain-derived neurotrophic factor (BDNF) mRNA expression was downregulated. BDNF is important for dendritic outgrowth and the formation of new synaptic connections and it was speculated that its downregulation could be one of the mechanisms by which phthalates affect the brain (Smith and Holahan 2014). Moreover, the role of BDNF was also acknowledged in the development of ASD (Qin *et al.* 2016). Dai *et al.* (2015) suggested another possible mechanism, analyzing the protein expressions for n-methyl-d-aspartate (NMDA) receptor subunits in the hippocampus after prenatal and early postnatal exposure to DEHP. NMDA receptors play a critical role in the establishment of synaptic connections and in the hippocampus, they are involved in long-term potentiation, a cellular model for learning and memory. Western blot analysis showed that the levels of receptor subunits were decreased in male brains and suggested that the development of the brain could be adversely affected (Dai *et al.* 2015). The role of NMDA receptors in ASD pathogenesis was also acknowledged (Sceniak *et al.* 2019). Moreover, an increase in synapsin-1 levels was seen in the hippocampus of both male and female rats after prenatal and early postnatal phthalate exposure (DeBartolo *et al.* 2016). Synapsins are important regulators of synaptic vesicle trafficking. Changes in their levels may skew excitatory/inhibitory synaptic transmission ratios (Gitler *et al.* 2004) and were implied in the pathogenesis of ASD (Chen *et al.* 2014).

Another possible pathomechanism was investigated in a study by Dong *et al*. (2018). Endoplasmic reticulum (ER) stress, oxidative stress, and apoptosis were studied in different regions of the autistic brain, including the hippocampus. These authors discovered that ER stress signals were activated, and that the occurrence of ER stress is possibly caused by oxidative stress and leads to apoptosis. It was suggested that these changes can play a role in the pathogenesis of autism (Dong *et al*. 2018).

The possibility of phthalate-induced apoptosis of hippocampal neurons was investigated in a study by Li *et al*. (2013). Rat pups of dams that were administered DBP from gestational day six and throughout lactation suffered cell apoptosis of the hippocampal neurons (Li *et al.* 2013). Furthermore, hippocampal neuron loss and structural alternations were reported (Smith *et al*. 2011, Li *et al.* 2013). Barakat *et al.* (2018), in their study, observed neurodegeneration of hippocampal neurons in mice prenatally treated with DEHP. These authors suggested that the neurodegeneration may be caused by neuronal inflammation and oxidative damage to DNA. This statement was supported by the elevated levels of inflammatory markers and DNA oxidation damage markers in the DEHP mice.

Abnormalities in the amygdala and prefrontal cortex

The amygdala is involved in the production and recognition of emotions. It reacts to emotionally potent

stimuli, assigns significance to stimuli, and is involved in the consolidation of stimuli into the memory (Anderson and Phelps 2001, Schultz 2005). The amygdala is functionally connected with the prefrontal cortex and together they have a role in the regulation of goal-directed behavior (Ghashghaei *et al.* 2007, Ochsner *et al.* 2012). Moreover, the amygdala and the prefrontal cortex were suggested as being important for social behavior and were implicated in the pathophysiology of ASD (Baron-Cohen *et al.* 2000, Liu *et al*. 2020).

When studies aimed to investigate a possible mechanism by which an autistic phenotype might be induced, often, abnormal functional connectivity was observed between the amygdala and cortical regions (Pitskel *et al.* 2014, Ibrahim *et al.* 2019, Shen *et al.* 2016, Gibbard *et al.* 2018, Sato *et al.* 2020). The authors suggested that diminished connectivity may play a role in emotion dysregulation (Pitskel *et al.* 2014, Ibrahim *et al.* 2019) and the atypicalities in social interaction seen in ASD (Sato *et al.* 2020). Furthermore, some findings indicate that weaker connectivity between the amygdala and prefrontal cortex is correlated with increased autism severity (Shen *et al.* 2016, Gibbard *et al.* 2018). Structural abnormality of the amygdala was observed in autistic brains in a study by Schumann and Amaral (2006). Significantly fewer neurons were counted in the autistic amygdala compared to the control and the authors concluded that the amygdala undergoes atypical development in individuals with autism (Schumann and Amaral 2006). Abnormal cytoarchitecture and disorganization of neurons was observed in prefrontal cortical tissue from children with ASD and the authors speculated that such neuropathological features may probably result from dysregulation of layer formation and layer-specific neuronal differentiation at prenatal developmental stages (Stoner *et al.* 2014).

The amygdala and medial prefrontal cortex were found to be affected by prenatal and early postnatal phthalate exposure. In a study by DeBartolo *et al.* (2016), molecular abnormalities in the amygdala were found after prenatal and early postnatal treatment with benzyl butyl phthalate. Levels of methyl CpG binding protein 2, which is important for normal synaptic plasticity and transmission (and its role in the pathology of ASD was recognized (Nagarajan *et al.* 2006)), were decreased in the amygdala of female rats. The authors suggested that this change may be related to altered learning processes (observed in the study). An environmentally relevant mixture of phthalates was administered in a dose presumably within the range of the estimated daily intake of humans in a study by Kougias *et al.* (2018a). The mixture caused a reduction in neuron number, synapse number, and size of the medial prefrontal cortex in laboratory rats across both sexes (Kougias *et al.* 2018a). Results from a study by Komada *et al.* (2016) suggested that prenatal exposure of mice to DEHP could inhibit neurogenesis, reduce proliferation, and induce cell death of neural stem cells as well as induce abnormal neuronal distribution in the neocortex. On the other hand, in a study by DeBartolo *et al.* (2016), neurohistological analysis found no adverse changes in neuronal migration and lamination of the neocortex.

Autistic-like traits observed in rodents exposed to phthalates

ASD is a range of childhood-onset neurodevelopmental disorders. They comprise a broad spectrum of heterogeneous disorders, including autistic disorder, Asperger's disorder, and pervasive developmental disorders not otherwise specified (PDD-NOS). The diagnosis is mostly based on behaviors and ASD is characterized by following core behavioral deficits: persistent deficits in social interaction and social communication (including restricted verbal and nonverbal communication) and restricted repetitive patterns of behavior, interests, or activities. These symptoms are present from early childhood and impair everyday functioning (American Psychiatric Association 2013). Additional associated features are common. A subset of patients with ASD tend to also display anxiety, depression, learning disability, reduction of spatial working memory abilities, hyperactivity, intellectual impairment, motor deficits, language impairment, self-injury, or disruptive behaviors (O'Brien and Pearson 2004, Steele *et al.* 2007, American Psychiatric Association 2013).

Evidence from twin and family studies suggest a strong genetic component in the etiology of ASD (Bailey *et al.* 1995, Luhrs *et al.* 2017). Despite this, concordance of the behavioral phenotype for autism in monozygotic pairs is not 100 %, and the degree of impairment and range of symptoms vary among concordant pairs, and no single gene or common genetic variants have been identified to explain the majority of ASD cases (Bourgeron 2016, Heavner and Smith 2020). This paradox has led to the notion that environmental influences, such as exposure to chemicals and pollutants, are most likely etiologically significant as well (Goodrich *et al.* 2018).

Results presented in several human studies suggest that increased prenatal and early postnatal phthalate metabolite concentrations are indeed connected to neurodevelopmental disability (Whyatt *et al.* 2012, Kim *et al.* 2018), behavioral problems (Minatoya *et al.* 2018, Hyland *et al.* 2019, Jankowska *et al.* 2019), and autism-like behavior in children (Miodovnik *et al.* 2011, Oulhote *et al.* 2020).

Together with human studies, experimental studies on laboratory animals have also highlighted the possible damage that can be done by prenatal and early postnatal exposure to phthalates; these studies are reviewed below (for more details and suggested pathomechanisms, see Table 1).

Table 1. Behavioral alterations in rodents observed after prenatal and early postnatal phthalate exposure.

Reference	Administered phthalates and dose	Days of administration	Model species	Tests	Results			Suggested pathomechanisms
					Both sexes	Males	Females	
Barakat *et al.* 2018	DEHP; 200 µg, 500 mg, or 750 mg/kg/day	GD 11 - birth	CD-1 mice	EPM, OFT, Y-maze, NOR	OFT: 200 µg, 500 mg, 750 mg/kg DEHP: fewer entries to the central area. EPM: 750 mg/kg DEHP: more time before making entries to the open arms. Y-maze: 200 µg/kg DEHP: the least alternation behavior and the fewest arm entries. NOR: 500 and 750 mg/kg DEHP: less time exploring the new object.			Neurodegeneration of hippocampal neurons caused by DEHP. The neurodegeneration was probably caused by the neuronal inflammation and oxidative damage to DNA.
Boberg *et al.* 2011	DiNP; 300, 600, 750 or 900 mg/kg/day	GD 7 - PND 17	Wistar rats	MWM		No significant difference found.	900 mg/kg DiNP: reduction in swim length and latency to reach the platform.	
Dai *et al.* 2015	DEHP; 10, 50, or 200 mg/kg/day	GD 7 - PND 21	ICR mice	OFT, MWM		OFT: 200 mg/kg DEHP: increased the grooming at age 6 weeks. MWM: 50 and 200 mg/kg DEHP: prolonged the time of searching the	OFT: 10 mg/kg DEHP: increased the grooming at age 12 weeks. 10 and 200 mg/kg DEHP: decreased the frequency of rearing at age 6 weeks,	Impact on behaviors was associated with the inhibition of n-methyl-D-aspartate receptor of the hippocampus. Authors speculated that the synaptic transmission might have been impaired.

						hidden platform in water maze and reduced the time staying in the target quadrant during a probe trial of 6-w-old male mice.	decreased the number of grid crossings at age 12 weeks.	
Hatcher *et al.* 2019	DEHP; 20 or 200 µg/kg/day, 500 or 750 mg/kg/day	GD 10.5 - 19.5	CD-1 mice	EPM		**F3:** No significant difference found.	**F3:** 750 mg/kg DEHP: increased time spent in the open arms.	Authors associated the transgenerational effect of DEHP with epigenetic changes.
Hoshi and Ohtsuka 2009	DBP; 10 or 1000 µg/kg/day	GD 8 - birth	Sprague-Dawley rats	Analysis of grooming behavior		10 µg/kg DBP: lower frequency of individual grooming bouts. 1.0 mg/kg DBP: no decrease in the frequency of individual grooming bouts.		
Kougias *et al.* 2018a	DEP, DEHP, DBP, DiNP, DiBP, BBP; 0, 200, or 1000 µg/kg/day	GD 2 - PND 10	Long–Evans hooded rats	Attentional set-shifting task	Phthalate mixture: decreased cognitive flexibility (less correct responses, more perseverative errors, more omission errors).			Neuroanatomical changes of the medial prefrontal cortex: reduction in neuron number, synapse number, and the size of the medial prefrontal cortex.
Kougias *et al.* 2018b	DEP, DEHP, DBP, DiNP, DiBP, BBP; 0, 200, or 1000 µg/kg/day	GD 2 - PND 10	Long-Evans hooded rats	Social play behavior		200 mg phthalates/kg: close to significant decrease in social play.		Authors speculated that the anti-androgenic effect of phthalates might have caused decreased social play behavior (social play is affected by androgens).
Lee *et al.* 2016	DEHP; 30 mg/kg/day	Pregnancy + lactation	Wild-type (WT) mice with C57BL/6 background	OFT, EPM, SIT and MWM	SIT: DEHP: decreased social preference to a stranger mouse.			Maternal DEHP exposure might have affected the prenatal differentiation of neurons in offspring and result in impaired ability for social interaction.

Li *et al.* 2013	DBP; 500 mg/kg/day	GD 6 - PND 21	Sprague Dawley rats	MWM	DBP: decreased numbers of platform crossings.	DBP: increased latency to escape.	No significant difference found.	DBP exposure might have induced synaptic loss and synaptic function failure, leading to cognitive impairment.
Lin *et al.* 2015	DEHP; 10 or 750 mg/kg/day	GD 12 - 21	Sprague Dawley rats	MWM	10 and 750 mg/kg DEHP: prolonged escape latencies, fewer times across a platform. 750 mg/kg DEHP: less search time in quadrant with the platform.			DEHP altered the expression of genes critical for neuron proliferation. Authors suggested that this might have partially contributed to the deficit of cognitive function.
Quinnies *et al.* 2015	DEHP; 150 or 200 mg/kg/day	GD 7 - 14	C57BL/6J mice	SIT, EPM		**F3:** SIT: 200 mg/kg DEHP: more time spent in digging and less time spent in self-grooming.		Authors associated the transgenerational effect of DEHP with epigenetic changes.
Quinnies *et al.* 2017	DEHP; 5, 40, or 400 μg/kg/day	GD 0.5 - PND 10	C57BL/6J mice	SIT, EPM	**F1:** SIT: 40, 400 μg/kg DEHP: less sitting alone. 40 μg/kg DEHP: sniffed their test partner more frequently. 400 μg/kg DEHP: more exploratory behaviors. EPM: 5, 40 μg/kg DEHP: more time in the closed arms. **F3:** less exploratory behaviors**.**	**F1:** SIT: 40, 400 μg/kg DEHP: less sitting side-by-side. **F3:** 400 μg/kg DEHP: more sitting side-by-side, more sitting alone.	**F3:** 400 μg/kg DEHP: more sitting alone.	Authors suggested possible impact of DEHP on neurodevelopment. Anxiety-like behavior might have been caused by the hypothalamic-pituitary-adrenal axis disturbance. The transgenerational effect of DEHP was associated with epigenetic changes.
Sellinger *et al.* 2020	DEP, DEHP, DBP, DiNP, DiBP, BBP; 0, 200, or 1000 μg/kg/day	GD 2 - PND 10	Long–Evans hooded rats	EPM		No significant difference found.	No significant difference found.	
Xu *et al.* 2015	DEHP; 10, 50, or 200 mg/kg/day	GD 7 - PND 21	ICR mice	OFT, Dark/light transition task, The mirrored maze task,		OFT: 200 mg/kg DEHP significantly increased the frequency of	OFT: 10 and 200 mg/kg DEHP: decreased the frequency of rearing at age	Authors suggested that anxiety- and depression-like behaviors were due to the downregulation of

	EPM, FST	grooming at age 6 weeks. EPM: 10, 50 mg/kg DEHP: decreased number of open arm entries and the time spent in them at age 6 weeks. Dark/light transition task: 200 mg/kg DEHP: decreased the time staying in the light chamber at age 6 weeks. 10, 50, 200 mg/kg DEHP: reduced the light chamber entries at age 6 weeks. The mirrored maze task: 200 mg/kg DEHP: decreased the time staying in the mirrored chamber at age 6 weeks. FST: 50 and 200 mg/kg DEHP: prolonged immobility time at age 6 weeks. 200 mg/kg DEHP: prolonged immobility time at age 12 weeks.	6 weeks and decreased the number of grid crossings at age 12 weeks. 10 mg/kg DEHP: increased the frequency of grooming at age 12 weeks. EPM: 10, 200 mg/kg DEHP: decreased number of open arm entries and the time spent in them at age 6 weeks. 10 mg/kg DEHP: decreased the number of total arm entries at age 6 weeks. 50 mg/kg DEHP: decreased the time spent in open arms at age 12 weeks. Dark/light transition task: 10, 50, 200 mg/kg DEHP: reduced the light chamber entries at age 12 weeks. The mirrored maze task: 10 mg/kg DEHP: decreased the mirrored chamber entries at age 12 weeks. FST: 10 mg/kg DEHP: prolonged immobility time at age 6 and 12 weeks.	androgen and estrogen receptors in the hippocampus. Therefore, the modulation of behaviors by gonadal hormones might have been disturbed.

Abbreviations: BBP – benzyl butyl phthalate, DBP – di-n-butyl phthalate, DEHP – di(2-ethylhexyl)phthalate, DEP – diethyl phthalate, DiBP – di-isobutyl phthalate, DiNP – diisononyl phthalate, EPM – Elevated plus maze, F1 – one generation removed from phthalate exposure, F3 – three generations removed from phthalate exposure, FST – Forced swim test, GD – gestational day, MWM – Morris water maze, NOR – Novel object recognition test, OFT – Open field test, PND – postnatal day, SIT – Social interaction test.

Impact on social behavior

Negative impact of prenatal and early postnatal exposure to phthalates on social behavior was recorded in several studies. Exposure to DEHP during pregnancy and lactation caused a decrease in social preference to a stranger mouse in young adult offspring mice (Lee *et al.* 2016). A negative impact on social behavior was also observed in a study by Kougias *et al.* (2018b), where prenatal and early postnatal exposure to a mixture of phthalates caused a decrease in social play in periadolescent rats. In a study by Quinnies *et al.* (2017), juvenile mice exposed to DEHP prenatally and early postnatally displayed fewer socially investigative behaviors.

In some studies, the transgenerational effect of phthalates was assessed to investigate if the impact of these substances persists through multiple generations, but results are inconclusive. Transgenerational DEHP exposure had no effect on social behavior in juvenile mice (only males were used) but they spent more time in nonsocial behavior (Quinnies *et al.* 2015). In another study, however, F3 male mice from a DEHP lineage were more interactive and spent less time in independent behavior (no difference was found in females) (Quinnies *et al.* 2017).

Anxiety-like behavior, depression-like behavior, and emotional deficits

Anxiety-like, as well as depression-like behavior (so-called because they resemble anxiety and depression behaviors in humans, though in rodents they might represent something else (Lezak *et al.* 2017)) were observed after prenatal phthalate exposure. In a study by Quinnies *et al.* (2017), mice had increased anxiety-like behavior after they were exposed to DEHP prenatally and early postnatally. These findings are in accordance with the results of another, earlier study (Xu *et al.* 2015), in which anxiety-like behavior was increased in pubertal mice regardless of sex, and in adults, when females were affected. On the other hand, Lee *et al.* (2016) and Sellinger *et al.* (2020) did not observe anxiety-like behavior in rodents after prenatal and early postnatal exposure to phthalates. Depression-like behavior was found to be induced in pubertal and adult mice after prenatal and postnatal phthalate exposure (Xu *et al.* 2015).

In transgenerational studies, no effect of DEHP lineage on anxiety-like behavior in F3 juvenile male or female mice was observed (Quinnies *et al.* 2015, Quinnies *et al.* 2017). In the most recent study, transgenerational DEHP exposure had an anxiolytic effect in adult mice from the F3 generation, but only in females. The authors suggest that this behavioral alteration indicates maladaptive anxiety-like behavior or hyperactivity rather than a therapeutic effect (Hatcher *et al.* 2019).

Adverse effects on emotional stability in a novel environment after phthalate exposure during prenatal development were indicated in a study by Hoshi and Ohtsuka (2009) and in a study by Dai *et al.* (2015). Adult male rats (Hoshi and Ohtsuka 2009) and pubertal male and adult female mice (Dai *et al.* 2015) exhibited changes in grooming behavior after they were placed in a test cage.

Impact on cognition, learning, and memory

Impairment of learning and spatial memory was observed in adult male rats after prenatal exposure to DEHP (Lin *et al.* 2015) and in male pubertal mice after prenatal and lactational exposure to this chemical (Dai *et al.* 2015). Learning and spatial memory was also impaired in young rats (postnatal day 22) exposed to DBP from gestational day six to postnatal day 21 (Li *et al.* 2013). On the other hand, prenatal and postnatal exposure to diisononyl phthalate (DiNP) resulted in better performance in tests for spatial learning in young adult rats, but this improvement was seen only in females. No changes were seen in males. As phthalates are endocrine disruptors, the authors suggested that hormonal imbalance during the critical periods of brain development could alter the default female outcome in a more masculine direction (Boberg *et al.* 2011). Lee *et al.* (2016) did not observe any changes in spatial learning and memory in young adult mice exposed to DEHP during lactation and gestation. In a more recent study, spatial and recognition memory were assessed in adult male mice after prenatal exposure to DEHP (Barakat *et al.* 2018). This exposure correlated with reduced spatial memory function and impaired recognition memory.

Kougias *et al.* (2018a) tested cognitive flexibility of adult rats in an attentional set-shifting task after prenatal and early postnatal phthalate exposure. Cognitive flexibility can be explained as the ability to learn a new strategy while inhibiting the execution of a strategy that was previously correct (Ragozzino *et al.* 1999). Rats were trained to enter a target arm of a testing apparatus using a specific cue based on color to obtain a food reward. The next day they were trained to enter a target arm using a cue based on texture. Whether a rat performed a correct choice or an error across the trials was analyzed. Vehicle-exposed rats performed better and made fewer errors than phthalate mixture-exposed rats in the testing apparatus.

On the initial discrimination training, however, phthalate-exposed rats did not perform worse. These authors suggest that prenatal and early postnatal exposure to an environmentally relevant mixture of phthalates resulted in a long-term deficit in cognitive flexibility rather than general learning ability (Kougias *et al.* 2018a).

Rodents prenatally and early postnatally affected by phthalates – An animal model of autism?

As we have already stated, ASD is a disorder whose etiology is not yet known. In order to elucidate the fundamental causes behind this disorder and to develop reliable diagnostic methods and medication or some other methods to control the symptoms, many animal models are currently being used. Animal models play a substantial role in understanding this disease by enabling investigators to carry out experiments that would be impractical or ethically prohibited with humans. Available models include those with genetic, physiological, and behavioral relevance to an autistic phenotype. Such a phenotype can be induced by generating genetic mutations, brain lesions, or by administrating chemical substances (e.g. valproic acid). The matter of available animal models for ASD was reviewed in detail elsewhere (Olexová *et al.* 2012, Ergaz *et al.* 2016, Möhrle *et al.* 2020). Here, we would like to discuss the autistic phenotype observed in rodents after prenatal exposure to phthalates and whether it would be possible to use prenatal exposure to these chemicals in the development of a new animal model of autism.

The defining criteria for ASD are behavioral, therefore, to evaluate external manifestations of this disorder in animal models (i.e. autistic-like traits), behavioral test techniques are used (Crawley 2012). Usually, social interaction tests are used to evaluate social behavior. In this test, social responses and time spent in social interactions are measured in pairs of animals (Quinnies *et al.* 2017). Anxiety-like behavior is often determined by the elevated plus maze test where time spent in open and closed arms of a maze is measured. Less time spent in open arms and more time spent in closed arms is indicative of anxiety-like behavior (Quinnies *et al.* 2017). Depression is evaluated by the forced swim test, where an animal is forced to swim in a container from which it cannot escape. Increased immobility then demonstrates depression-like behavior (Xu *et al.* 2015). For analyzing motor activity and repetitive behaviors, the open field test is used. An animal is allowed to move freely in an arena with walls and its motor behavior is monitored (Barakat *et al.* 2018). Memory, learning, and cognitive ability are often measured by the Morris water maze (an animal has to swim to find the hidden platform and then across trials, it is expected to remember its position (Lin *et al.* 2015)) or the novel object recognition test (one of two known objects is replaced by a novel object and the amount of time taken to explore the novel object provides an index of recognition memory (Barakat *et al.* 2018). There is no doubt that prenatal exposure to phthalates affects the behavior of rodents. However, it yet remains unanswered whether the altered behavior can really be considered as an autistic phenotype. In animal models of autism, the behavior that is most frequently studied is social behavior. Social behavior was affected, and sociability was decreased after prenatal and early postnatal exposure to phthalates in a number of studies mentioned above (Lee *et al.* 2016, Quinnies *et al.* 2017, Kougias *et al.* 2018b). The occurrence of motor stereotypes and repetitive behaviors is also being examined when phenotyping mouse and rat models of autism. Regarding motor stereotypes, we were unable to find any studies addressing this parameter. Self-grooming is sometimes considered a repetitive behavior (Kalueff *et al.* 2016). Hoshi and Ohtsuka (2009) and Dai *et al.* (2015) investigated self-grooming after prenatal and postnatal phthalate exposure, however, it was to estimate the emotional state of the animals rather than to assess repetitive behavior. Moreover, results from these studies were contradictory, and no definitive conclusion can be drawn at this point. Behaviors relevant to associated symptoms of ASD are also being investigated. Anxiety-like and depression-like behaviors were observed in several animal studies assessing exposure to phthalates during prenatal and postnatal phthalate exposure (Xu *et al.* 2015, Quinnies *et al.* 2017). Cognitive abilities and cognitive flexibility were negatively affected by prenatal and early postnatal phthalate exposure (Lin *et al.* 2015, Kougias *et al.* 2018a). Hyperactivity might also be related to phthalate exposure, but results are inconclusive. Ishido *et al.* (2005) administered phthalates to male rats in early postnatal development and observed an increased spontaneous motor activity during the nocturnal phase later in life. On the other hand, motor activity levels of exposed male and female rodent offspring were not affected in studies by Boberg *et al.* (2011) and Lee *et al.* (2016), moreover, Dai *et al.* (2015) found that horizontal and vertical motor activity was decreased in female mice after prenatal and postnatal exposure to phthalates.

From the studies we have reviewed so far, we can say that this possible animal model of autism has only one out of three types of validity that are sought from animal models (Bourin *et al.* 2007). First is face validity, where the model is phenotypically similar and implies that the response observed in the animal model should be identical to the behavioral and physiological responses observed in humans. So far, it seems that in both humans and rodents, the behavioral and physiological impact of prenatal phthalate exposure is mostly the same. Second is construct validity, requiring that the etiology of the behavioral and biological factors underlying the disorder may be similar in animals and humans. The cause for abnormalities in reproductive tract development observed in rodents and humans after prenatal phthalate exposure seems to be a reduction of steroidogenesis and therefore, reduced function of steroids (Hannas *et al*. 2012). However, it is not yet clear how phthalates induce an autistic-like phenotype in rodents and humans. Reduced function of testosterone may play a role, especially in prenatal development when this hormone is essential for brain development (Negri-Cesi *et al.* 2004). Changes in brain structure and function described above in section 2 had a negative impact on cognition, learning, memory (Li *et al.* 2013, Dai *et al.* 2015, DeBartolo *et al.* 2016, Kougias *et al.* 2018a), and motor activity (Ishido *et al.* 2005). Moreover, interference with normal synaptogenesis and connectivity as well as morphological changes in different brain areas were suggested as potential causes for ASD (Ha *et al.* 2015, Guang *et al.* 2018). Therefore, changes caused by prenatal and early postnatal phthalate exposure in the development of the brain could indeed be responsible for an autistic phenotype, but more studies would be necessary to clearly address the construct validity of this model. Predictive validity (entails that the model should be sensitive to clinically effective pharmacological agents) is even harder to assess. One can say that, for now at least, it is impossible as there is no standard effective pharmacological treatment for ASD yet (Reichow *et al.* 2018).

One final point we would like to consider is that for ASD to manifest, it seems that genetic abnormalities and certain environmental conditions must be present. This means that we need not only understand the consequences of the genetic and environmental perturbations as such, we also need to examine their interactions. If, for example, a model based on genetic manipulations were to be combined with the environmental one and tested and described thoroughly, such a model could meet the criteria for all three types of validity, and this could lead to the development of one universal model of autism which could reflect this condition accurately and reliably.

Conclusions

In this review, we presented the existing body of evidence regarding the effect of prenatal and early postnatal exposure to phthalates on neurodevelopment and behavior and highlighted the possibility of phthalates having a role in the emergence of ASD. Autistic-like traits observed in animal studies, as well as the prospect of using developmental exposure to phthalates as a new animal model of autism, were also discussed.

Prenatal and early postnatal exposure to phthalates in rodents resulted in abnormalities in brain development and behavior observable in individuals with ASD. This could indicate the possibility of creating a new animal model of autism. However, it should be noted that not all tests used in reviewed studies are unanimously accepted for the measurement of ASD-like behaviors. Considering the information on validity, more studies would be necessary to determine if the “phthalate animal model of autism” has high translational value. Still, considering the results of reviewed studies, we believe that this matter should be further investigated, and more importantly, developmental phthalate exposure might be considered as an ASD risk factor.

Conflict of Interest

There is no conflict of interest.

Acknowledgements

This work was supported by grants VEGA 2/0166/16, VEGA 2/0154/20 and UK/73/2018.

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