

**The effects of early-life phthalate exposure on prefrontal cortex mediated
behaviours**

by

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**A thesis submitted to the Faculty of Graduate and Post-Doctoral Affairs in partial
fulfillment of the requirements for the degree of:**

Master of Science

in

Neuroscience

Carleton University

Ottawa, Ontario

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ABSTRACT

Human exposure to environmental toxicants, such as phthalates, occurs throughout the lifespan. Phthalates are a group of synthetic chemicals added to plastics to increase their flexibility and durability, however they easily migrate out of the plastic product into the environment. Children and infants have a higher level of exposure to phthalates than adults, which is concerning as the majority of neurodevelopment occurs during this time period. Previous research has shown that early life exposure to di-(2-ethylhexyl) phthalate (DEHP), the most commonly used phthalate worldwide, has resulted in altered neurodevelopment. DEHP was administered once daily from postnatal day (P) 18-23, a critical period of development of dopaminergic innervation to the PFC. Wildtype C57Bl/6 mice were used to assess behavioural effects. Transgenic AldH11-L10:GFP mice were used to measure changes in astrocyte number and activation in the PFC. Behavioural and histological measurements were assessed at P29 and P49, to examine both short-term and long-term responses. Both males and females were assessed to determine whether sex differences exist in response to early-life DEHP exposure. Our results suggest that male mice exposed to DEHP exhibit a short-term hyperactive phenotype paralleled by a decrease in anxiety-like phenotype. The majority of these effects were not seen at P49, suggesting these effects are not long-lasting. No differences were seen at either time point in females, suggesting that males and females differentially respond to early-life DEHP exposure. No changes in astrocyte number or astrocyte activation were observed, suggesting astrocytes in the PFC are not affected by early-life DEHP exposure. In conclusion, early-life DEHP exposure appears to have acute anxiolytic effects only in males, and these effects do not seem to be mediated through astrocytic changes in the PFC.

ACKNOWLEDGEMENTS

I would like to extend my most sincere thanks to my two supervisors, Matthew Holahan and Natalina Salmaso, for their constant support and guidance throughout the last two years. This process has been both challenging and rewarding and I would not have made it through this project without their encouragement.

I would also like to express my gratitude towards my committee members, Alfonso Abizaid, Michael Hildebrand, and Maria DeRosa for their time and input on this project.

Finally I'd like to thank my friends and family for their support. Thank you Angelo for happily talking about phthalates with me. Special thanks to the close friends I made throughout this journey who always kept a smile on my face during the countless hours in the lab.

TABLE OF CONTENTS

ABSTRACT	ii
ACKNOWLEDGEMENTS	iii
TABLE OF CONTENTS	iv
LIST OF TABLES	v
LIST OF FIGURES	vi
ABBREVIATIONS	vii
INTRODUCTION	8
Overview	8
Phthalates	9
Properties of Phthalates.....	9
Endocrine Disruption	11
Toxic Potential of Phthalates in Humans	14
Prefrontal Cortex	15
PFC Anatomy and Development	15
PFC network connectivity and DEHP interference	17
PFC and Dopaminergic Implications in ADHD	18
Hippocampus	19
Hippocampal Anatomy and Development.....	20
Interference from DEHP.....	21
Astrocytes	22
Hypothesis	25
METHODS	28
Experimental Animals	28
Genotyping.....	28
Phthalate injection schedule and dosing	29
Behavioural testing	31
Locomotor test/Open field test.....	31
Elevated Plus Maze	32
Adapted One-Day Morris Water Maze.....	32
Immunohistochemistry	33
Statistical analysis	36
Experimental Timeline	38
RESULTS	39
Behavioural Analyses	39
<i>Locomotion</i>	39
<i>Open Field Test</i>	41
<i>Elevated Plus Maze</i>	44
<i>Adapted One-Day Morris Water Maze</i>	47
Immunohistological results	55
DISCUSSION	59
Behaviour	59
Immunohistochemistry	63
CONCLUSIONS	70
REFERENCES	71

LIST OF TABLES

Table 1. *Experimental animal groups.*

30

Table 2. *List of antibodies used for immunohistochemical analysis in the PFC.*

35

LIST OF FIGURES

Figure 1. <i>Chemical structure of di-(2-ethylhexyl) phthalate</i>	10
Figure 2. <i>Experimental timeline for wildtype C57Bl/6 mice.</i>	38
Figure 3. <i>Experimental timeline for transgenic AldHL1-L10:GFP mice.</i>	38
Figure 5. <i>Locomotion at P25.</i>	40
Figure 6. <i>Locomotion at P45.</i>	41
Figure 7. <i>Anxiety behaviours on the open-field test in males and females at baseline at P25.</i>	41
Figure 8. <i>Open field test at P25.</i>	43
Figure 9. <i>Open field test at P45.</i>	44
Figure 10. <i>Anxiety-like behaviours on the EPM at baseline at P25.</i>	45
Figure 11. <i>Elevated plus maze at P25.</i>	46
Figure 12. <i>Elevated plus maze at P45.</i>	47
Figure 13. <i>Learning ability in males and females at baseline at P25.</i>	47
Figure 14. <i>Adapted one-day Morris Water Maze at P27.</i>	48
Figure 15. <i>Adapted one-day Morris Water Maze at P47.</i>	49
Figure 16. <i>Memory during probe trial of the MWM at P27.</i>	50
Figure 17. <i>Memory during probe trial of the MWM at P47.</i>	51
Figure 18. <i>Learning over trials on the adapted one-day MWM at P27.</i>	53
Figure 19. <i>Learning across trials on the adapted one-day MWM at P47.</i>	54
Figure 20. <i>Representative photo of PFC sections in females at P49.</i>	55
Figure 21. <i>Representative photo of a single astrocyte.</i>	55
Figure 22. <i>Astrocyte analysis in the PFC at P29.</i>	56
Figure 23. <i>Astrocyte density in the PFC at P29.</i>	57
Figure 24. <i>Astrocyte analysis in the PFC at P49.</i>	58
Figure 25. <i>Astrocyte density in the PFC at P49.</i>	58

ABBREVIATIONS

ADHD – attention deficit hyperactivity

disorder

ANOVA – analysis of variance

CA – *cornu ammonis*

DCC – deleted in colorectal cancer

DEHP – di-(2-ethylhexyl)-phthalate

DG – dentate gyrus

ED – embryonic day

EDC – endocrine disrupting chemicals

EPM – elevated plus maze

FGF2 – fibroblast growth factor 2

GD – gestational day

GFAP – Glial fibrillary acidic protein

GFP – green fluorescent protein

i.p. – intraperitoneal

LH – luteinizing hormone

mPFC – medial prefrontal cortex

MRI – magnetic resonance imaging

NSC – neural stem cell

MWM – Morris Water Maze

P – postnatal day

PBS – phosphate buffered solution

PCR – polymerase chain reaction

PFC – prefrontal cortex

PVC – polyvinyl chloride

RGC – radial glial cell

SEM – standard error of the mean

SVZ – subventricular zone

TH – tyrosine hydroxylase

VTA – ventral tegmental area

INTRODUCTION

Overview

Humans are exposed to environmental toxicants throughout the lifespan – one of these toxicants includes a group of synthetic chemicals, phthalates, that are added to polyvinyl chloride (PVC) plastics to increase their flexibility and durability (Graham, 1973). Phthalates are found in everyday items, including adhesives, building materials, medical devices, children's toys, paints, pharmaceuticals, and food products (Green et al., 2005; Sathyanarayana et al., 2008a). Since phthalates are found in such a wide variety of products, humans are likely exposed to continuous low levels throughout the lifespan. Because phthalates are not bound to the plastic polymer, they can easily migrate out of the plastic into the environment over time leading to human exposure.

While the incorporation of phthalates into children's toys has been restricted in the European Union and Australia since 1999 and 2010, respectively, no such restrictions have been put in place in Canada. Numerous animal studies and limited human studies have been conducted in an effort to examine the effects of phthalates on health, but the neurological toxic potential of phthalates has not yet been fully elucidated. An important question in the study of environmental toxicants is whether human exposure to phthalates can result in abnormal neurodevelopment and/or long-lasting health problems. While neurodevelopment begins prenatally, it continues after birth and is marked by the appearance of several sensitive periods for different brain regions. The prefrontal cortex (PFC) and the hippocampus are two such regions that have sensitive postnatal developmental periods. The PFC is one of the last brain regions to mature (Teffer and

Semendeferi, 2012), with ongoing dopaminergic innervation occurring throughout adolescence (Kalsbeek et al., 1988). The sensitive hippocampal period of development is marked by extensive synaptic growth and remodeling, with increases in mossy fiber projections from the dentate gyrus (DG) to the pyramidal neurons (Holahan et al., 2007; Keeley et al., 2010). As will be discussed in further detail in the following sections, exposure to environmental insults during any of these critical periods could disrupt these developmental processes, resulting in long-lasting deficits. The mechanism through which phthalates interfere with neurodevelopment is the focus of the current thesis.

Phthalates

Properties of Phthalates

Phthalates are a group of colorless, odorless, synthetic chemicals that were first produced in the 1920s to add flexibility to brittle plastics (Graham, 1973). They consist of a benzene ring with a di-ester structure and are synthesized from phthalic anhydride and an alcohol in a two-step process. Phthalates can be categorized into three groups based on the length of their ester side chain: low molecular weight, transitional or mid-molecular weight, and high molecular weight (NICNAS, 2010). The side chains of high molecular weight phthalates can have either a ring structure or a long, straight side chain with seven or more carbon atoms. High molecular weight phthalates with di-ester side chains in an *ortho* configuration on the benzene ring show the greatest toxic potential, as these phthalates do not undergo complete metabolism, whereas phthalates in a *para* conformation are completely metabolized (Barber et al., 1994). While the classification for phthalate toxicity is not yet completely understood, it is thought that a side chain with a backbone length of 4-6 carbons shows the most toxicity (Fabjan et al., 2006).

Di-(2-ethylhexyl)-phthalate (DEHP) is the most commonly used phthalate worldwide (Lyche et al., 2009) and is the main phthalate used in PVC plastics (Afshari et al., 2004; Clausen et al., 2004). It has a molecular formula of $C_{26}H_{44}O_4$ and a molecular weight of 390.57 g/mol (Agency for Toxic Substances and Disease Registry, 2002), and is thus classified as a high molecular weight phthalate with potentially high toxicity. DEHP has a lipophilic character and is thus able to quickly pass through biological membranes such as the placenta and the blood-brain barrier. DEHP can also cross the placenta into the fetus and can be transferred to neonates through the breast milk (Dostal et al., 1987; Fromme et al., 2011). Due to this toxicity potential, long-term exposure to DEHP may lead to negative outcomes on human health and thus will be the phthalate examined in this thesis.

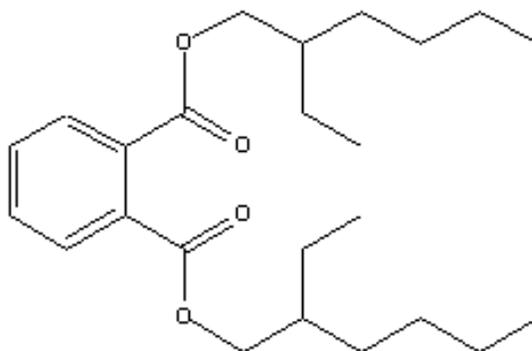


Figure 1. Chemical structure of di-(2-ethylhexyl) phthalate

Human exposure to phthalates can occur through inhalation, dermal contact, and ingestion (Engel et al., 2010). Adults are mainly exposed to DEHP through consumption of contaminated food whereas children are exposed to DEHP through both consumption of contaminated food or mouthing plastic toys (Wormuth et al., 2006). Concentrations of DEHP in plastic toys have been reported to be as high as 40% and can easily migrate out of the toys into saliva (Lyche et al., 2009). A study examined levels of DEHP exposure across different age groups in the European Union and found that the maximum daily

exposure to DEHP for infants and toddlers was 100 µg/kg of body weight per day, whereas the maximum daily exposure for adults was 10 µg/kg of body weight (Koch et al., 2003, 2005; Wormuth et al., 2006; Sathyanarayana et al., 2008b). Neonates may be exposed to even higher levels of DEHP, reaching 2.5mg/kg (2500 µg/kg) per day due to the use of medical devices including intravenous tubing, which is known to contain a high level of DEHP (Schettler, 2005).

There are two methods used to estimate DEHP exposure in humans. The first is via measurement of environmental levels of DEHP including levels found in food, dust, household products, toys, etc. This method is limited as estimating the exposure from different sources and different routes can become complicated. The second method is to measure the concentration of DEHP metabolites in urine. After DEHP is ingested, gut lipases break it down to the main metabolite, monoester 5-OH-MEHP (Tickner et al., 2001). The half life of DEHP is 6-12 hours, and 24 hours following DEHP ingestion, 67% of DEHP is excreted in the urine in the form of several metabolites, including 23% 5-OH-MEHP, 18.5% 5cx-MEPP, 15% 5oxo-MEHP, and 4.2% 2cx-MEHP (Koch et al., 2006). This method of measuring DEHP exposure is limited as well, since it assumes that all individuals metabolize DEHP at the same rate and it only gives a point estimate of DEHP exposure rather than chronic exposure levels.

Endocrine Disruption

Phthalates are classified as endocrine disrupting chemicals (EDCs): they are synthetic chemicals that mimic endogenous hormones and thus interfere with the proper functioning of the endocrine system (Crisp et al., 1998). As these compounds can cross the placental barrier and the blood-brain barrier, they have the potential for causing serious negative consequences for proper development.

Evidence for the endocrine-disrupting properties of phthalates comes from studies examining reproductive development (Swan et al., 2005; Foster, 2006; Pocar et al., 2012). *In utero* and lactational exposure to DEHP resulted in both altered gonadal weight and decreased germ cell quality and viability in mature male and female mice (Pocar et al., 2012). Prenatal exposure to DEHP resulted in reproductive abnormalities, including reduced anogenital distance, impaired testicular descent, and reduced genital size in male rats (Foster, 2006). These anatomical abnormalities are associated with changes in hormonal levels, as prenatal exposure to DEHP also resulted in decreased levels of serum testosterone and luteinizing hormone (LH) compared to controls when measured on both postnatal day (P) 21 and P35 (Akingbemi et al., 2001). This study also showed that male rats exposed to DEHP daily from P21-35 showed a 77% decrease in steroidogenic enzyme 17 β -hydroxysteroid dehydrogenase and a 50% decrease in Leydig cell testosterone production compared to controls. No changes in androgen biosynthesis were seen when DEHP exposure occurred from P62-89 (Akingbemi et al., 2001), suggesting that the timing of DEHP exposure is critical in determining whether any deficits will result.

One proposed mechanism for these endocrine-disrupting properties suggests that DEHP acts via an anti-androgenic mechanism (Parks et al., 2000). In this study, DEHP did not display affinity for testosterone receptor *in vitro*, but instead, prenatal exposure to DEHP resulted in decreased testosterone production in male rats down to female levels, suggesting that this anti-androgenic mechanism is not through interaction with the androgen receptor, but instead occurs through the inhibition of testosterone production. This supports the previously stated result that exposure to DEHP results in decreased Leydig cell function (Akingbemi et al., 2001), as Leydig cells are responsible for the

production of testosterone. These results suggest that exposure to DEHP during critical periods of reproductive development can lead to improper sexual differentiation.

Another proposed mechanism for the endocrine-disrupting properties of DEHP is that DEHP may disrupt estrogen metabolism through the suppression of aromatase, an enzyme that catalyzes the conversion of testosterone to estradiol and is thus critical for masculinization of the brain during development (Gorski, 1986). Aromatase activity can be disrupted by DEHP activity in both the male and female brains, though the effects occur at different times. In the hypothalamic/preoptic area of male rats, DEHP inhibits aromatase activity at low doses and increases it at high doses at P1, whereas in female rats, aromatase activity is disrupted at P22 (Andrade et al., 2006). These results suggest that there may be different sensitive periods for the disruptive effects of DEHP activity in males and females.

While the endocrine-disrupting properties of DEHP are not yet fully understood, sufficient evidence exists to suggest the potential for negative effects of early-life DEHP exposure on the function of the endocrine system. The majority of studies that have examined the endocrine-disrupting properties of phthalates were carried out in animals, however, progressively more human research is being conducted. One of the first studies to examine the endocrine disrupting properties of DEHP in humans demonstrated that maternal urinary metabolite levels of phthalates were correlated with reduced anogenital distance in male infants (Swan et al., 2005), thus supporting the data found in animal studies. This provides possible evidence that human exposure to DEHP can also result in reproductive and/or endocrine abnormalities.

Toxic Potential of Phthalates in Humans

Attention deficit hyperactivity disorder (ADHD) is a neurodevelopmental disorder characterized by an inappropriate pattern of inattention, hyperactivity, and/or impulsivity that interferes with functioning or development (American Psychiatric Association, 2013). ADHD is one of the most common childhood disorders (Dirlikov et al., 2015), with males being 2:1 to 9:1 more likely to have ADHD than females (Rucklidge, 2010). In recent years, an association between prenatal and childhood/adolescent exposure to phthalates, as measured by metabolites, and subsequent ADHD symptoms has been shown (Kim et al., 2009; Engel et al., 2010; Chopra et al., 2014). However the mechanisms that underlie this relationship have yet to be determined.

The fact that infants and children are exposed to higher levels of phthalates is concerning, as many critical phases of brain development occur during this period. A study examining the association between third trimester phthalate metabolites in maternal urine showed that increased high molecular weight phthalate metabolite levels (including DEHP metabolites) were correlated with decreased orientation and levels of alertness in girls within 5 days after birth (Engel et al., 2009). A follow-up study of these children when aged 4-9 years showed a correlation between increased levels of maternal urinary low molecular weight phthalates and boys with attention problems, decreased executive functioning, and increased scores on the Behavioural Symptoms Index (Engel et al., 2010). Boys aged 6-15 diagnosed with ADHD showed significantly elevated concentrations of DEHP metabolites in their urine compared with age-matched controls (Park et al., 2015). This study also found a negative correlation between the concentration of DEHP metabolites and cortical thickness in the right middle and superior temporal gyri, suggesting a possible relationship between DEHP exposure, development of

ADHD, and neuroanatomical changes. In a nationally representative survey of the United States population, a child between the ages of 6-15 showing a 10-fold increase in urinary DEHP metabolite concentrations was found to have twice the odds of being diagnosed with ADHD (Chopra et al., 2014). An association between levels of urinary DEHP metabolites and teacher-rated ADHD symptoms in children ages 8-11 has also been shown (Kim et al., 2009). Increased urinary concentration of MEHP in children with ADHD was associated with increased omission and commission errors on the Continuous Performance Test (CPT), a widely used measure of inattention and impulsivity in children with ADHD (Park et al., 2014). Interestingly, this relationship was only found in children possessing the DRD4 4/4 genotype, indicating the presence of polymorphisms at the dopamine receptor D4, the dopamine transporter, α -2A-adrenergic receptor, and norepinephrine transporter genes. This suggests the possibility of dopaminergic involvement in the onset and maintenance of ADHD that may interact with DEHP.

While our understanding of the relationship between early-life DEHP exposure and the subsequent development of ADHD remains limited, additional work has been conducted in animal models. These data are discussed in the following sections.

Prefrontal Cortex

PFC Anatomy and Development

The human cerebral cortex consists of VI granule cell layers and is organized such that the most superficial layer is layer I, and the layer closest to the ventricular zone is layer VI. The PFC is the anterior-most part of the frontal lobe of the cerebral cortex in mammals. The PFC is implicated in many higher cognitive functions grouped together under the term “executive function” including planning, decision-making, monitoring

information in working memory, and attention (Teffer and Semendeferi, 2012). It is suggested that the orbital and medial regions of the PFC are involved in regulation of emotional behaviour, whereas the lateral PFC is more tightly implicated in language and executive functioning (Fuster, 2001). The PFC undergoes progressively greater expansion and development in higher animals, reaching its most complex state in humans. Because anatomical studies of human PFC development are limited in resolution, rodents have been often employed as subjects to delineate the unfolding patterns and mechanisms of PFC development and maturation. As such, the following discussion of PFC development will consider findings from studies in rodents.

Development of the central nervous system begins when the ventral ectoderm matures into the neural plate and eventually folds into the neural tube (Osakada and Takahashi, 2011). Microglia are born in the yolk sac and are the first cellular migrants to the cortical region (Ginhoux and Merad, 2011). Neurons and macroglia (astroglia and oligodendrocytes) arise from radial glial cells (RGCs) that populate the ventricular zone and extend their endfeet to the pial matter, creating radial extensions which act as guides for migrating neuroblasts. Migration of these neuroblasts occurs from gestational day (GD) 16-21, with cortical layer VI forming first on GD16 and each subsequent layer developing from neural stem cells (NSCs) migrating through the previously formed layer to reach a more superficial end point (Berry and Rogers, 1965). New neurons travel to their final cortical destination through two modes of migration: somal translocation and locomotion (Nadarajah et al., 2001). Importantly, the majority of these neuroblasts will commit to an excitatory phenotype. Cortical inhibitory cells arise from a different population of NSCs located in the medial ganglionic eminence and migrate in a tangential pattern towards their final destination (Tan et al., 1998). Following migration

of neuroblasts, RGCs retract their radial extension from the pial matter and migrate to their own destinations throughout the layers of the cerebral cortex, forming individual, non-overlapping “tiled” domains (Halassa et al., 2007), where they will support the neurons within this region through various functions that will be outlined in the section on astrocytes below.

The subdivisions of the PFC can first be distinguished on P6 (Van Eden and Uylings, 1985). The subdivisions within the medial PFC are the medial precentral area, the dorsal anterior cingulate areas, and the prelimbic area. The PFC can be clearly distinguished from the rest of the cerebral cortex as rodents lack granule cell layer IV. Differentiation of neurons within the cortical plate and formation of the cortical layers occurs from P1 to P18 in the rodent brain (Van Eden and Uylings, 1985).

Lesion studies have shown that while PFC development begins prenatally, it has relatively late functional maturity compared to the rest of the cortex, as development continues throughout adolescence and into adulthood (Kolb and Nonneman, 1976). Synaptic pruning, a process of structural synaptic refinement, occurs throughout adolescence, with 15-20% of prefrontal dendritic spines being eliminated in adolescent (1-month-old) mice compared to 3-5% elimination occurring in adult (4 to 6-month-old) mice (Zuo et al., 2005).

PFC network connectivity and DEHP interference

The PFC receives dopaminergic projections from cell bodies that are located in the ventral tegmental area (VTA) and the substantia nigra; this is one of the only cortical areas to do so. The first dopaminergic fibers to reach what will become the PFC appear on embryonic day (ED) 16 (Verney et al., 1982) and this dopaminergic innervation continues to form and mature throughout adolescence (Kalsbeek et al., 1988). Adult

levels of medial PFC tyrosine hydroxylase (TH) are reached by P45, and juvenile rats (P25) showed only 40% of adult levels of dopaminergic innervation (Naneix et al., 2012). TH is the enzyme responsible for catalyzing the conversion of L-tyrosine to L-DOPA during the synthesis of dopamine. This ongoing innervation is mediated by Netrin-1, a protein that acts as a guidance cue and determines the target for growing neurons, either by attracting them via DCC receptors or by repelling them via UNC5 receptors (Hoops and Flores, 2017).

Exposure to DEHP early in development results in disrupted dopaminergic function in the brain. In vitro addition of MEHP to PC12 cells (used as a model of neurodifferentiation) resulted in the down-regulation of TH mRNA. A single injection of DEHP given to rats on P5 significantly increased hyperactivity and significantly decreased dopamine D1A receptor expression in these rats when measured at four weeks old (Ishido et al., 2005). Four-week old female mice exposed to various daily doses of DEHP for 2 weeks resulted in significant decreases in dopamine D2 receptor levels in the striatum at all doses (Wang et al., 2016). Male and female rats exposed to either 10 mg/kg or 20 mg/kg once daily from P16-22 showed reduced TH density in the substantia nigra, and increased locomotor activity (Holahan et al., 2018). These data suggest that alterations of dopaminergic development and function occur after early-life exposure to DEHP.

PFC and Dopaminergic Implications in ADHD

One area of research focuses on the potential involvement of PFC dysfunction in the development and maintenance of ADHD. Boys and girls with ADHD show impairments in executive function and planning, as well as impairments in cognitive control. Adolescent boys with ADHD have been shown to have decreased prefrontal

tissue volume compared to age-matched controls (Castellanos et al., 1996; Mostofsky et al., 2002), providing a possible explanation for the differences in the behavioural phenotype observed. Contradictory results exist, as MRI studies suggest that girls with ADHD show reductions in total PFC surface area, whereas boys show reductions only in the right anterior cingulate and the left medial PFC (Dirlikov et al., 2015). These results, along with the increased prevalence of ADHD diagnoses among boys, suggest there are sex-specific mechanisms involved in the pathology of ADHD, although additional research is needed to fully parse these out. Additional support for the hypothesis of dopaminergic involvement in ADHD comes from successful treatment of ADHD symptoms with methylphenidate, a dopamine transporter antagonist (Dresel et al., 2000).

Hippocampus

In addition to receiving dopaminergic projections from the VTA, the PFC also receives projections from the hippocampus proper (Swanson, 1981). Additionally, the hippocampus and medial PFC display coordinated firing through theta rhythms (4- to 12-Hz oscillations) during complex tasks, including during spatial exploration and decision-making (Jones and Wilson, 2005). The hippocampus is functionally connected to the PFC in their common role in the implementation of working memory. Working memory is the way through which the brain temporarily stores and manipulates information, processes that are critical for complex cognitive tasks such as reasoning and learning (Baddeley, 2010). In addition to the hippocampus, the PFC is critically involved in this function, as it functions in higher cognitive control. Specifically, the medial PFC is involved in working memory. Neural activity in the theta range in the PFC becomes synchronized with activity in the hippocampus during working memory tasks (Jones and Wilson, 2005).

Hippocampal Anatomy and Development

The hippocampus proper is functionally connected to surrounding brain regions including the DG, containing the *fascia dentate* and the hilus, subiculum, and entorhinal cortex, which together make up the hippocampal formation (Khalaf-Nazzal and Francis, 2013). The hippocampus proper is called the *cornu ammonis* (CA), which is subdivided into CA1, CA2, and CA3 regions. The CA3 region is innervated through three pathways (Amaral and Witter, 1989; Witter, 2007). The first is the mossy fiber pathway, in which mossy fibers from the DG project to the CA3 apical dendrites. The second consists of projections from the entorhinal cortex to the CA1 and CA3 areas, called the perforant pathway. The third pathway is through recurrent collaterals within the CA3 region itself.

Development of the hippocampus proper follows an inside-out pattern of development and migration (Deguchi et al., 2011), much in the same way as the cerebral cortex. This is due to the fact that both the PFC and the hippocampus are brain regions within the telencephalon. The hippocampus forms in the dorso-medial region of the telencephalon starting on ED8.5. Different subregions of the hippocampus follow different developmental time courses. The CA fields are laminated and form discrete subregions with distinct morphology, connectivity, and electrophysiological properties. KA1, a marker specific to CA3 neurons, and SCIP, a marker specific to CA1 neurons, are detectable in the developing hippocampus on ED14.5 and ED15.5, respectively (He et al., 1989; Tole et al., 1997). These time points represent the peak of neurogenesis in these respective areas (Angevine, 1965; Stanfield and Cowan, 1979).

Cells that will form the DG begin developing from the ventricular zone. The ventricular zone is a transient embryonic layer of tissue containing neural stem cells, principally radial glial cells, which lines the ventricles. These cells undergo tangential

migration to reach their final location (Nakahira and Yuasa, 2005). A period of neurogenesis occurs in the granule cell layer of the DG from P15 to P18, during which time the cell density of the granule cells increases (Bayer, 1980).

Following this period of neurogenesis in the DG, the mossy fibers projecting to the CA3 region undergo extensive remodeling that appears to be completed by P24 (Amaral and Dent, 1981). Thus, the period from P18-24 is hypothesized to represent a critical period of development in the CA3 region (Holahan et al., 2007).

Interference from DEHP

Since phthalates have been shown to be able to reach the developing fetus by crossing the placenta, and since infants and children have higher levels of exposure to phthalates, one important question is whether phthalates can affect the developing hippocampus. Animal models have been used to study this question. Offspring born to dams exposed to DEHP from GD2-21 were reported to have increased path length to find the hidden platform on the Morris Water Maze (MWM) during acquisition and spent less time in the target area during the probe trials than the controls, suggesting impairments in learning and memory (Sun et al., 2012). In addition to negatively affecting spatial learning and memory, early-life exposure to DEHP may also increase anxiety-like behaviours. DEHP exposure from GD7 to P21 resulted in both male and female 6 week-old mice spending less time in the open arms of the elevated plus maze (EPM), suggesting an anxiogenic effect of perinatal DEHP exposure (Xu et al., 2015).

The behavioural deficits seen following early-life DEHP exposure are associated with structural changes within the hippocampus. Male mice exposed to 10mg/kg DEHP from P16-22 had reduced axonal innervation in the CA3 region, decreased CA3 cell density, and decreased neurogenesis occurring in the DG (Smith et al., 2011). These

results were seen only in male rats, with no differences observed between females exposed to DEHP and controls. This provides further evidence that exposure to environmental toxicants such as phthalates during sensitive developmental periods adversely affects neurodevelopment such that structural and behavioural deficits appear. Important to note is the sex differences seen in these studies, as this suggests that phthalates differentially affect developing males and females.

Astrocytes

Although several studies have suggested that neurodevelopment is negatively affected by early-life exposure to phthalates, the exact mechanism by which this occurs is still unclear. However, many of the perturbations induced by phthalates, such as improper network connectivity, decreased neuronal spine development, and impaired endocrine functioning, include functions that are generally governed by astroglial cells. Therefore, it is possible that astroglial cells themselves might be affected by early-life DEHP exposure.

Astroglia were long thought to be passive support cells, simply the “glue” holding the neurons together. Astroglia are the most abundant cell type in the mammalian brain, representing approximately 50% of the cells in the mammalian brain (Wiese et al., 2012). Recent work has demonstrated that astroglial cells show similar levels of heterogeneity as neurons, as there are subtypes of astroglial cells that differ in morphology, protein expression patterns, location, and function. These subtypes include the typical star-shaped protoplasmic astrocytes, astroglial stem cells with a more radial morphology, reactive, and stellate subtypes, amongst others. Protoplasmic astrocytes are the most common astrocytes observed in the intact mature cortex. They are tightly associated with synapses, from formation to pruning and maintenance of synaptic function. In the mature

brain, protoplasmic astrocytes occupy functional domains, such that there is limited overlap between the territory of two astrocytes (Bushong et al., 2004). Research on astroglia has increased significantly in recent years, and it is now widely accepted that astroglia are very important active players in the nervous system, as will be outlined below.

Astrocytes have a major role in synaptogenesis. This was first demonstrated in cultured rodent retinal ganglion cells: neurons had a sevenfold increase in the number of mature, stable synapses as well as increased functionality of these synapses in the presence of astrocytes as compared to neurons cultured without these glia (Ullian et al., 2001). This role of astrocytes in synaptic development has been found in both cortical (Diniz et al., 2012) and hippocampal cell cultures (Xu et al., 2010), with accelerated synaptogenesis occurring after the addition of thrombospondins or transforming growth factor β , respectively, both astrocyte-secreted proteins, and has also been demonstrated in *ex vivo* and *in vivo* models.

As mentioned previously, the cerebral cortex follows a laminar pattern of development, with new layers migrating through previously formed layers in an inside-out pattern, via either somal translocation or locomotion. Somal translocation occurs in neurons with a long leading process that extends out towards the pial surface; the neuron travels to its final destination through shortening of this process, thus moving the entire soma (Nadarajah et al., 2001). In contrast, a distinct population of cells travels through the cortex via locomotion. These cells are flattened on one side due to their apposition against a radial glial cell guiding the neuronal migration. These migration processes occur during both prenatal (Nadarajah et al., 2001) and postnatal development (Stitt et al., 1991). In addition to simply guiding cortical development, it was discovered that radial

glia have neural stem cell potential, with the ability to differentiate into oligodendrocytes, astrocytes and neurons. These radial glial cells are involved in first generating cortical neurons and then guiding them to their proper location in the cortical lamina (Nadarajah, 2003). Furthermore, a small subset of astrocytes found in the dentate gyrus of the hippocampus and the subventricular zone (SVZ) remain neurogenic throughout the lifespan. Mature cortical astroglial cells are also capable of de-differentiating and regaining neural stem cell capabilities following various injuries such as hypoxia and lesion models. Since astroglial cells play such an important role in typical cortical development, early-life insults that affect astroglial cell function can potentially lead to long-lasting detrimental consequences for cellular (neuronal) and cortical network development.

Astrocytes release several trophic factors in their role of modulating neurodevelopment, including fibroblast growth factor 2 (FGF2), a growth factor that functions in hippocampal neurogenesis, neurite outgrowth, and synapse formation (Flores et al., 2002). Importantly, the hippocampus expresses the highest levels of FGF2 and its receptors. Additional work has shown that FGF2 regulates neuronal density in the cerebral cortex (Dono et al., 1998) and that FGF2 is essential for cerebral cortex development, as FGF2 knockout mice showed a 45% reduction in cortical neuron number (Raballo et al., 2000).

Interestingly, astrocytes show expression of steroid hormone receptors including estrogen and progesterone receptors; their expression has been observed both throughout development and in adulthood. These steroid hormones are thought to affect astrocytic function, as administration of estrogen to female rats changed the number of astrocytic processes in contact with neurons and resulted in synaptic remodeling (Garcia-Segura et

al., 1986). Further, ovariectomized rats showed a significant increase in glial fibrillary acidic protein (GFAP) density, an intermediate filament protein expressed by astrocytes, after administration of estradiol compared with ovariectomized rats without estradiol treatment (Tranque et al., 1987). Ovariectomy has also been shown to increase levels of FGF2 in dopaminergic projection regions of the PFC (Flores et al., 1999), providing further evidence that steroid hormone levels affect astrocytic function. Neuronal deficits have also been observed following changes in levels of steroid hormone receptors, as ovariectomy in rats leads to decreased CA1 dendritic spine density, deficits which are rescued with estradiol administration (Gould et al., 1990). As astrocytes are known to mediate synaptic function, it has been suggested that changes in neuronal structure and function that arise due to changing steroid hormone levels are mediated through the effects of these hormones on astrocytes (Flores et al., 1999). Since DEHP is classified as an EDC, it is possible that it has the ability to bind to estrogen receptors on astrocytes, thus influencing their function, potentially leading to adverse effects on neurons and even resulting in neurodevelopmental deficits if exposure occurs during early life.

Hypothesis

Humans have ubiquitous exposure to phthalates throughout the lifespan, with the most adverse effects resulting if the exposure occurs early in development, either pre- or postnatally. While research has begun investigating the neurotoxic effects of early-life phthalate exposure, the exact toxic potential, and the mechanism through which these effects arise have not yet been elucidated. The process of neurodevelopment includes sensitive developmental periods for specific brain regions, and it is thought that insult or injury during these sensitive periods could result in abnormal neurodevelopment. Previous studies have shown that early-life exposure to DEHP, the most commonly used

phthalate worldwide, may result in abnormal hippocampal development (Smith et al., 2011), alterations in the development of the dopaminergic system (Holahan et al., 2018), and human research has also linked this exposure with the onset of ADHD (Kim et al., 2009; Chopra et al., 2014). However, these adverse effects of DEHP may be sex-specific, perhaps due to different critical periods of development or due to neuroprotective effects of differing hormone levels.

Previous research has shown that the hippocampus undergoes a period of critical development beginning on P18 and ending on P24 (Holahan et al., 2007), and daily exposure to DEHP during this time resulted in abnormal hippocampal development in male rats (Smith et al., 2011). This critical period of hippocampal development is paralleled by increased dopaminergic innervation in the PFC. The PFC is implicated in higher-order cognitive functions, including attention, planning, and behavioural inhibition. In addition, dopaminergic innervation in the PFC has been implicated in neurodevelopmental disorders in humans, especially ADHD. The development of ADHD has also been linked to early-life phthalate exposure, again with sex-specific effects. Finally, DEHP exposure has been suggested to decrease proper dopaminergic innervation throughout the brain. These data together lead to the hypothesis that DEHP exposure could alter dopaminergic innervation in the PFC, which then leads to a hyperactive phenotype in rodents, suggestive of an ADHD phenotype.

The process of neurodevelopment occurs through a variety of mechanisms, but one of the most important contributors to neurodevelopment is astroglial cells. These cells are responsible for ensuring proper migration of neurons through the laminar structure of the cortex, as well as proper hippocampal development. Because 1) the perturbations in neurodevelopment that are induced with DEHP exposure represent

functions that are typically regulated by astroglia, and 2) it is believed that DEHP is an endocrine disrupting chemical, and 3) astroglial cell function and protein expression patterns are altered in response to changes in steroid hormone levels, it is hypothesized that the developmental effects of exposure to DEHP may be regulated through direct actions of DEHP on astroglia. Specifically, it was hypothesized that astrocytes in the PFC will have altered morphology or altered levels of functioning as a result of DEHP exposure during the critical period of prefrontal dopaminergic innervation. It was further hypothesized that these neuroanatomical deficits will be complemented with behavioural abnormalities, including decreased learning ability, increased locomotion, and increased anxiety-like behaviours. Finally, it was hypothesized that any alterations seen in response to early-life DEHP exposure will be sex-specific.

METHODS

Experimental Animals

Wildtype C57Bl/6 mice were ordered from Charles River Laboratories. Mice arrived on P11 in order to ensure one week of acclimation prior to the beginning of the experiment. Mice arrived with a surrogate mother, and all mice were housed in the standard (27 cm x 21 cm x 14 cm) fully transparent polypropylene cages until they were weaned on P21. After weaning, mice were group housed with 2 or 3 same-sex mice per cage.

AldHL1-L10GFP transgenic mice were bred in house (Breeding Colony, Carleton University, Ottawa) on a C57Bl/6 background. These transgenic mice have green fluorescent protein (GFP) fused to the ribosomal protein L10a under the control of Aldh-11, a pan astrocytic promoter. On P14, ear notches were taken from the pups, and Polymerase Chain Reaction (PCR) was conducted so as to determine which pups expressed the transgene.

Genotyping

Lysis buffer was made using 10mL sterile water (Thermo Scientific #R0581), 14 μ L 50% sodium hydroxide, and 14 μ L ethylenediamine tetraacetate acid (EDTA; 0.5 molar, pH 8.0; Fisher Scientific BP2482100). Ear notches were lysed with 75 μ L of this lysis buffer and were heated to 90°C for 40 minutes. Lysis buffer was then deactivated by adding 75 μ L of Tris buffer, made up of Tris Hydrochloric Acid (Fisher Scientific #BP153-500) and Tris Hydroxymethyl Aminomethane (BioShop® #77-86-1), to each sample and placing samples on ice for 30 minutes. A master mix was created using the following ingredients: 6.25 μ L HotStart MasterMix (BRAND), 1 μ L of GFP forward primer (EGFPFOR aagttcatctgcaccaccg; Invitrogen #280187G06), 1 μ L of GFP reverse

primer (EGFPREV tgctcaggtagtggtgtcg; Invitrogen #208187G07), and 2.25 μ L nuclease-free H₂O (Thermo Scientific #R0581). PCR thin-wall tubes (Ayxgen PCR-0208FCP-C) were filled with 11 μ L of this solution along with 1 μ L of sample, and were centrifuged for 30 seconds. Tubes were placed in the Thermal Cycler (VWR, #THERM-1001) following GFP protocol for 2 hours and 58 minutes. During this time, a 2% gel for electrophoresis was prepared using 200mL 1xTAE with 4 grams of agarose (Fisher Scientific, #BP160-500), This solution was heated 3x 45 seconds in the microwave, 4 μ L of ethidium bromide 1% solution (Fisher Scientific BP-1602-10) was added, and gel was poured into a mold. Once the amplification cycle was completed and the gel had set, samples were loaded into wells and gel electrophoresis was conducted at 120 V for 25 minutes to allow for visualization of GFP bands.

All AldHL1-L10GFP pups were housed in the standard (27 cm x 21 cm x 14cm) fully transparent polypropylene cages with the mother until they were weaned on P21, at which point they were separated based on sex. GFP-positive mice were retained and housed in groups of 2 or 3 same-sex mice per cage. GFP-negative mice were euthanized via rapid decapitation and were not included in this study.

All mice had *ad libitum* access to chow and water and were maintained on a 12-hour light/dark cycle in a temperature-controlled environment (21°C).

Phthalate injection schedule and dosing

Male and female C57Bl/6 wildtype mice and Aldh-11-L10:GFP transgenic mice were randomly injected intraperitoneally with 0mg/kg, 10mg/kg, or 20mg/kg Bis-(2-ethylhexyl)-phthalate PESTANAL® (Sigma-Aldrich #36735; DEHP) once daily from P18-23. These doses were chosen based on previous research showing changes in brain

lipid composition following exposure to 10 mg/kg of DEHP (Smith et al., 2015). These doses are also relevant to the “no observable adverse effect levels” (NOAELs), or the level of toxin exposure that has no biological consequences on an organism (Faustman and Omenn, 2001). The NOAEL for DEHP is 4.8 mg/kg/day (Lyche et al., 2009), and the estimated exposure level for DEHP is up to 9.8 mg/day in children and infants from ages 2-14 (Wittassek et al., 2011). Injections were given intraperitoneally so as to increase bioavailability by avoiding absorption in the intestines and by bypassing the first effect of hepatic metabolism (Holahan et al., 2018).

A)

	Vehicle	10 mg/kg	20 mg/kg
Male	10 wildtype mice 5 transgenic mice	10 wildtype mice 5 transgenic mice	10 wildtype mice 5 transgenic mice
Female	10 wildtype mice 5 transgenic mice	10 wildtype mice 5 transgenic mice	10 wildtype mice 5 transgenic mice

B)

	Vehicle	10 mg/kg	20 mg/kg
Male	11 wildtype mice 5 transgenic mice	10 wildtype mice 5 transgenic mice	10 wildtype mice 5 transgenic mice
Female	11 wildtype mice 5 transgenic mice	11 wildtype mice 5 transgenic mice	11 wildtype mice 5 transgenic mice

Table 1. *Experimental animal groups.* A) depicts the groups for the early time point B) depicts the groups for the late time point

All injections were given between 9:00 and 10:30 am. Because DEHP is lipid-soluble, different concentrations were made by diluting stock DEHP with corn oil, which was also used as vehicle for control injections. Weights were taken the day of the first

injection to ensure proper dosage. Tails were marked with permanent marker in order to differentiate between the different doses; black was used for vehicle, red for 10 mg/kg, and blue for 20mg/kg. Animals were monitored throughout the duration of the experiment for signs of illness or distress, however none were noted. The breakdown of animals listed in Table 1 represent the number of animals used for all behavioural and Immunohistochemical results displayed below.

Behavioural testing

C57Bl/6 wildtype mice were used for behavioural measures. As described in Table 1 above, 10 mice per group (N=120) were tested in order to ensure sufficient power to identify behavioural effects.

Locomotor test/Open field test

The open field test is a commonly used measure of rodent exploratory locomotion and innate anxiety in a novel environment (Salmaso et al., 2016). Mice were placed in the center of a brightly lit (650 lux) white polypropylene box (50 cm x 50 cm x 35 cm) and were videotaped with AnyMaze Video Tracking System for 20 minutes. Although all behavioural measures were calculated for 20 minutes, the anxiety portion of the open field focuses only on the first 5 minutes of the test when group differences in anxiety behavior are typically observed (Salmaso et al., 2016). In contrast, data from the full 20 minutes was used as the test for general locomotor activity. Distance travelled, average speed, and percentage of time spent in the central vs. peripheral zones of the field were calculated by AnyMaze software. The test apparatus cleaned with Acell wipes between each animal and were wiped dry.

Elevated Plus Maze

The elevated plus maze (EPM) is another commonly used and widely validated measure of innate rodent anxiety (Osborne et al., 2009). The EPM is considered to be an ethnologically-based approach-avoidance task that measures the conflicts between the natural exploratory tendency of rodents in a novel environment and their natural aversion to brightly lit open areas. The EPM provides a measure of innate anxiety in the absence of conditioning or strongly aversive stimulus effects and has the additional advantage of no reliance on memory or pain thresholds and has no lengthy training or observation periods. In the EPM, mice were allowed to freely explore a novel, elevated, plus-shaped maze in which two opposing arms have walls (the closed arms) and the other two arms are devoid of walls (the open arms). Rodents generally spend less time exploring the open arms compared to the closed arms, and display behavioural and physiological indices of fear during open arm exposure. Avoidance of the open arms is considered to reflect anxiety.

The EPM used has four 30 x 5 cm arms, 2 enclosed with 25 cm walls and was elevated 30 cm from the floor. Illumination on all parts of the maze was 40 lux. Initially, animals were placed in the center of the maze facing an open arm. They were subsequently allowed to explore the maze for 5 minutes. The testing sessions were videotaped and behavioural outputs including the number of arm entries and time spent on the arms were analyzed with the AnyMaze software. The maze was cleaned with Acell Wipes and wiped dry between each animal.

Adapted One-Day Morris Water Maze

The Morris Water Maze is a task used to assess cognitive abilities, such as learning and memory, in rodents (Nunez, 2008). A one-day adapted version of the task

was used so as to decrease variability introduced by changes in cognitive abilities across days of the female estrous cycle. The pool was filled with water maintained at 25°C. A platform was placed in the pool just below the surface of the water, and the water was coloured with non-toxic children's paint so the platform was hidden from view. The pool was visually divided in quadrants; a quadrant was picked randomly as a starting location, and the starting location and order were both conserved across testing cohorts. Each mouse was placed in the first quadrant and allowed to swim until they found the platform or until 60 seconds had passed. At the end of the 60 seconds, the mice were placed on the platform for 15 seconds for them to associate location cues with the escape platform location. Once all mice underwent the first trial, the first mouse was placed in a different quadrant, and again was allowed to find the platform, which did not change location. This continued for 12 trials, and the amount of time each mouse took to find the platform in each trial was recorded. There was an hour break after both the 4th and 8th trials to prevent fatigue. The 13th trial was the probe trial, in which the platform was removed, and the mice were allowed to swim for 60 seconds. This trial was videotaped and analyzed using AnyMaze Software. The following variables were analyzed during the probe trial: the amount of time spent in the target zone (the location of the platform during the first 12 trials), number of entries into the zone, path length, and first latency to enter the target zone. At the end of the task, mice were dried and returned to their home cages.

Immunohistochemistry

C57BL/6 wildtype mice were euthanized on P29 for the short-term group and P49 for the long-term group via rapid decapitation. Upon decapitation, trunk blood was immediately collected in an Eppendorf tube, which was coated in EDTA (0.5 molar, pH 8.0; Fisher Scientific BP2482100) and centrifuged for 6 minutes at 4°C; the serum was

extracted and the samples stored at -80°C . Upon extraction, the brains were flash frozen at -80°C .

Aldh-L1-L10GFP transgenic mice were similarly euthanized and intra-cardially perfused on P29 and P49. Briefly, an intraperitoneal injection of 0.10-0.20 mL sodium pentobarbital was administered and a toe pinch was used to ensure proper level of anesthetic. Perfusions were performed via administration of saline into the left ventricle of the heart to flush out the blood, followed by administration of 4% paraformaldehyde to fix the tissue. Brains were collected and stored in the fridge in 4% paraformaldehyde for 24 hours before being transferred to 30% sucrose (Fisher Chemical #173322) solution for 48 hours. The brains were then stored at -80°C , until they were sectioned on a Leica (Leica™ CM 1900) cryostat (25 μm thick). The whole brain was sliced, with 20 sister sections slide mounted on electrostatic slides, resulting in 20 slides each with a representation of the whole brain for stereological reconstruction as per our previous studies (Salmaso et al., 2016). Sections were then processed for immunohistochemistry and to analyze total astrocyte number and astrocyte activation.

One complete set of representative sister sections from each subject was employed for each immunohistochemical stain. All slides were prepared using identical procedures and processed simultaneously to minimize batch effects.

Prior to staining the sections, a stock solution of 1X phosphate buffered solution (PBS) with 0.3% Triton 100-X (Fisher Scientific) and mixed with 10% horse serum (Gibco™) was prepared. Each slide was incubated with 500 μL of the PBS/serum solution for 1 hour at room temperature. The primary antibodies were diluted in the PBS/serum solution and 500 μL was applied to each slide (refer to Table 2 for list of primary antibodies used). The slides were covered and left overnight at room temperature. The

following day, the slides were washed 3 times with 1X PBS for 5 minutes each time to remove any residual unbound antibodies. Conjugated secondary antibodies were diluted as appropriate and sections were incubated for 1.5-2 hours at room temperature (refer to Table 2 for list of secondary antibodies used). Following incubation with the appropriate secondary antibodies, the slides were washed 3 times with 1X PBS for 5 minutes each time, again to remove unbound antibodies. The slides were then mounted using 3 drops of mounting solution with DAPI (VectaShield Hard Set) and then covered with a cover slip (Fisher Scientific).

Analysis of astrocytes was conducted using StereoInvestigator software in order to measure number of astrocytes, astrocyte activation as measured through GFAP expression, and total volume.

Antibody	Supplier	Species	Dilution	Clone information
GFP	Abcam	Chicken	1:1000	Polyclonal
GFAP	Proteintech	Rabbit	1:500	Polyclonal
Anti-chicken	Invitrogen	Donkey	1:1000	Polyclonal
Anti-rabbit	Invitrogen	Donkey	1:500	Polyclonal

Table 2. List of antibodies used for immunohistochemical analysis in the PFC.

Total cell counts were conducted in one hemisphere of the PFC using Zeiss AxioImager M2 with Apotome motorized fluorescent microscope (Carl Zeiss, Thornwood, NY, USA) as well as with StereoInvestigator™ Software (MicroBrightfield, Colchester, VT, USA). Contours encompassing the entire prefrontal cortex were drawn as boundaries, and cells within these contours were counted for expression of GFP and/or GFAP at 40x magnification. Sampling grips were optimized to ensure a minimum of 3 sampling sites per contour, allowing for unbiased stereological sampling. This allows for

the estimation of cell density and total volume regardless of cell shape, size, spatial distribution, or post-mortem tissue shrinkage (Schmitz & Hof, 2005). All analysis was conducted blind to experimental condition.

Representative confocal images were obtained using ZEN software (Zeiss™) with the Airyscan 800 microscope (Zeiss™).

Statistical analysis

The data collected was analyzed using a 2 (male vs. female) x 3 (0mg/kg vs. 10mg/kg vs. 20mg/kg) between subjects analysis of variance (ANOVA) design. The statistical analyses were conducted using IBM SPSS Statistics software (Version 20). The data were analyzed independently for both the early and late time-points.

Interactions were considered statistically significant if they were observed at $p \leq 0.05$, in which case post-hoc tests using a Bonferroni correction were conducted. If no interactions were observed, main effects or simple effects were considered to be statistically significant at $p \leq 0.05$, in which case two-tailed t-tests were conducted and again were reported to be statistically significant at $p \leq 0.05$.

Differences were observed between males and females in the vehicle group for the open field test and elevated plus maze results. As such, these data are expressed as z-scores. The formulae for calculating z-scores are as follows:

$$Z\ score_{OF} = \frac{X - \bar{X}}{std(X)}$$

$$Z\ score_{EPM} = \frac{X - \bar{X}}{std(X)}$$

Data from the adapted one-day MWM and Immunohistochemical analyses are expressed as mean \pm standard error of the mean (SEM).

Experimental Timeline

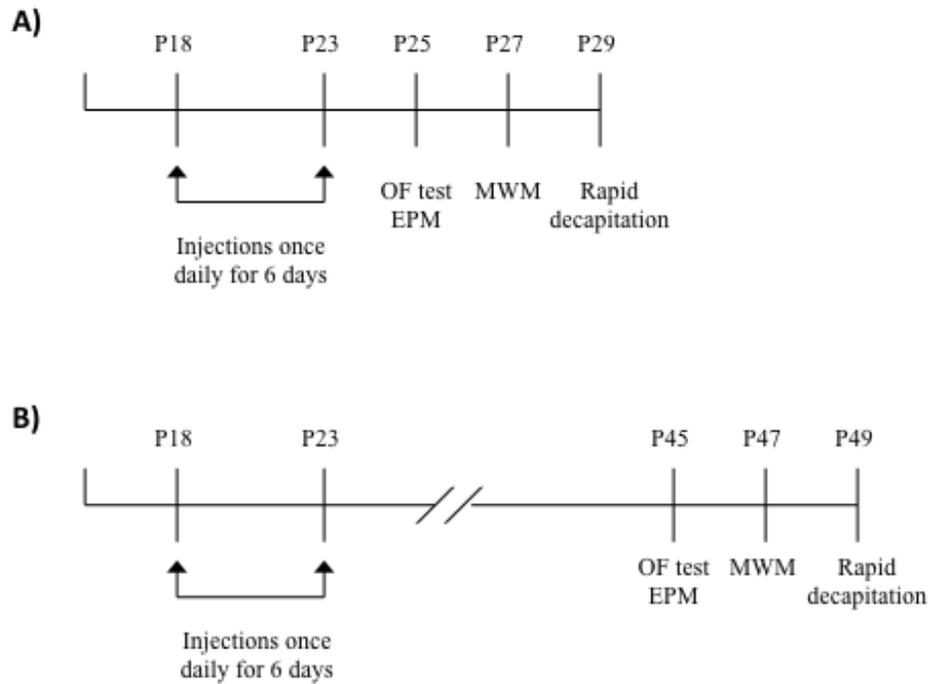


Figure 2. Experimental timeline for wildtype *C57Bl/6* mice. A) depicts the early time point; B) depicts the late time point

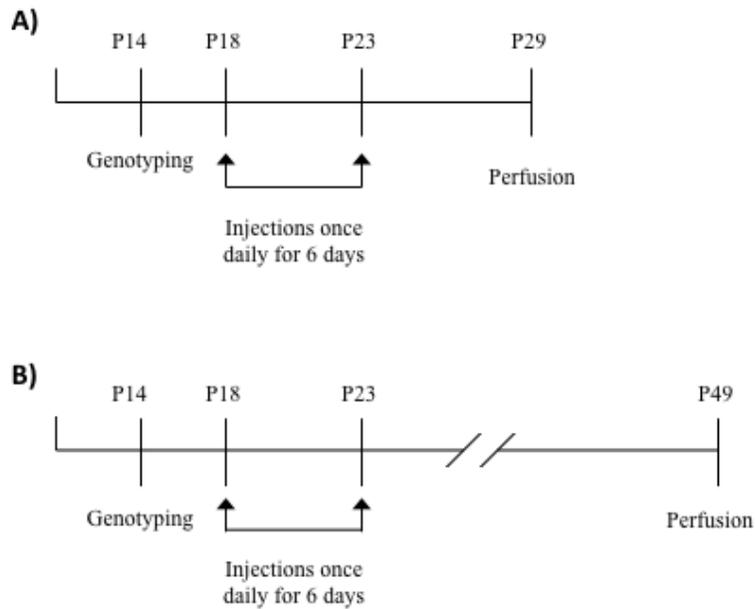


Figure 3. Experimental timeline for transgenic *AldHL1-L10:GFP* mice. A) depicts the early time point; B) depicts the late time point.

RESULTS

Behavioural Analyses

Locomotion

A two-way analysis of variance test was conducted to assess the effect of treatment (vehicle vs. 10 mg/kg DEHP vs. 20 mg/kg DEHP) and sex (male vs. female) on locomotor activity.

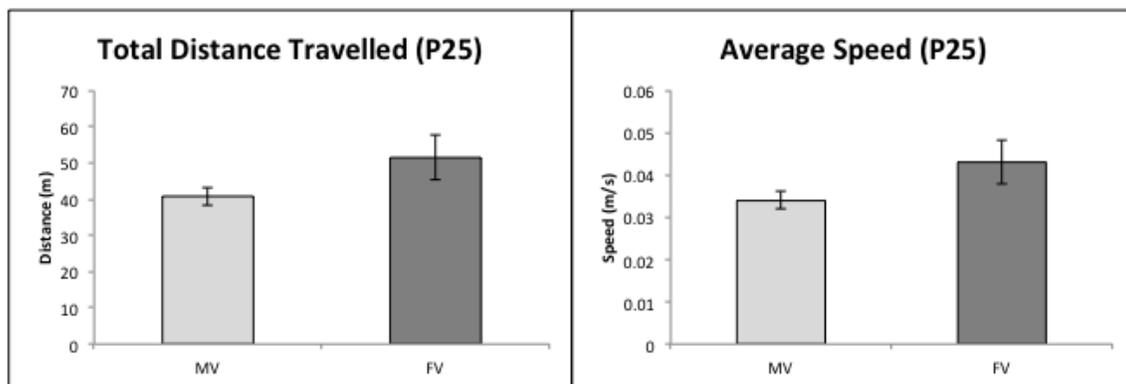


Figure 4. *Locomotion activity in males and females at baseline at P25.*

At P25, males and females administered vehicle differed in their behaviour on the locomotor test, such that females travelled a greater total distance and had a faster average speed than males. Therefore, data were transformed into Z-scores, and are reported as such below.

No significant interactions were observed between treatment and sex at P25 for total distance travelled ($F=5.224$, $p>0.05$) or for average speed ($F=5.073$, $p>0.05$).

No significant main effect of treatment was observed at P25 for total distance travelled ($F=2.208$, $p>0.05$) or for average speed ($F=2.251$, $p>0.05$).

A significant main effect of sex was observed on total distance travelled ($F=4.531$, $p<0.05$) and on average speed ($F=4.327$, $p<0.05$).

At P25, males administered 10 mg/kg DEHP travelled a significantly farther distance ($p < 0.05$) and had a significantly faster average speed ($p < 0.05$) than females administered 20 mg/kg.

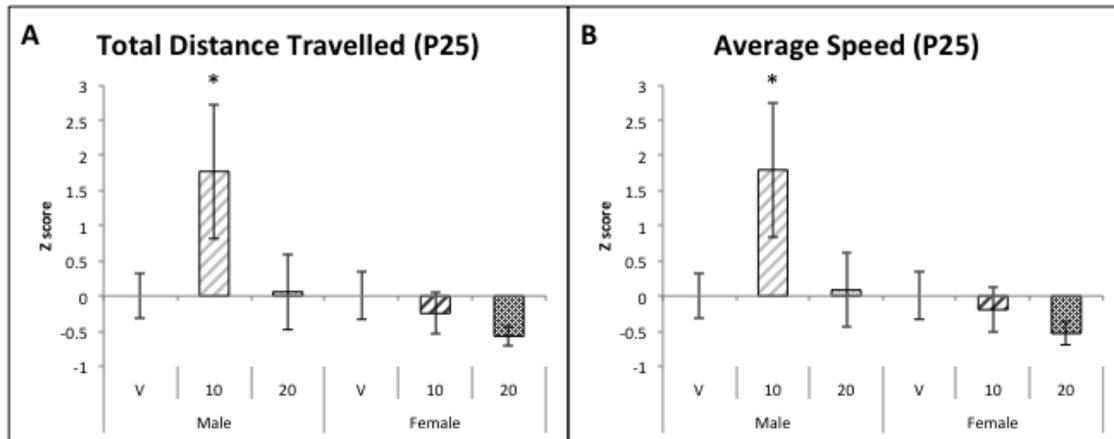


Figure 5. Locomotion at P25. A) Z-transformation of distance travelled over 20 minutes; B) Z-transformation of average speed. Data expressed at Z-score \pm SEM. *represents significant difference from F20

No significant interactions were observed between treatment and sex at P45 for either total distance travelled ($F=0.815$, $p > 0.05$) or for average speed ($F=0.757$, $p > 0.05$).

No significant main effect of treatment was seen at P45 for either total distance travelled ($F=0.772$, $p > 0.05$) or for average speed ($F=0.819$, $p > 0.05$). No significant main effect of sex was seen at P45 for either total distance travelled ($F=0.170$, $p > 0.05$) or for average speed ($F=0.116$, $p > 0.05$).

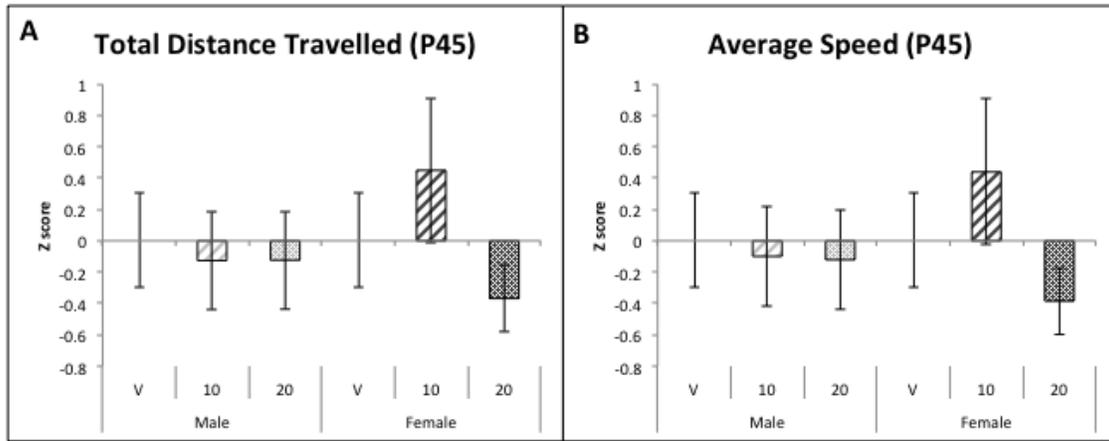


Figure 6. Locomotion at P45. A) Z-transformation of distance travelled over 20 minutes; B) Z-transformed average speed . Data expressed as Z-score +/- SEM.

Open Field Test

A two-way analysis of variance test was conducted to assess the effect of treatment (vehicle vs. 10 mg/kg DEHP vs. 20 mg/kg DEHP) and sex (male vs. female) on performance on the open field test.

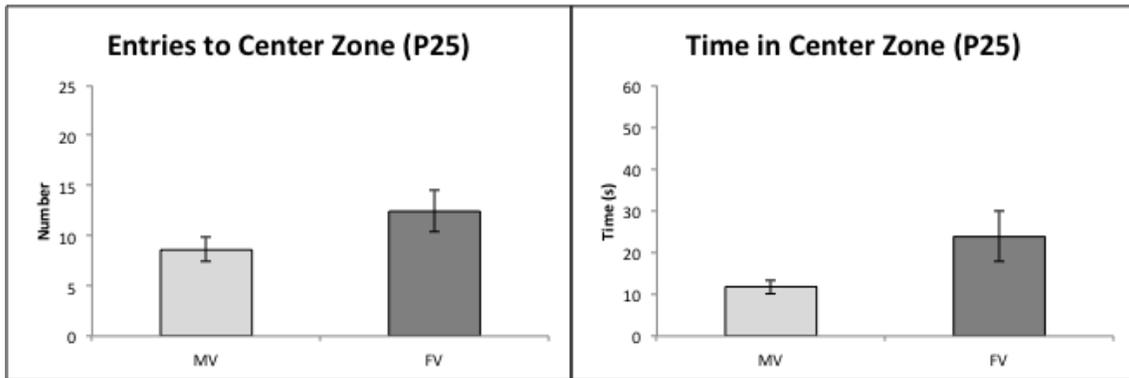


Figure 7. Anxiety-like behaviours on the open-field test in males and females at baseline at P25.

At P25, males and females administered vehicle differed in their behaviour on the open field test, such that females had a greater number of entries to the center zone and

spent more time in the center zone than males. Therefore, data were transformed into Z-scores, and are reported as such below.

No significant interactions were observed between treatment and sex at P25 for entries to the center zone ($F=1.025$, $p>0.05$), for time spent in the center zone ($F=3.092$, $p>0.05$), for latency to first enter the center zone ($F=0.361$, $p>0.05$), or for proportion of distance travelled in the center zone divided by total distance travelled ($F=0.890$, $p>0.05$).

A significant main effect of treatment was observed at P25 on time spent in the center zone ($F=3.616$, $p<0.05$). No significant main effect of treatment was observed for number of entries to the center zone ($F=1.231$, $p>0.05$), for latency to first enter the center zone ($F=0.039$, $p>0.05$), or for proportion of distance travelled in the center divided by total distance travelled ($F=1.532$, $p>0.05$). A significant main effect of sex was found on time spent in the center zone ($F=11.927$, $p=0.001$) and on number of entries to the center zone ($F=4.115$, $p<0.05$). No significant main effect of sex was found for latency to first enter the center zone ($F=0.263$, $p>0.05$) or for proportion of distance travelled in the center divided by total distance travelled ($F=2.849$, $p>0.05$).

At P25, males administered both 10 mg/kg and 20 mg/kg DEHP spent significantly more time in the center zone than males given vehicle ($p<0.01$, $p<0.05$), than females given vehicle ($p<0.05$), females given 10 mg/kg ($p<0.05$), and females given 20 mg/kg ($p<0.01$, $p<0.05$). Additionally, males given 10 mg/kg DEHP had significantly more entries to the center zone than males given vehicle ($p<0.05$), females given vehicle ($p<0.05$), and females given 20 mg/kg ($p<0.05$).

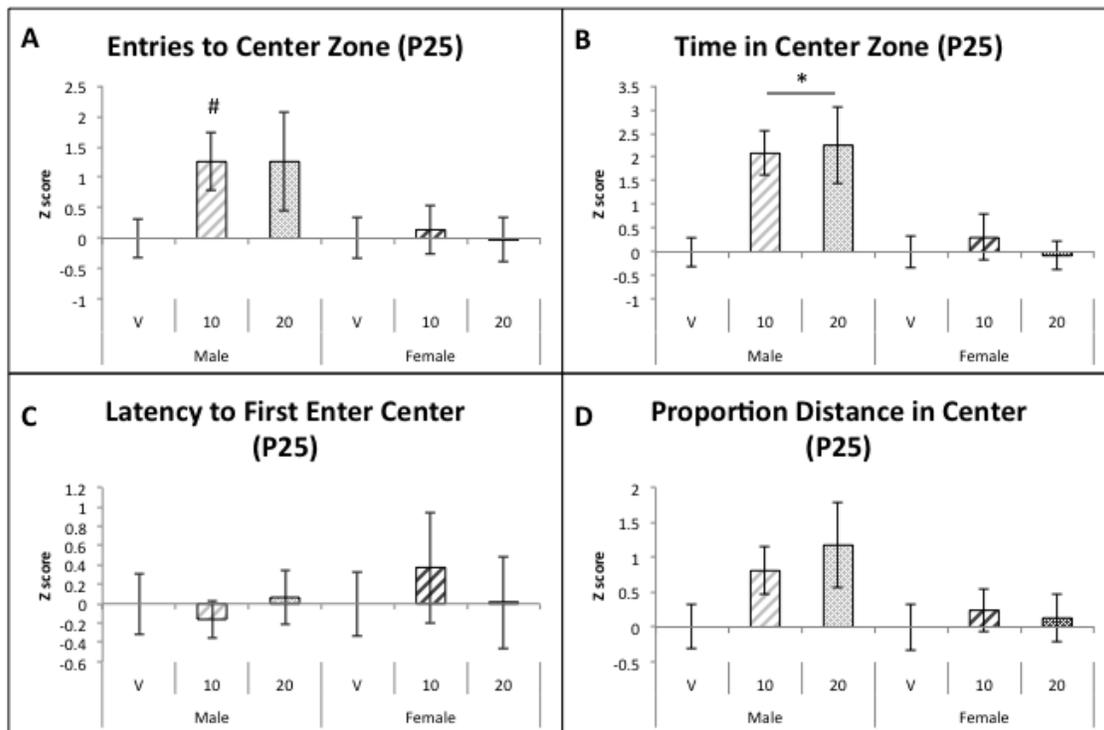


Figure 8. Open field test at P25. Z-transformed data representing A) number of entries to the center zone; B) time spent in the center zone; C) latency to first enter the center zone; and D) proportion of distance travelled in the center zone divided by total distance travelled. Data expressed at Z-score \pm SEM. * represents significant difference from all groups; # represents significant difference from MV, FV, and F20.

At P45, no significant interaction was observed between treatment and sex for entries to the center zone ($F=0.863$, $p>0.05$), for time spent in the center zone ($F=1.256$, $p>0.05$), for latency to first enter the center zone ($F=2.346$, $p>0.05$), or for proportion of distance travelled in the center zone divided by total distance travelled ($F=2.626$, $p>0.05$).

No significant main effect of treatment was found for entries to the center zone ($F=0.067$, $p>0.05$), for time spent in the center zone ($F=0.283$, $p>0.05$), for latency to first enter the center zone ($F=0.629$, $p>0.05$), or for proportion of distance travelled in the center zone divided by total distance travelled ($F=0.231$, $p>0.05$).

A significant main effect of sex was found on proportion of distance travelled in the center over total distance travelled ($F=4.188$, $p<0.05$). No significant main effect of treatment was found for entries to the center zone ($F=1.073$, $p>0.05$), for time spent in the center zone ($F=2.106$, $p>0.05$), for latency to first enter the center zone ($F=3.235$, $p>0.05$), or for proportion of distance travelled in the center zone divided by total distance travelled ($F=0.890$, $p>0.05$).

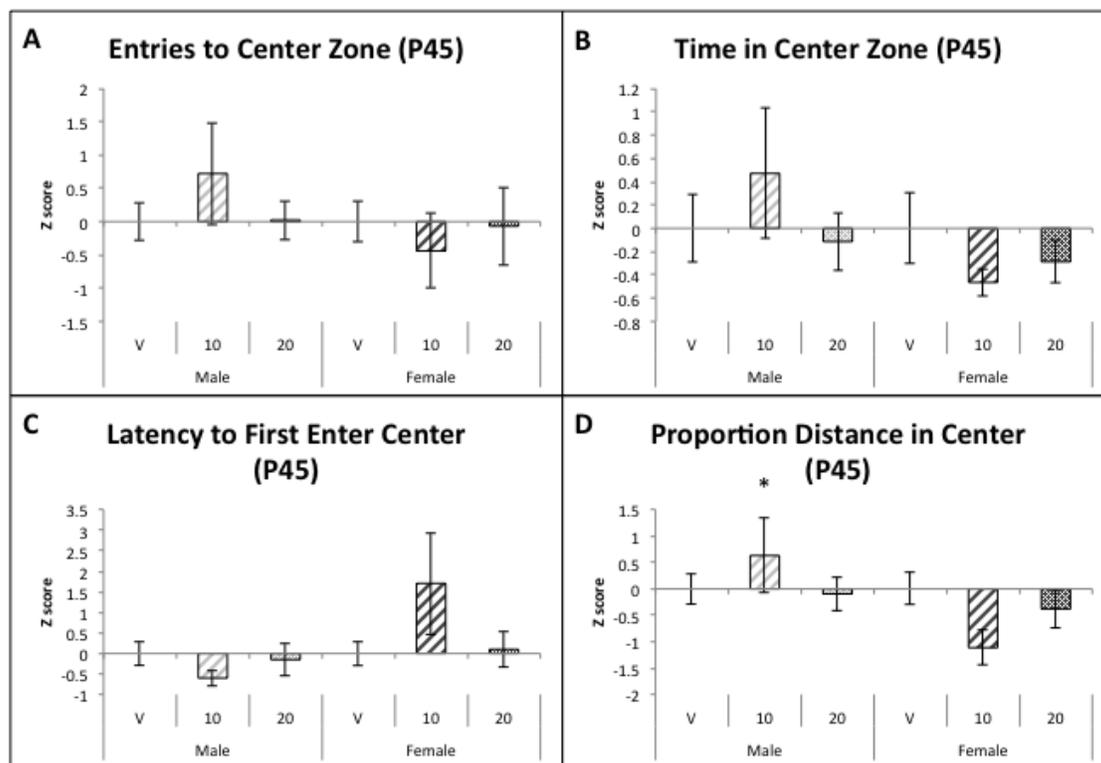


Figure 9. Open field test at P45. Z-transformed data representing A) number of entries to the center zone; B) time spent in the center zone; C) latency to first enter the center zone; D) proportion of distance travelled in the center zone divided by total distance travelled. Data expressed at Z-score +/- SEM. *represents significant difference from F10.

Elevated Plus Maze

A two-way analysis of variance test was conducted to assess the effect of treatment (vehicle vs. 10 mg/kg DEHP vs. 20 mg/kg DEHP) and sex (male vs. female), on performance on the elevated-plus maze.

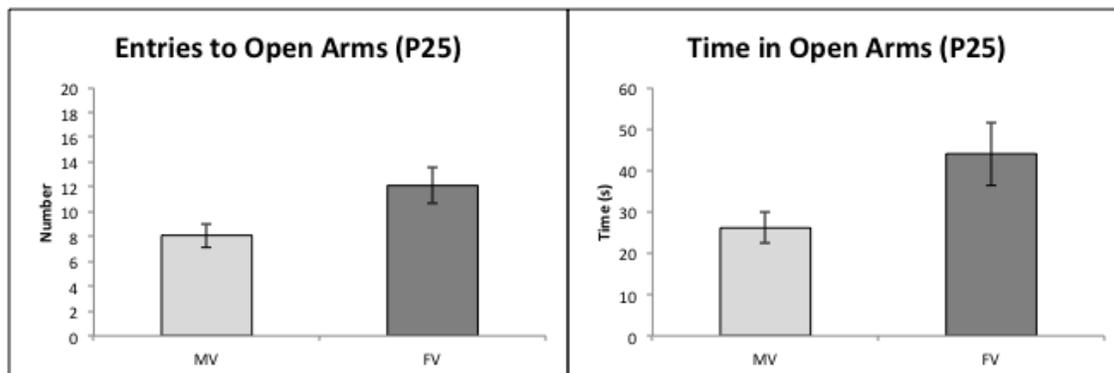


Figure 10. *Anxiety-like behaviours on the EPM at baseline at P25.*

At P25, males and females administered vehicle differed in their behaviour on the elevated plus maze, such that females had a greater number of entries to the open arms and spent more time in the open arms than males at baseline. Therefore, data were transformed into Z-scores, and are reported as such below.

A significant two-way interaction was observed between treatment and sex on number of entries to the open arms ($F=7.685$, $p<0.05$) and on time spent in the open arms ($F=4.179$, $p<0.05$).

At P25, males administered 10 mg/kg and males administered 20 mg/kg had significantly more entries to the open arms ($p<0.05$, $p<0.05$, $p<0.001$, $p<0.01$) and spent significantly more time in the open arms ($p<0.05$, $p<0.05$, $p<0.01$, $p<0.05$) compared to MV, FV, F10, and F20, respectively.

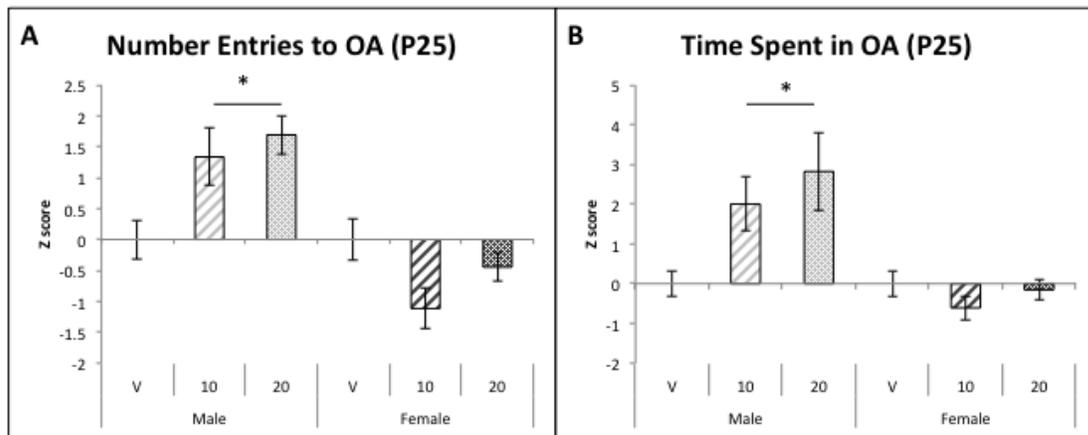


Figure 11. Elevated plus maze at P25. Z-transformed representation of A) number of entries to the open arms; B) time spent in the open arms. Data expressed at Z-score \pm SEM. *represents significant differences from all groups.

At P45, a significant interaction between treatment and sex was found for time spent in the open arms of the EPM ($F=4.182$, $p<0.05$). No significant interaction between treatment and sex was found for number of entries to the open arms ($F=0.195$, $p>0.05$). No significant main effect of treatment was found for number of entries to the open arms ($F=0.324$, $p>0.05$). No significant main effect of sex was found for number of entries to the open arms ($F=0.304$, $p>0.05$).

At P45, males administered 10 mg/kg spent significantly more time in the open arms ($p<0.05$, $p<0.05$) than males and females administered vehicle, respectively.

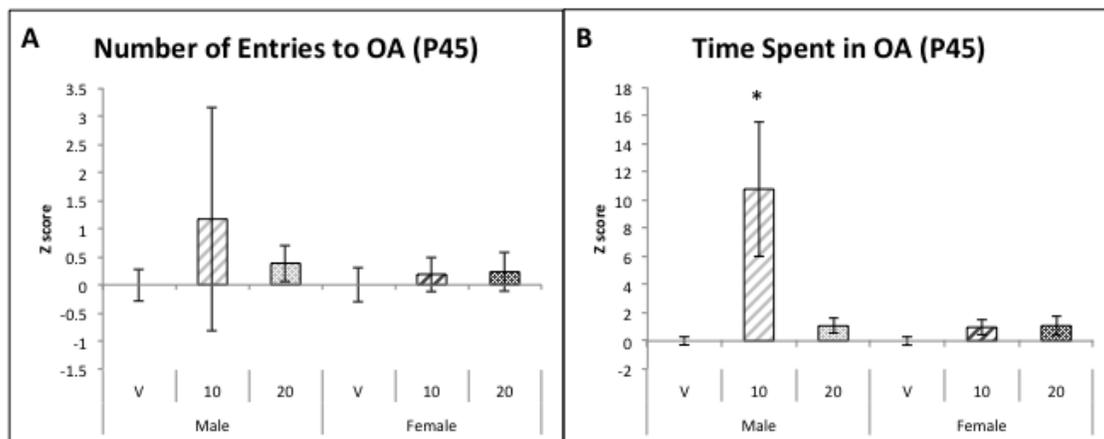


Figure 12. Elevated plus maze at P45. Z-transformation of data representing A) number of entries to the open arms; B) time spent in the open arms. Data expressed as Z-score +/- SEM. *represents significant difference from MV and FV.

Adapted One-Day Morris Water Maze

Latency and success

A two-way analysis of variance test was conducted to assess the effect of treatment (vehicle vs. 10 mg/kg DEHP vs. 20 mg/kg DEHP) and sex (male vs. female) on performance on the adapted one-day Morris Water Maze.

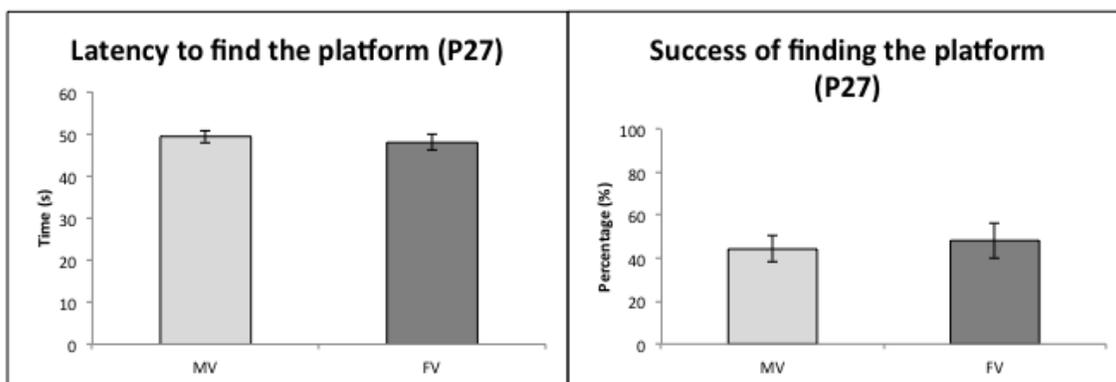


Figure 13. Learning ability in males and females at baseline at P25.

At P25, no differences were seen between males and females at baseline on latency to find the platform or on success of finding the platform ($p>0.05$). Therefore data for the MWM were expressed as original data.

At P27 no significant interaction was found between treatment and sex on either latency to find the platform ($F=1.090$, $p>0.05$) or on success of finding the platform ($F=1.248$, $p>0.05$). A significant main effect of treatment was found on latency to find the platform ($F=5.602$, $p<0.05$) or on success of finding the platform ($F=4.089$, $p<0.05$). No significant main effect of sex was found on latency to find the platform ($F=0.153$, $p>0.05$), or for success of finding the platform ($F=0.603$, $p>0.05$).

At P27, males administered 20 mg/kg of DEHP were significantly faster at finding the platform ($p<0.01$, $p<0.01$) and had significantly greater success of finding the platform ($p<0.01$, $p<0.01$) than males administered vehicle or 10 mg/kg DEHP, respectively. No significant differences were found between females at P27 ($p>0.05$).

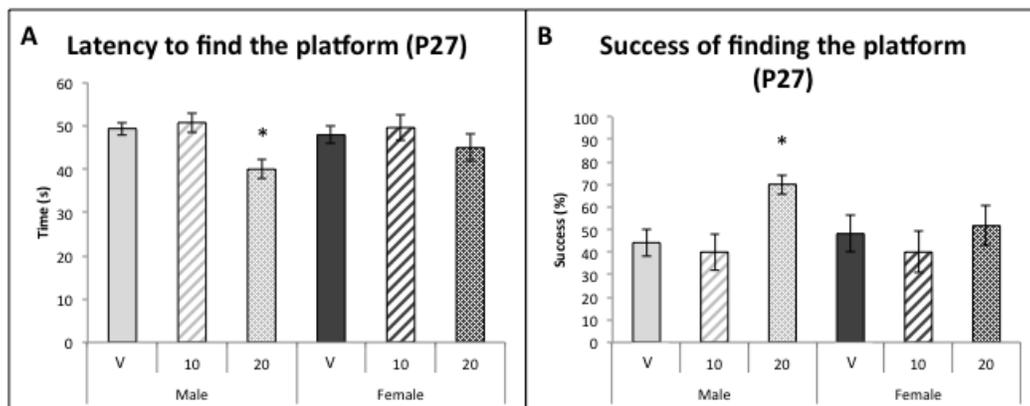


Figure 14. Adapted one-day Morris Water Maze at P27. A) Latency to find the platform over 12 learning trials; B) Success of finding the platform over 12 learning trials. Data expressed as mean \pm SEM. *represents significant difference from all groups except F20.

No significant interaction was found between treatment and sex at P47 for latency to find the platform ($F=0.001$, $p>0.05$) or for success of finding the platform ($F=0.029$, $p>0.05$). No significant main effect of treatment was seen for latency to find the platform ($F=0.763$, $p>0.05$) or for success of finding the platform ($F=0.640$, $p>0.05$). No significant main effect of sex was seen for latency to find the platform ($F=0.190$, $p>0.05$) or for success of finding the platform ($F=0.332$, $p>0.05$).

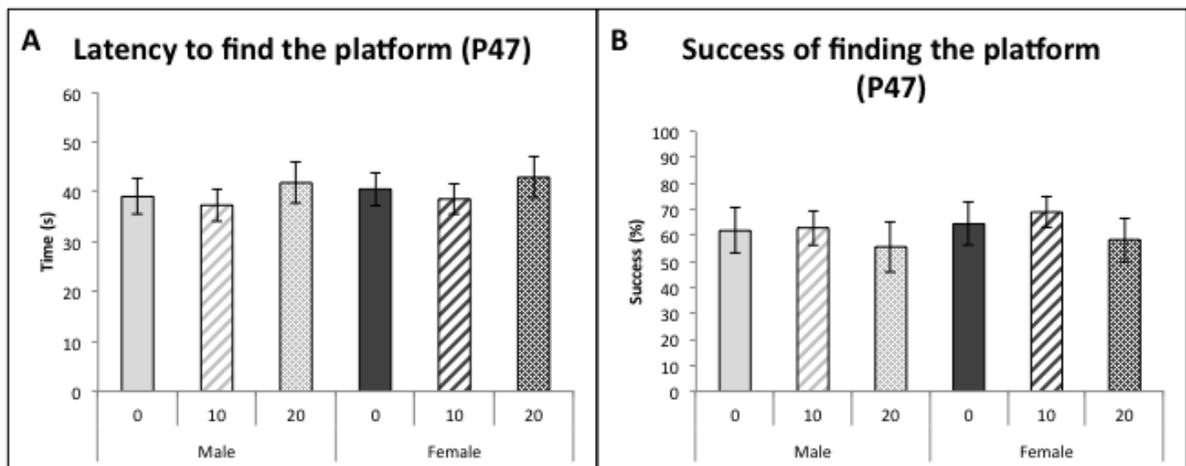


Figure 15. Adapted one-day Morris Water Maze at P47. A) Latency to find the platform over 12 learning trials; B) Success of finding the platform over 12 learning trials. Data expressed as mean \pm SEM.

Probe Trial

A two-way analysis of variance test was conducted to assess the effect of treatment (vehicle vs. 10 mg/kg DEHP vs. 20 mg/kg DEHP) and sex (male vs. female) on performance on the probe trial of the adapted one-day Morris Water Maze.

No significant interaction was found between treatment and sex at P27 on time spent in the target zone ($F=0.062$, $p>0.05$), time spent in the off target zone ($F=0.194$, $p>0.05$), number of entries to the target zone ($F=0.216$, $p>0.05$), or number of entries to the off target zone ($F=0.054$, $p>0.05$).

No significant main effect of dose was found at P27 on time spent in the target zone ($F=2.144$, $p>0.05$), time spent in the off target zone ($F=0.447$, $p>0.05$), number of entries to the target zone ($F=1.785$, $p>0.05$), or number of entries to the off target zone ($F=1.281$, $p>0.05$).

No significant main effect of sex was found at P27 on time spent in the target zone ($F=0.991$, $p>0.05$), time spent in the off target zone ($F=0.350$, $p>0.05$), number of entries to the target zone ($F=0.577$, $p>0.05$), or number of entries to the off target zone ($F=0.414$, $p>0.05$).

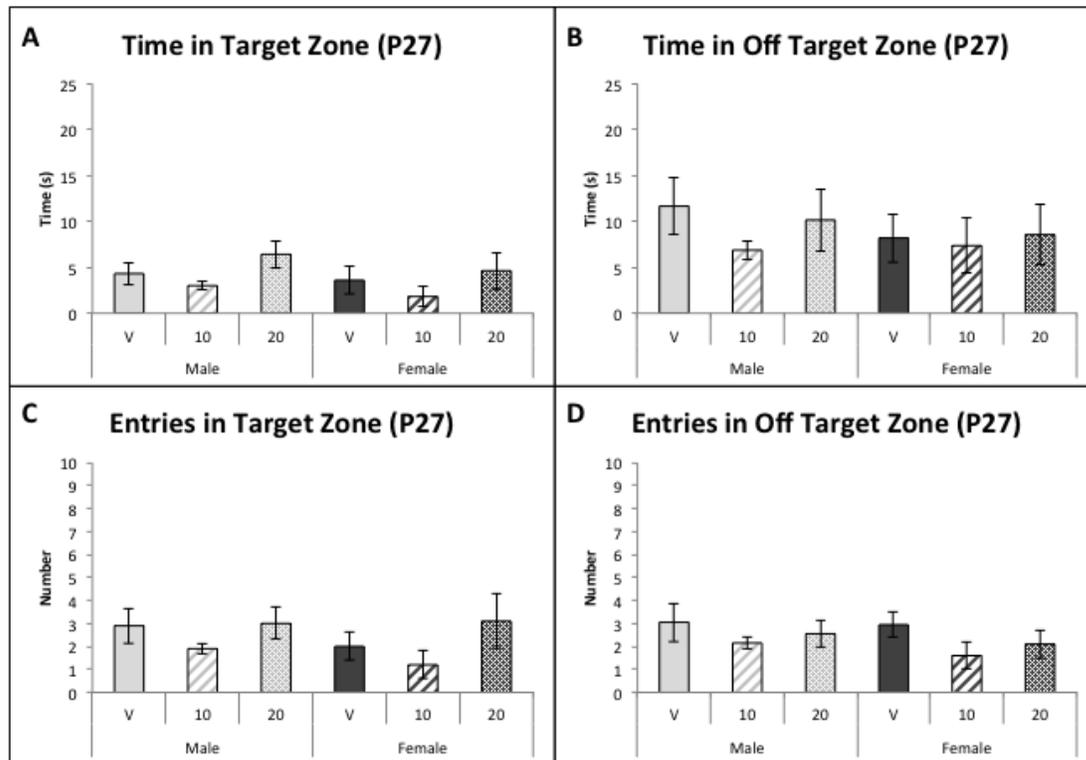


Figure 16. Memory during probe trial of the MWM at P27. A) Time spent in the target zone; B) Average time spent between the two off target zones; C) Number of entries in the target zone; D) Average number of entries between the two off target zones. Data expressed at mean \pm SEM.

At P47, no significant interaction was found between treatment and sex on time spent in the target zone ($F=0.354$, $p>0.05$), time spent in the off target zone ($F=0.072$,

$p > 0.05$), number of entries to the target zone ($F = 1.367$, $p > 0.05$), or number of entries to the off target zone ($F = 2.530$, $p > 0.05$).

No significant main effect of dose was found at P47 on time spent in the target zone ($F = 0.175$, $p > 0.05$), time spent in the off target zone ($F = 0.229$, $p > 0.05$), number of entries to the target zone ($F = 2.759$, $p > 0.05$), or number of entries to the off target zone ($F = 1.281$, $p > 0.05$).

No significant main effect of sex was found at P27 on time spent in the target zone ($F = 0.3245$, $p > 0.05$), time spent in the off target zone ($F = 0.1687$, $p > 0.05$), number of entries to the target zone ($F = 3.245$, $p > 0.05$). A significant main effect of sex was seen for number of entries to the off target zone ($F = 12.205$, $p < 0.05$).

At P47, males administered 10 mg/kg DEHP had significantly more entries to the off target zone than females administered any dose ($p < 0.05$).

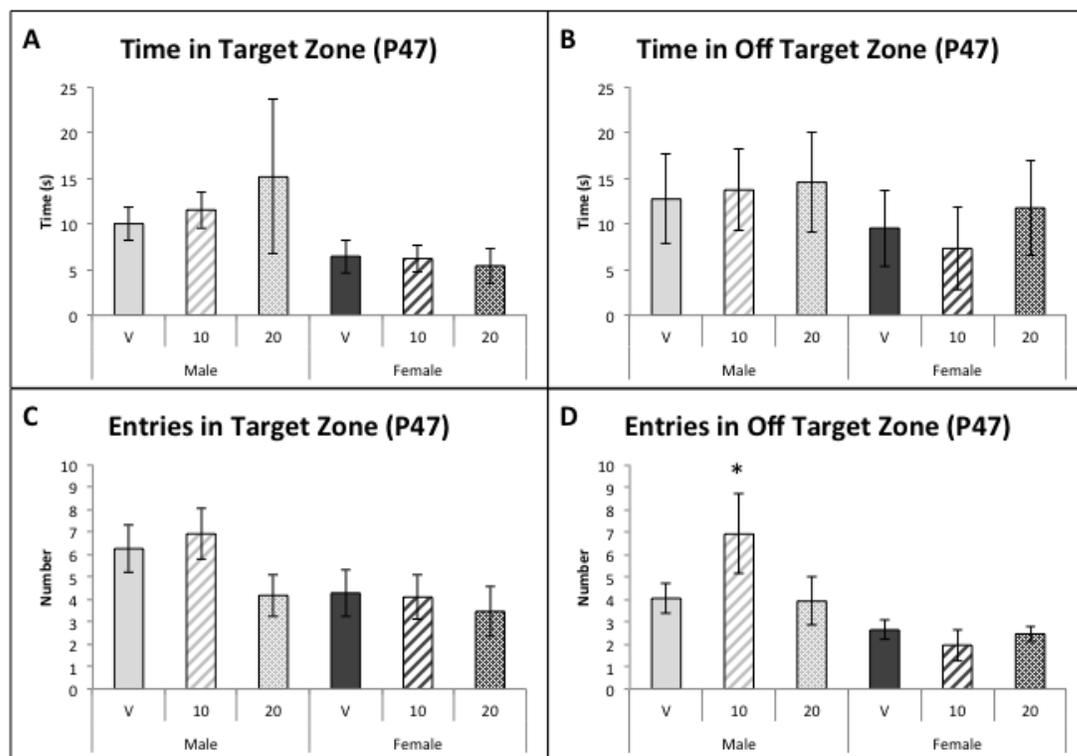


Figure 17. Memory during probe trial of the MWM at P47. A) Time spent in the target zone; B) Average time spent between the two off target zones; C) Number of entries in the target zone; D) Average number of entries between the two off target zones. Data expressed at mean \pm SEM.

Learning and success across trials

A repeated measures analysis was conducted to assess the effect of treatment (vehicle vs. 10 mg/kg DEHP vs. 20 mg/kg DEHP), sex (male vs. female) and trial block on latency to find the platform at P27.

No significant three-way interaction was found between trial block, treatment, and sex ($p > 0.05$).

No significant interaction was found between trial block and sex, or between trial block and treatment ($p > 0.05$).

At P27, males given 20 mg/kg DEHP were significantly faster at finding the platform during trials 4-6 ($p < 0.01$) and during trials 10-12 ($p < 0.01$) compared to males given vehicle and were significantly faster at finding the platform during trials 4-6 ($p < 0.01$), trials 7-9 ($p < 0.05$), and trials 10-12 ($p = 0.01$) compared with males given 10 mg/kg DEHP.

A repeated measures analysis was also conducted to assess the effect of treatment (vehicle vs. 10 mg/kg DEHP vs. 20 mg/kg DEHP), sex (male vs. female) and trial block on success of finding the platform across trials. No significant three-way interaction between trial group, treatment, and sex ($p > 0.05$).

No significant interaction was found between trial block and treatment ($p > 0.05$).

A significant interaction was found between trial block and sex ($F = 2.960$, $p < 0.05$).

At P27 males given 20 mg/kg had significantly more success of finding the hidden platform during trials 4-6 ($p < 0.01$, $p < 0.01$) and during trials 10-12 ($p < 0.01$,

p<0.05) compared to males given vehicle and males given 10 mg/kg, respectively. No significant differences were found between females at P27.

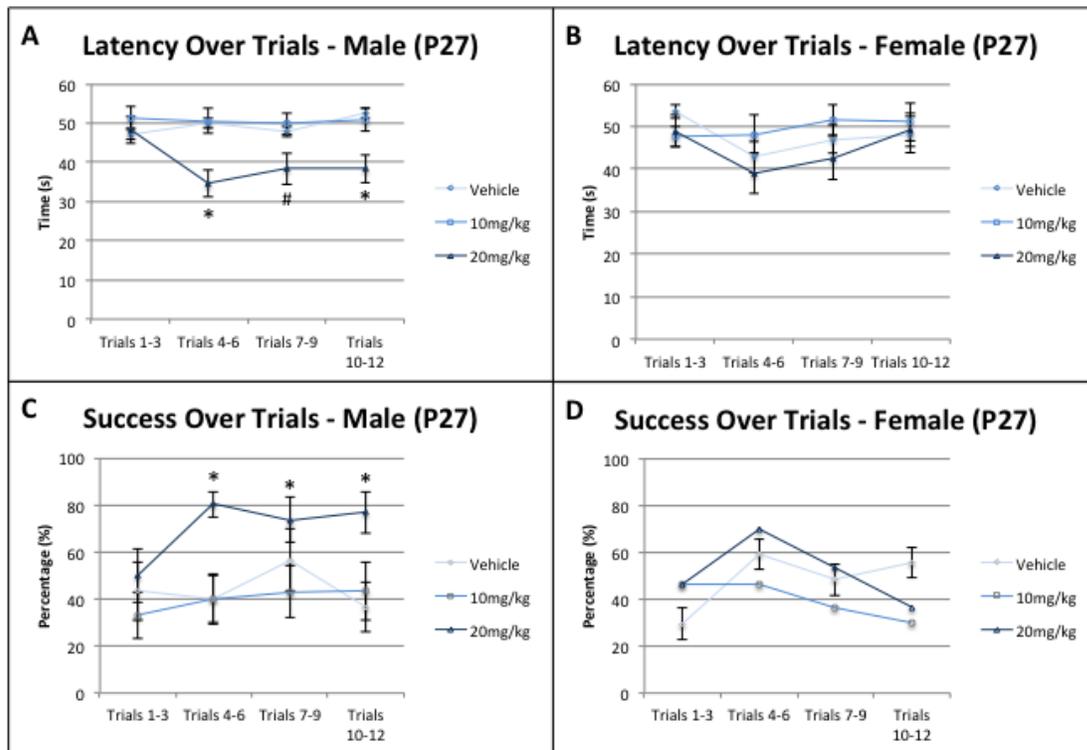


Figure 18. Learning over trials on the adapted one-day MWM at P27. A) Latency to find the platform across trials in males; B) Latency to find the platform across trials in females; C) Success of finding the platform across trials in males; D) Success of finding the platform across trials in females. Data expressed as mean +/- SEM. *represents significant difference from all groups; #represents significant difference from M10 only.

A repeated measures analysis was conducted to assess the effect of treatment (vehicle vs. 10 mg/kg DEHP vs. 20 mg/kg DEHP), sex (male vs. female) and trial block on latency to find the platform at P47.

No significant three-way interaction was found between trial block, treatment, and sex (p>0.05). No significant interaction was found between trial block and sex, or between trial block and treatment (p>0.05).

No significant main effects of trial block, treatment, or sex were found in either males or females at P47 ($p>0.05$).

A repeated measures analysis was also conducted to assess the effect of treatment (vehicle vs. 10 mg/kg DEHP vs. 20 mg/kg DEHP), sex (male vs. female) and trial block on success of finding the platform across trials. No significant three-way interaction between trial block, treatment, and sex ($p>0.05$).

No significant interaction was found between trial block and sex, or between trial block and treatment ($p>0.05$).

No significant main effects of trial block, treatment, or sex were found in either males or females at P47 ($p>0.05$).

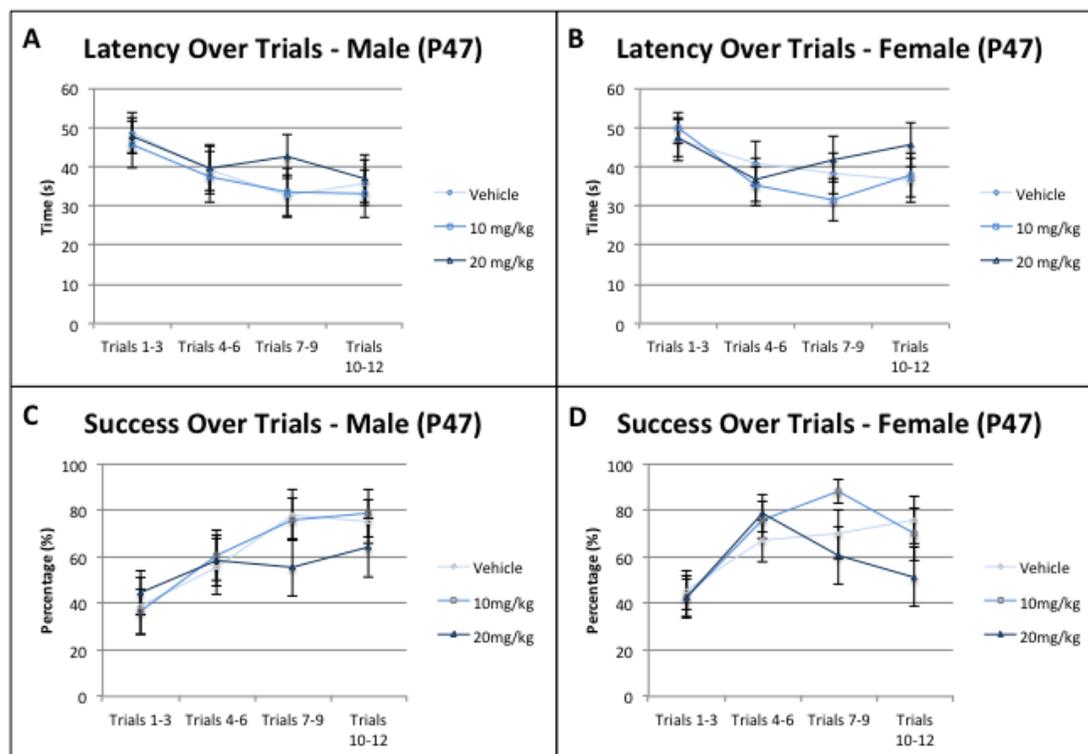


Figure 19. Learning across trials on the adapted one-day MWM at P47. A) Latency to find the platform across trials in males; B) Latency to find the platform across trials in females; C) Success of finding the platform across trials in males; D) Success of finding the platform across trials in females. Data expressed as mean +/- SEM.

Immunohistological results

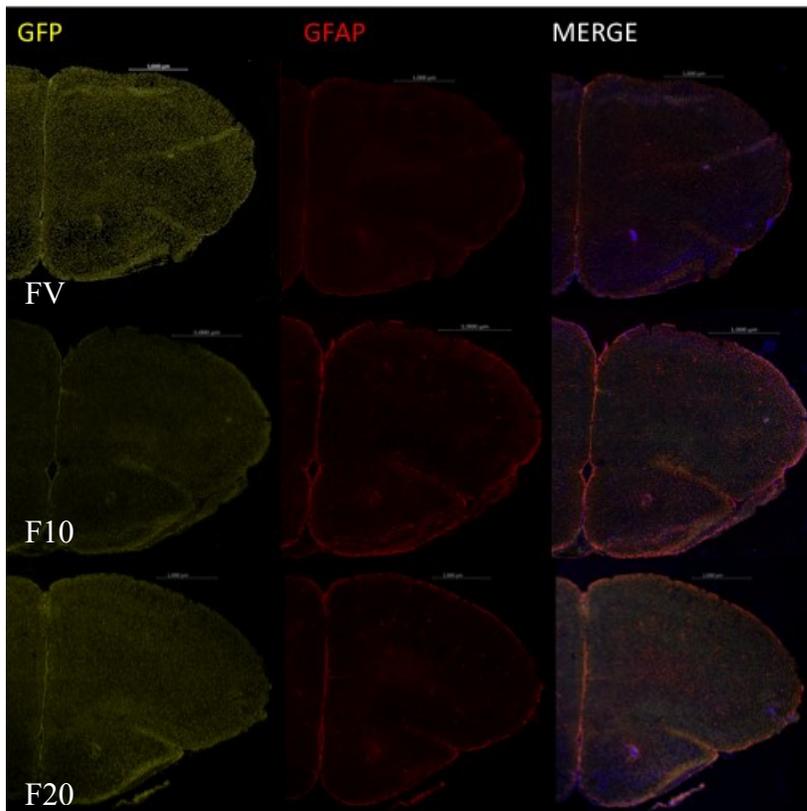


Figure 20. *Representative photo of PFC sections in females at P49.* Individual photos of GFP and GFAP staining followed by a merged image of all stains in female vehicle, 10mg/kg, and 20 mg/kg DEHP treatment groups, respectively.

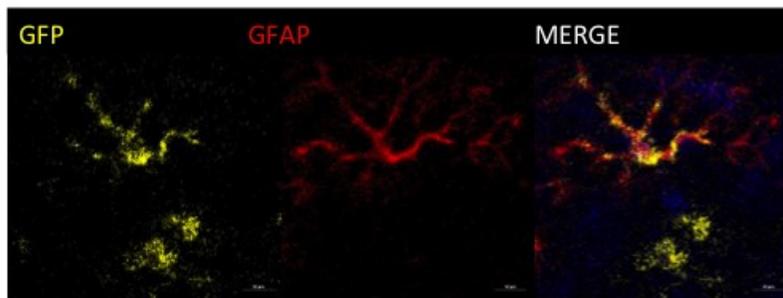


Figure 21. *Representative photo of a single astrocyte.* Individual photos of GFP and GFAP staining followed by a merged image of all stains.

A two-way analysis of variance was conducted to assess the effects of treatment (vehicle vs. 10 mg/kg DEHP vs. 20 mg/kg DEHP) and sex (male vs. female) on average GFP, average GFAP, proportion of GFAP to GFP, density of GFP, and density of GFAP at P29. No significant interactions were found on any of these measures at this time point ($F=0.561$, $p>0.05$; $F=0.344$, $p>0.05$; $F=0.991$, $p>0.05$; $F=1.663$, $p>0.05$; $F=1.819$, $p>0.05$).

No significant main effect of treatment was observed at P29 for average GFP ($F=0.339$, $p>0.05$), average GFAP ($F=0.375$, $p>0.05$), proportion of GFAP to GFP ($F=0.539$, $p>0.05$), density of GFP ($F=1.622$, $p>0.05$), or for density of GFAP ($F=1.494$, $p>0.05$).

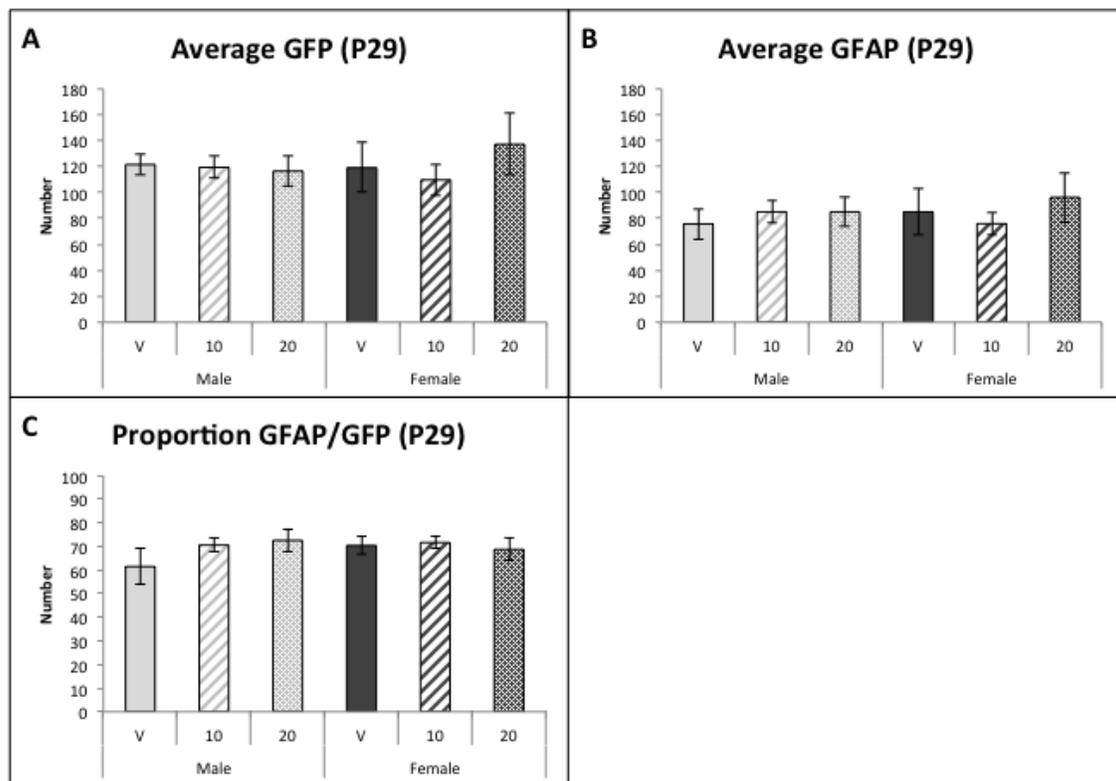


Figure 22. Astrocyte analysis in the PFC at P29. A) Average GFP counts in the PFC; B) Average GFAP counts in the PFC; C) Proportion of GFAP counts divided by GFP counts. Data expressed as mean \pm SEM.

No significant main effect of sex was observed at P29 for average GFP ($F=0.051$, $p>0.05$), average GFAP ($F=0.110$, $p>0.05$), proportion of GFAP to GFP (0.109 , $p>0.05$), density of GFP ($F=1.813$, $p>0.05$), or for density of GFAP ($F=1.865$, $p>0.05$).

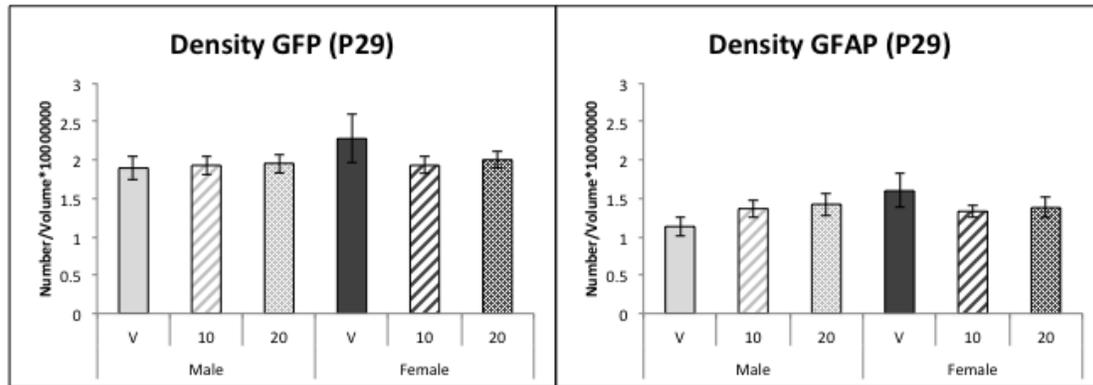


Figure 23. Astrocyte density in the PFC at P29. A) Density of GFP in the PFC; Density of GFAP in the PFC. Data expressed as mean \pm SEM.

A two-way analysis of variance was conducted to assess the effects of treatment (vehicle vs. 10 mg/kg DEHP vs. 20 mg/kg DEHP) and sex (male vs. female) on average GFP, average GFAP, proportion of GFAP to GFP, density of GFP, and density of GFAP at P49. No significant interactions were found on any of these measures at this time point ($F=0.474$, $p>0.05$; $F=0.061$, $p>0.05$; $F=0.787$, $p>0.05$; $F=2.015$, $p>0.05$; $F=0.046$, $p>0.05$).

No significant main effect of treatment was observed at P49 for average GFP ($F=0.224$, $p>0.05$), average GFAP ($F=0.195$, $p>0.05$), proportion of GFAP to GFP (0.784 , $p>0.05$), density of GFP ($F=0.099$, $p>0.05$), or for density of GFAP ($F=0.502$, $p>0.05$).

No significant main effect of sex was observed at P49 for average GFP ($F=3.501$, $p>0.05$), average GFAP ($F=0.165$, $p>0.05$), proportion of GFAP to GFP (1.766 , $p>0.05$), density of GFP ($F=0.096$, $p>0.05$), or for density of GFAP ($F=0.519$, $p>0.05$).

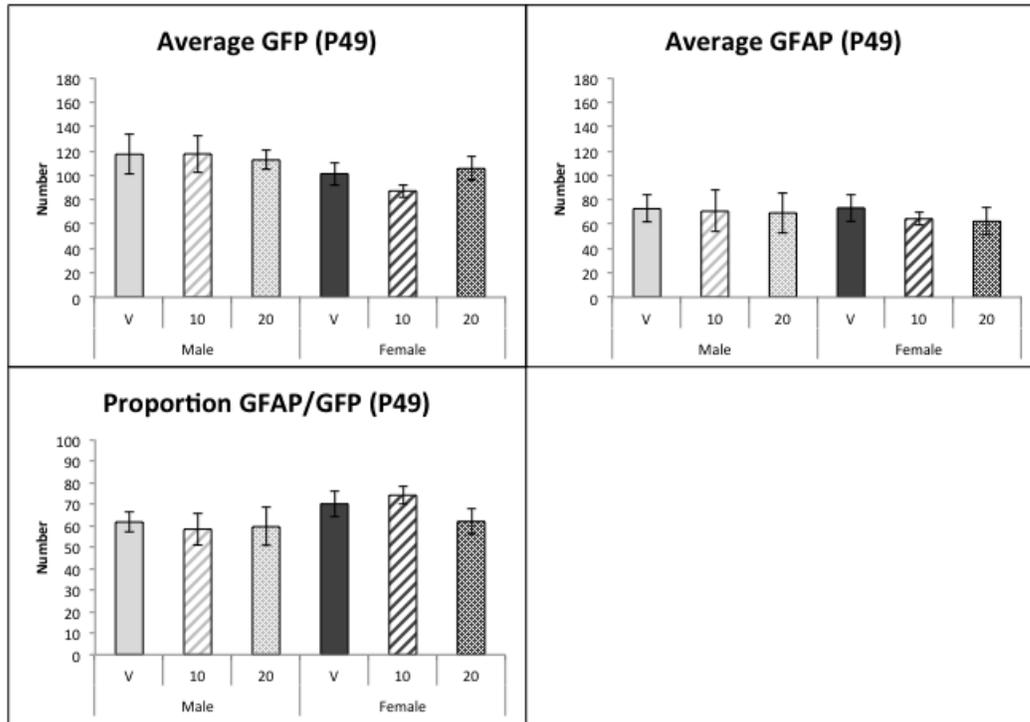


Figure 24. Astrocyte analysis in the PFC at P49. A) Average GFP counts in the PFC; B) Average GFAP counts in the PFC; C) Proportion of GFAP counts divided by GFP counts. Data expressed as mean \pm SEM.

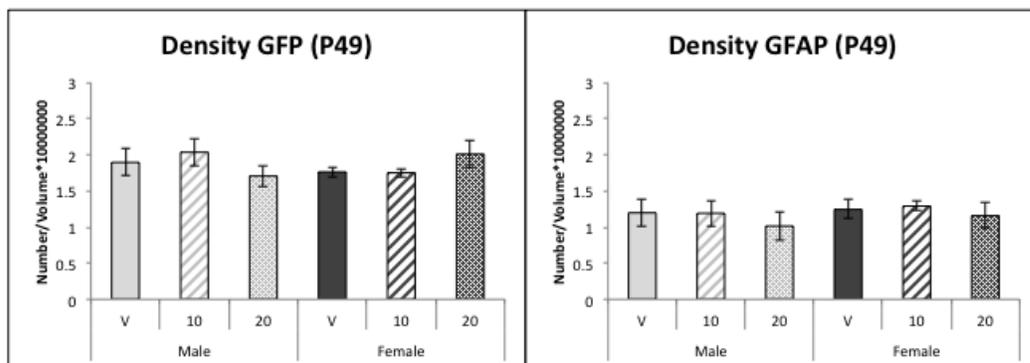


Figure 25. Astrocyte density in the PFC at P49. A) Density of GFP in the PFC; Density of GFAP in the PFC. Data expressed as mean \pm SEM.

DISCUSSION

Behaviour

The present thesis examined whether early-life exposure to DEHP would affect PFC-mediated behaviours including locomotion, anxiety, and learning and memory and whether DEHP administration would result in changes in astrocyte number and/or activation in the PFC.

The PFC is involved in cognitive functions, such as attention, planning, and behavioural inhibition – all aspects of executive function (Teffer and Semendeferi, 2012). The PFC has also been shown to play an important role in mediating anxiety and stress responses (Schubert et al., 2015). Previous research has suggested that PFC dysfunction is involved in the etiology of ADHD, since it is a neurodevelopmental disorder characterized by inattention, hyperactivity, and/or impulsivity (Castellanos et al., 1996; Mostofsky et al., 2002; Association, 2013). A correlation has also been found between early-life DEHP exposure and increased risk of being diagnosed with ADHD. Therefore, it was hypothesized that exposure to DEHP once daily from P18-P23 would alter PFC-mediated behaviours, resulting in increased locomotion and anxiety-like behaviours, and decreased learning and memory. It was also hypothesized that changes in these PFC-mediated behaviours would impact males more than females, as research suggests that DEHP exposure detrimentally affects males more than females (Smith et al., 2011) and the prevalence of ADHD is higher in males than in females (Rucklidge, 2010).

The data from the present thesis show that at P25, males administered 10mg/kg DEHP showed significantly more entries to the center zone of the open field test than males administered vehicle. Males administered either 10 mg/kg or 20 mg/kg DEHP spent significantly more time in the center zone of the open field test than males

administered vehicle. Since the open field test is a measure of innate anxiety in rodents (Salmaso et al., 2016), these results suggest an anxiolytic effect of early-life exposure of DEHP in males. This pattern of behaviour was not seen mice were tested on the open field at P45, suggesting that the potential anxiolytic effects of juvenile DEHP administration did not persist beyond the transition to adulthood. Importantly, there were no differences observed within the female group at either time point.

A similar pattern of results was observed in the EPM, another measure of innate anxiety in rodents (Osborne et al., 2009). Males administered either 10 or 20 mg/kg DEHP showed significantly more entries into the open arms and spent more time in the open arms than males administered vehicle, again suggesting an anxiolytic effect of DEHP administration. At P45, there were no differences detected in the number of entries into the open arms, but males administered 10 mg/kg DEHP spent significantly more time in the open arms than males administered either vehicle or 20 mg/kg DEHP. Once again, no significant changes were observed within the female group at either P25 or P45.

While the open field test and the elevated plus maze are both considered measures of innate rodent anxiety (Osborne et al., 2009; Salmaso et al., 2016), there are some alternate interpretations as to whether they measure the exact same underlying constructs. It has been suggested that the open field test might be more appropriately used to measure exploratory behaviours in novel environments, whereas the elevated plus maze might more accurately assess innate anxiety (Ennaceur et al., 2016). It is also possible the EPM is more sensitive to changes in anxiety-like behaviour than the open field test. The results of these two behavioural measures in the present thesis were similar at the early time point, as males administered both doses of DEHP showed increased time spent in the center zone of the open field test and in the open arms of the elevated plus maze,

suggesting a decrease in anxiety-like behaviours. The results of elevated plus maze suggests that this anxiolytic effect of DEHP administration persists to P45 in males administered 10 mg/kg, whereas the open field test results suggest this anxiolytic effect is resolved at P45. As these results are inconsistent at the late time point, more research is needed to fully elucidate the underlying psychological constructs measured by each of these tests.

The adapted one-day Morris Water Maze was used to assess learning and memory in response to early-life DEHP administration. Males administered 20 mg/kg DEHP were significantly faster at finding the platform and had increased success of finding the platform at P27 than males administered either vehicle or 10 mg/kg DEHP, suggesting an enhancement learning ability. These enhancements were no longer apparent at P47, suggesting that these changes dissipate with time. No differences in learning abilities were noted in females at either time point.

The probe trial was used as a measure of memory, to assess whether the mice remembered the location of the platform. The only significant difference noted was that males administered 10 mg/kg DEHP had significantly more entries to the off target zone than females administered any dose of DEHP. No significant differences were found in males or females for number of entries to the target zone at either the early or late time point, suggesting that DEHP administration does not affect memory capabilities.

The results seen during the acquisition trials of the MWM could potentially be explained due to the anxiolytic effects of DEHP administration. As males administered 20 mg/kg DEHP displayed a decreased anxiety-like phenotype and also were the only treatment group to show changes on the MWM, it is possible these results are both arising due to a common mechanism of decreasing anxiety in response to DEHP

administration. This mechanism may be dose-dependent, as males administered 10 mg/kg DEHP also showed a decreased anxiety-like phenotype but showed no changes on the MWM. Females did not show any reductions in anxiety on the open field test or on the EPM and similarly did not show changes on the MWM. Correlations with serum corticosterone levels to assess anxiety could be an avenue for future studies.

An important result from this study is that females with early-life DEHP exposure did not show changes on any of the behavioural measures assessed, whereas at the early time-point males administered either 10 mg/kg or 20 mg/kg DEHP showed changes in the open field test and EPM, and males administered 20 mg/kg DEHP showed changes on the acquisition trials of the MWM. Previous research has suggested differences in vulnerability to DEHP in males and females (Andrade et al., 2006) so it was hypothesized that males would be more sensitive to the effects of DEHP administration. However, the mechanism responsible for these sex-specific effects has yet to be elucidated. One clue into the mechanism is based on the anti-androgenic effects of DEHP (Akingbemi et al., 2001; Andrade et al., 2006). It has been shown that DEHP suppresses aromatase activity, thus suppressing estrogen metabolism through decreasing the conversion of androgens to estrogens (Davis et al., 1994). Estrogenic activity is critical for masculinization of the brain in rodents (Gorski, 1986) and alterations to these neurodevelopmental processes might manifest as changes in sex-specific behaviour. Estrogen activity is also implicated in anxiety-like behaviours in both rodents and humans (for review, please see Borrow & Handa, 2018). While more research is required to fully explain this relationship, it provides a possible explanation for the changes in anxiety-like behaviours seen in the present thesis. If DEHP acts to suppress aromatase activity, thus decreasing estrogen

levels in the male brain, this might account for the anxiolytic effect of juvenile DEHP exposure seen in males but not females on the open field test and elevated plus maze.

A possible explanation for the reversal of behavioural effects seen in males at the later time point is puberty. Puberty in rodents can be defined as ongoing from P30-P50 (Spear, 2000), and with the onset of puberty comes a significant increase in testosterone levels in male mice (Gillies, 2010). As the mice tested at P45 are well into the process of puberty, one possibility is that this increase in testosterone is the mechanism behind this behavioural reversal. Testosterone has been suggested to exert a protective effect against the actions of DEHP in previous studies (Carbone et al., 2013), however this has been in the context of reversing an anxiogenic, not anxiolytic, effect of DEHP. Future studies should examine whether exposure of DEHP from P18-23 results in abnormal testosterone levels, and whether testosterone administration can reverse the behavioural effects observed in the present thesis.

It may also be that the timing of DEHP exposure is critical for observing sex-specific effects. Andrade et al. (2006) showed that DEHP exposure more greatly affects hypothalamic/preoptic area (HPOA) aromatase activity in males on P1 and in females on P22. While these dates of exposure and results do not correlate perfectly with the dates of exposure in the present thesis, these results certainly suggest that the timing of DEHP exposure may be critical in elucidating certain sex-specific outcomes.

Immunohistochemistry

The present thesis also examined whether early-life DEHP exposure affects astrocyte number and astrocyte activation in the PFC through the use of immunohistochemistry. The transgenic mouse line used, AldH11-L10:GFP, is a mouse

line that constitutively expresses GFP in all astroglial cells. GFP was thus used as a marker of astrocyte number. Astrocytic activation was measured through GFAP, a protein associated with glial plasticity (Torres-Platas et al., 2011). The results of the present thesis show that there were no differences in GFP or GFAP counts in either males or females at either time point in response to early-life DEHP exposure, suggesting no changes in astrocyte number or activation. As GFAP is an intermediate filament protein that is typically associated with a reactive state or with recent morphological changes, these data also suggest that there are no changes in astroglial morphology in response to DEHP administration. However, astroglial morphology was not explicitly examined in this study, and could be an avenue for future studies.

One possible explanation for this lack of astroglial changes is that development of the PFC is not affected by DEHP exposure during the time points examined in this study. The behavioural changes observed in this study following DEHP administration suggested that the PFC might be affected, since increased locomotion and decreased anxiety-like behaviours were observed in males administered either 10 mg/kg or 20 mg/kg DEHP. The PFC has been shown to mediate these behaviours (Fuster, 2001; Teffer and Semendeferi, 2012) and has also been implicated in ADHD behaviours in humans (Mostofsky et al., 2002; Dirlikov et al., 2015). Additionally, males administered 20 mg/kg DEHP showed changes on the one-day adapted MWM, a test of learning and memory. The PFC has also been shown to function in working memory (Caballero et al., 2016), again providing evidence that changes in this brain region might explain the changes in behaviour observed in this study. While the PFC does play an important role in mediating these behaviours, it is certainly not the only brain region to do so. The hippocampus also contributes to both learning and memory (Jarrard, 1993) and

anxiety/stress responses (McEwen, 1999, 2016). The MWM has often been used as a measure of learning and memory to assess hippocampal function (Nunez, 2008). Research has shown that development of the hippocampus is altered in response to DEHP exposure during the time examined in the present thesis (Smith et al., 2011). While the behavioural changes seen in the present thesis suggested possible PFC alterations, perhaps the hippocampus is more so affected by DEHP exposure. Future studies will be needed to examine changes in astrocyte number, activation, and protein expression in the hippocampus, as this might help explain the changes in hippocampus-dependent behaviours that were seen in this study. Immunohistochemical analysis would be useful in parsing out potential differences in the response of different hippocampal regions to DEHP administration. Importantly, because the behaviours measured in the present thesis included both anxiety-like behaviours and cognitive behaviours, immunohistochemistry could be used to quantify changes in the dorsal vs. ventral areas of the hippocampus. This might also provide explanation for the results seen in the present study, as the dorsal hippocampus is responsible for cognitive behaviours, such as those seen in the MWM, whereas the ventral hippocampus is more involved in emotive behaviours, such as those seen in anxiety phenotypes (Bannerman et al., 2003). The importance of studying astrocyte number and activation in specific regions of the hippocampus is further highlighted by research showing region specific changes in the hippocampus following DEHP administration from P16-P22 such that decreased spine density was observed only in the CA3 region of the dorsal hippocampus (Smith et al., 2011).

Since astrocytes are the key cells involved in modulating neurodevelopment, it was hypothesized in the present thesis that deficits in astrocytic number and/or function would result from early-life DEHP exposure. While previous research has demonstrated

neurodevelopmental changes in response to DEHP exposure (Ishido et al., 2005; Smith et al., 2011), limited research exists on the direct effects of DEHP exposure on astrocytes. It was hypothesized that perturbations in neurodevelopment as a consequence of early-life DEHP exposure might arise as a direct result of DEHP interference with astrocyte number or function. This hypothesis is supported by evidence that DEHP is an EDC (Carbone et al., 2013) and thus DEHP might bind directly onto estrogen receptors expressed by astrocytes. No changes in GFP number were observed in this study across DEHP dose or in males and females, suggesting DEHP administration does not affect astrocyte number in the PFC. No changes were observed in expression of GFAP across groups either, suggesting that DEHP administration does not affect astrocyte activation in the PFC. While GFP and GFAP were the markers quantified in the present thesis, perhaps other markers should be quantified to measure astrocytic changes in response to DEHP exposure. A major function of astrocytes during development is synaptogenesis and synaptic remodeling (Diniz et al., 2012). Previous research has demonstrated that DEHP administration results in decreased spine density in the CA3 region of the hippocampus (Smith et al., 2011) thus altering synapse number. Since astrocytes are closely involved in regulating synapse expression, perhaps DEHP acts on astrocytes to limit their functional ability. Future studies might measure astrocyte-specific synaptic proteins such as connexins 30 and 43 (Gosejacob et al., 2011; Chever et al., 2014) to examine whether these are affected after early-life DEHP exposure.

Timing of DEHP exposure was discussed previously in terms of explaining sex-specific effects in response to this exposure, and it must also be discussed in terms of affecting PFC development. It is possible that the timing of exposure in the present thesis was not optimal to disrupt PFC development. DEHP administration in this study occurred

once daily from P18-P23 based on research suggesting this is a critical period of dopaminergic development in the PFC (Hoops and Flores, 2017), and research suggesting DEHP interference with dopaminergic development and alterations in behaviours governed by the PFC (Ishido et al., 2005; Kim et al., 2009). Additional research has shown that dopaminergic innervation in the PFC continues well beyond this time period, as rats at P25 show only 40% of the TH levels that will be reached in the PFC by P45 (Naneix et al., 2012). While dopaminergic innervation was ongoing during the time of DEHP exposure during the present study, it is possible that DEHP exposure needed to be more chronic or continue past P23 to observe astrocytic changes in the PFC. While the present thesis chose to examine astrocytic changes in response to DEHP administration due to their important role in mediating cortical development, as mentioned previously, it is possible that astrocytes themselves are not affected by DEHP exposure. Dopaminergic neurons were not examined in this study, and it is possible that DEHP can disrupt the dopaminergic innervation to the PFC without any impact on astrocytic integrity. Future studies might quantify TH in the PFC through either immunohistochemistry or through Western blot analysis to examine whether dopaminergic innervation is disrupted by early-life DEHP exposure, which perhaps could account for the behavioural changes noted in this study.

A limitation of the present thesis is the route of administration of DEHP. Mice were injected i.p. with either vehicle (corn oil), 10 mg/kg DEHP, or 20 mg/kg DEHP. Although the present thesis attempted to understand the mechanisms through which DEHP disrupts neurodevelopment to relate the findings back to humans, this is not a representative method of exposure, as most human exposure is through ingestion of contaminated food and water or mouthing plastic toys for infants (Wormuth et al., 2006).

I.p. injections were chosen as route of administration instead of oral gavage or adding DEHP to the food or water to be able to control the dose administered. This itself comes with the limitation that an i.p. injection is a peripheral route of administration, thus administering a specific dose of DEHP does not ensure that the full concentration crosses the BBB and reaches the brain. Although DEHP is a lipophilic compound and has been shown to cross the BBB (Agency for Toxic Substances and Disease Registry, 2002), we did not measure central DEHP levels, and therefore cannot be sure of the dose that enters the brain.

An additional limitation of the present thesis is that the mice are continuously exposed to DEHP in their surroundings. The plastic cages in which the mice live and the plastic water bottles from which the mice obtain their water also contain phthalates. This might pose a concern as the mice might be exposed to phthalates in addition to DEHP administered in this study and might thus increase the dose of exposure. As all animals live in the same cages and use the same water bottles, animals in the control group were also exposed to phthalates through their surroundings. However, this is representative of the human condition, as humans are exposed to phthalates through a variety of venues throughout the lifespan. The concern for the impact of phthalates on human health arises because some humans are exposed to higher doses than others. Correlational research examining the association between human levels of urinary phthalate metabolites and the development of ADHD suggests that children with high levels of phthalate metabolites have increased odds of ADHD diagnosis or symptoms including attention problems and decreased executive function (Engel et al., 2010), whereas low levels of urinary phthalate metabolites are correlated with lower risk. There are no children in these studies with zero exposure to environmental phthalates, thus research has examined how increased

levels of phthalate exposure from baseline negatively impacts human health. This is similar to the experimental set up in the present thesis, as the mice administered either 10 mg/kg DEHP or 20 mg/kg DEHP have increased phthalate exposure from a baseline level.

CONCLUSIONS

Human exposure to phthalates is ubiquitous throughout the lifespan, however the potential impacts this exposure has on human health is not fully understood. Exposure levels are higher in infants and children than in adults, which is concerning given that an abundance of neurodevelopment continues to occur into adolescence. While some research exists examining the relationship between early-life DEHP exposure and neurodevelopmental consequences, much more research is needed to explain the mechanisms underlying these consequences. The present study examined the effects of early-life DEHP exposure on both male and female mice and found short-term decreases in anxiety-like behaviours and increases in learning abilities in male mice exposed to DEHP. These changes were absent when assessed at a later time period, suggesting the effects of DEHP are not long-lasting. No behavioural changes were seen in females at either time point, suggesting sex-specific effects of early-life DEHP exposure. No astrocytic changes were seen in the PFC in either males or females at either time point, suggesting perhaps DEHP is not acting on astrocytes, or it is not acting in the PFC. In conclusion, early-life DEHP exposure appears to have acute anxiolytic effects only in males, and these effects do not seem to be mediated through astrocytic changes in the PFC. Future studies should examine additional astrocytic markers as well as astrocytes in different brain regions to attempt to parse out the mechanisms underlying the behavioural changes seen following early-life DEHP exposure.

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