

**The evaluation of neurodevelopmental and behavioural
correlates of acute postnatal exposure to di(2-
ethylhexyl) phthalate in rats.**

by

Catherine A. Smith

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Carleton University
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Abstract

Phthalates are synthetically derived chemicals used as plasticizers in a variety of common household products. They are not chemically bound to plastic polymers and over time easily migrate out of these products and into the environment. Experimental investigations evaluating the biological impact of phthalate exposure on developing organisms are critical given that estimates of phthalate exposure are considerably higher in infants and children compared to adults. Extensive growth and re-organization of neurocircuitry occurs during development leaving the brain highly susceptible to environmental insults. The primary goals of this dissertation were to evaluate the effects of early developmental phthalate exposure on brain structure and function, and to explore what changes in neurobiology were associated with changes in performance using behavioural measures of cognitive function. Widespread disruptions in hippocampal and dopaminergic neurocircuitry were reported in DEHP-treated male rats while only minimal changes in neurobiology were observed in DEHP-treated female rats. The cognitive effects of postnatal DEHP exposure were marginal and were only evident in female rats. The biological contributors underlying DEHP-induced changes in neurodevelopment and behaviour are not fully understood, but it is likely that the effects of DEHP are mediated by different mechanisms in male and female rats. Decreased BDNF expression may be a potential candidate for the near-selective detrimental effect of DEHP exposure on neurodevelopment in male rats. The up-regulation of hippocampal lipids may serve a neuroprotective role in DEHP-treated female rats. Comprehensive investigations which simultaneously assess the neurodevelopmental and behavioural correlates of DEHP exposure are needed and will provide an opportunity to thoroughly evaluate the toxic potential of DEHP.

Dedication

I dedicate this thesis to my wonderful boyfriend Mike. Thank you for all of your support and encouragement. I could not have made this journey without you.

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I would like to express my sincere appreciation to my supervisor Dr. Matthew Holahan for his direction and advice throughout my time in graduate school. I would not be where I am today without his mentorship and support. I would like to thank my mom for all of her love, support, and encouragement along the way. To my sister Jen, thank you for being so supportive over the years, and providing an ear for when I needed to vent. You ensured that I remained sane for the past six years. I would like to thank my Dad for all of his advice and encouragement. To my friends, thank you for your friendship and advice over the years. Thank you.

Abbreviations

2cx-MMHP: mono[2-(carboxylmethyl)hexyl] phthalate; 5cx-MEPP: mono(2-ethyl-5-carboxylpentyl) phthalate; 5OH-MEHP: mono(2-ethyl-5-hydroxyhexyl) phthalate; 5oxo-MEHP: mono(2-ethyl-5-oxohexyl) phthalate; AA: arachidonic acid; ADHD: attention deficit-hyperactivity disorder; AFB: animal-free blocker; AMPA: α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid; AR: androgen receptor; BBP: benzylbutyl phthalate; BDNF: brain-derived neurotrophic factor; CA: cornu ammonis; cAMP: cyclic adenosine monophosphate; CR: conditioned response; CS: conditioned stimulus; DCHP: dicyclohexyl phthalate; DCX: doublecortin; DEP: diethyl phthalate; DEHP: di(2-ethylhexyl) phthalate; DG: dentate gyrus; DHA: docosahexaenoic acid; DINP: diisononyl phthalate; EPSP: excitatory postsynaptic potential; ER: estrogen receptor; FSH: follicle stimulating hormone; FI: fixed interval; FR: fixed ratio; HPLC: high-performance liquid chromatography i.a.: intra-arterial; i.p.: intraperitoneal; LH: luteinizing hormone; LPC: lysophosphatidylcholine; LTP: long-term potentiation; LXR α : liver X receptor alpha; MEHP: mono(2-ethylhexyl) phthalate; NMDA: *N*-methyl-D-aspartate; NOAEL: no observable adverse effect levels; PND: postnatal day; PBS: phosphate buffered saline; PC: phosphatidylcholine; PFC: prefrontal cortex; PPAR: peroxisome proliferator-activated nuclear hormone receptor family; PSP: post-synaptic potentials; PR: progressive ratio; PVC: poly-vinyl chloride; RfD: reference dose; SL: stratum lucidum; SM: sphingomyelin; SNc: substantia nigra pars compacta; SO: stratum oriens; SP: stratum pyramidale; SR: stratum radiatum; SREBP: sterol regulatory element-binding protein; T-PBS: Triton-X/phosphate buffered saline; T-TBS: Triton-X/tris-buffered saline; TBS: tris-buffered saline; TDI: tolerable daily intake; TG: triglyceride; TH: tyrosine hydroxylase; UCR: unconditioned response; UCS: unconditioned stimulus; VI: variable interval; VR: variable response; VTA: ventral tegmental area.

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Chapter I

Introduction

Overview

Environmental experiences have the capacity to shape neurocircuitry and structure throughout the lifespan of an organism (Greenough et al., 1987; Shonkoff et al., 2009). Organisms are especially vulnerable to environmental influences during critical or sensitive development periods, which can occur both pre- and postnatally. The increased capacity of the developing brain for modification by environment experience is related to the overproduction and the flexibility of newly formed synaptic connections (Greenough et al., 1987; Shonkoff et al., 2009). These connectivity-based changes in the brain are activity-dependent – neural connections that are consistently activated are strengthened over time, while unused (or rarely used) connections are eliminated or ‘pruned’ (Citri & Malenka, 2008). Fully developed neurocircuitry maintains the capacity to be altered by environmental experiences; however, these adjustments are more restricted (Greenough et al., 1987; Shonkoff et al., 2009).

Extensive growth and re-organization of neurocircuitry occurs during postnatal development, leaving the brain highly susceptible to environmental insults at this time (Anderson et al., 2011). Exposure to adverse environmental experiences, such as toxicants, can negatively alter normal neurodevelopment by producing long-lasting changes in neurocircuitry, and in the behaviours controlled by those brain circuits (Anderson et al., 2011).

A Sensitive Period for Hippocampal Development

The hippocampus is one brain region that undergoes extensive synaptic growth and remodeling postnatally (Burgess, 2008; Cotman et al., 1973; Holahan et al., 2007; Nadel & Moscovitch, 2001). Mossy fibers are a set of hippocampal synaptic projections which relay information from granule cells in the dentate gyrus to pyramidal neurons in the *cornu ammonis* (CA) 3 region (Gluck & Meyers, 1997; Goodrich-Hunsaker et al., 2008; Squire & Kandel, 1999). During postnatal development, mossy fiber axonal projections show significant expansion and remodelling suggestive of a sensitive period for hippocampal development (Holahan et al., 2007; 2010).

In Long Evans rats, the axonal terminal fields in the hippocampal CA3 region show a change in their distribution from the stratum lucidum (SL) to the stratum oriens (SO) beginning on postnatal day (PND) 18 and ending by PND24. By PND24, the axonal staining patterns to SL and SO reflect the connectivity patterns observed in adulthood (Holahan et al., 2007). These morphological adjustments also coincide with the emergence of cognitive function, with Long Evans rats showing a remarkable improvement in performance on a spatial water maze task between PND19 and PND20 (Keeley et al., 2010). Together these findings indicate a sensitive developmental period between PND18 and PND21 for both neuroanatomical connectivity and behavioural-based cognitive performance.

Effects of Phthalate Exposure on the Developing Brain

Phthalates are a particular class of industrial chemicals used primarily to increase the flexibility and durability of plastic products. The worldwide consumption of phthalates exceeds three million metric tons annually, with the majority of these chemicals used in common household

products including food storage containers, children's toys, pharmaceuticals, cosmetics, and personal care products (Lyche, et al., 2009). Phthalates have very weak covalent bonds to polymers and often leach out of these products contaminating those who are in close contact (Heudorf, et al., 2007; Schettler, 2005). It has been estimated that exposure to phthalates is twice as high in children as adults, with 40 % of children (age two to six) showing higher urinary concentrations of phthalate metabolites than the maximum concentration measured in adults (Koch et al., 2004; 2006).

There has been increasing concern over the dangers of exposure to phthalates – especially in developing organisms as key organ systems, such as the brain, undergo rapid development at this time (Anderson et al., 2011). Studies that have investigated the effects of phthalates on brain development are limited; although it has become apparent that phthalate exposure can adversely affect many aspects of neurodevelopment in animals, particularly rodents (see 'Phthalate' section below). This dissertation evaluated the effects of acute phthalate exposure during a sensitive developmental period (PND16 to PND22) on brain structure and function, as well as explored how these changes in neurobiology were associated with performance on behavioural measures of cognitive function.

Specific Aims

The primary goals of this dissertation were:

1. Evaluate the severity of the disruptions to hippocampal connectivity following acute phthalate treatment in developing and young adult rats (Chapter II).

2. Characterize the impact of acute postnatal phthalate exposure on hippocampal development in developing rats, including alterations in neurogenesis, synaptogenesis, apoptosis, and lipid composition (Chapter II, III, and IV).
3. Identify long-term consequences of acute postnatal phthalate exposure on brain structure and function in young adult rats, particularly the hippocampus, and dopaminergic pathways (Chapter V and VI).
4. Establish whether phthalate-induced changes in neurobiology were associated with performance deficits on spatial learning and associative learning tasks in developing and young adult rats (Chapter V and VI).
5. Examine whether acute postnatal phthalate treatment differentially altered neurodevelopment and behavioural performance in male and female rats (Chapter II, II, IV, V, and VI).

The remainder of this chapter will be dedicated to the review of relevant topics for understanding the specific aims of the above studies, and will include an overview of neuroplasticity, hippocampal anatomy and development, brain lipids, spatial learning and memory, operant conditioning, and the biological properties, pharmacokinetics, and toxic potential of phthalates.

Neuroplasticity

The mammalian brain is an extremely adaptive organ that can undergo changes in organization and function with experience; a property referred to as plasticity (Citri & Malenka, 2008). This unique characteristic is a bidirectional phenomenon responsible for the formation of new neural pathways, the strengthening of existing connections, and the weakening or elimination of rarely used pathways (Citri & Malenka, 2008). Given the malleability and flexibility of synaptic connections in response to experience, researchers believe that synaptic plasticity may represent a valid biological mechanism of learning and memory (Kopp, 2007). Therefore, adverse experiences, such as exposure to environmental toxicants, have the capacity to not only interfere with the organization and formation of neurocircuitry, but disrupt behaviours underlying that neurocircuitry producing cognitive dysfunction.

Synaptic Plasticity

Synaptic plasticity is a specific form of neuroplasticity that is expressed by changes in the strength of synaptic connections (i.e. the efficiency whereby one neuron excites or inhibits another neuron) as a function of neural activity (Citri & Malenka, 2008; Tsanov & Manahan-Vaughan, 2008). The synaptic connection between two neurons can increase in strength when the pre-synaptic neuron repeatedly causes an action potential in the post-synaptic neuron (Hebb, 1959). The inability to consistently and sufficiently depolarize the post-synaptic neuron can lead to a weakening (and occasionally the elimination/ pruning) of synaptic connections. It is the combination of synaptic activity and efficacy of signal propagation that determines the overall synaptic strength between neurons.

Synaptic plasticity can be subdivided into two main categories: short-term and long-term plasticity. Short-term synaptic plasticity refers to structural or functional modifications to the synapse that influence the likelihood of signal propagation, such as alterations in synaptic transmission or dendritic spine density (Citri & Malenka, 2008). Long-term plasticity represents more permanent alterations to synaptic strength and includes long-term potentiation (LTP). LTP can be described as an increase in synaptic strength following high-frequency stimulation of a neural pathway that remains after the stimulus has been removed (Bliss & Lomo, 1973).

Synaptic plasticity is closely linked to alterations in intracellular Ca^{+2} levels by the *N*-methyl-D-aspartate (NMDA) receptor (an ionotropic glutamate receptor) located post-synaptically on dendritic spines (Citri & Malenka, 2008). At resting membrane potential (-70 mV), the NMDA receptor is impermeable to ion influx due to a voltage-dependent Mg^{+2} block within the channel pore (Evans et al., 1977; Seeburg et al., 1995). Removal of the Mg^{+2} block requires the depolarization of the postsynaptic membrane (by means other than the NMDA receptor) in concert with the co-activation of the receptor complex by glycine and glutamate (Foster & Fagg, 1987; Mayer et al., 1984; Nowak, 1984). Temporary membrane depolarization (i.e., excitatory postsynaptic potential or EPSP) can occur from the influx of Na^{+} through the activation of α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptors or through nicotinic receptors (Mayer et al., 1984; Nowak et al., 1984). Once the Mg^{+2} block is displaced, the receptor channel opens leading to an influx of Na^{+} and Ca^{+2} ions causing further postsynaptic depolarization. This process increases synaptic strength and the likelihood of generating LTP (Foster & Fagg, 1987; Mayer et al., 1984; Nowak et al., 1984). The potentiation generated by the influx of Ca^{+2} ions through NMDA receptors degrades relatively quickly and thus, long-term

changes in synaptic potentiation requires the establishment of long-term or permanent changes at the synapse.

The metabotropic glutamate receptors 1 and 5 are also thought to play a role in the potentiation of NMDA receptor synaptic currents by elevating levels of intracellular Ca^{+2} and activating second messengers, such as protein kinase C, Ca^{+2} / calmodulin-dependent kinase II, and phospholipase A_2 (Lea et al., 2002; Sabatini et al., 2001; Skeberdis et al., 2001). It is alterations in these Ca^{+2} -dependent second messenger systems that may be responsible for the long-lasting alterations in synaptic plasticity.

On the presynaptic side, an influx of Ca^{+2} initiates the release of neurotransmitters into the synaptic cleft (Katz, 1969). The amount of neurotransmitter that is released and the size of the resulting postsynaptic potentials can be altered by changes in the levels of intracellular Ca^{+2} , leading to short-term modifications in synaptic plasticity. Long-term changes in plasticity can be attributed to Ca^{+2} / calmodulin-dependent kinase II, and presynaptic metabotropic glutamate receptors 2-4 and 6-8 (Llinas et al., 1985; Ambrosini et al., 1995). Elevated levels of Ca^{+2} / calmodulin-dependent kinase II in the presynaptic neuron increases the amplitude and decreases the latency of postsynaptic potentials (Llinas et al., 1985), whereas metabotropic glutamate receptors 2-4 and 6-8 are important for the attenuation of NMDA receptor-mediated synaptic responses on the post-synaptic side by inhibiting the formation of the second messenger cyclic adenosine monophosphate (cAMP; Ambrosini et al., 1995). Together, these alterations in second messenger systems in the pre- and post-synaptic neuron are important for maintaining long-term changes in synaptic plasticity.

Structural Plasticity

There are a number of important structural modifications associated with changes in synaptic plasticity, including the formation of new connections, re-structuring or elimination of existing connections, and changes in dendritic complexity. Animals that are placed in enriched environments compared to standard laboratory environments show an increase in the volume of the cerebral cortex, which can be attributed to an increase in the number of new synapses (synaptogenesis) along with an increase in the number of glial cells (Rosenzweig et al., 1962; Diamond et al., 1964). New synapses or connections can be generated via the formation of new axon terminals on the post-synaptic neuron or through the formation of new axon collaterals on the pre-synaptic side. Existing connections are also modified through the formation of new synapses, but in an activity-dependent manner, where unused connections are eliminated (synaptic pruning) and frequently activated connections are divided and strengthened (Cowan et al., 1984; Kantor & Kolodkin, 2003; Purves & Lichtman, 1980).

Other important structural modifications include alterations in dendritic length, branching and spines (Greenough & Volkmar, 1973; Chen et al., 2004). Enriched animals show greater dendritic complexity than animals in standard laboratory conditions with increases in dendritic length and branching, and enhanced spine growth (Greenough & Volkmar, 1973; Volkmar and Greenough, 1972; Greenough et al., 1973; 1979; Globus et al., 1973). Since dendrites are the main site on the post-synaptic neuron where synapses are formed, persistent structural changes to dendrites can lead to significant functional changes in the efficacy of synaptic transmission (see Edwards, 1995 for review). Neurons with greater dendritic arborisation have the potential to form more synaptic connections with other neurons and thus, may have a greater impact on synaptic transmission and signal propagation.

Plasticity and Learning

The idea that synaptic plasticity is at the very core of learning and memory has considerable support within the literature (Blokland & Boess, 2008; Martin et al., 2000; Kopp, 2007). Morphological and physiological modifications to neuronal architecture are strong candidates for biological mechanisms underlying these cognitive processes (Blokland & Boess, 2008). The activation of a neural circuit can promote the development of new synaptic connections between neurons as well as the rearrangement of existing connections (Globus et al., 1973; Engert & Bonhoeffer, 1999; Maletic-Savatic et al., 1999). It is these new connections and circuits that are thought to underlie the storage of information.

LTP shares many properties with what researchers have described as the ‘ideal memory process’ (Martin et al., 2000; Remy & Spruston, 2007). Once LTP has been induced, the increase in synaptic strength can remain for hours or days after the removal of the stimuli. If electrical stimulation is repeated, the increase in synaptic strength may last for several weeks (Blokland & Boess, 2008; Remy & Spruston, 2007). LTP has also been implicated in structural changes in the brain, such as enhanced spine growth (Engert & Bonhoeffer, 1999; Maletic-Savatic et al., 1999; Sur & Rubenstein, 2005). LTP appears to be critical in post-synaptic spine outgrowth as no changes in spine density can be observed when LTP is chemically blocked (Engert & Bonhoeffer, 1999). It is these persistent and long-lasting changes in synaptic strength and neuronal architecture that are believed to be indicative of memory storage. For this reason, it is strongly believed that LTP represents one of the main mechanisms of information processing and storage within the brain.

Plasticity in the Hippocampus

Synaptic plasticity is most prolific throughout an individual's lifetime in the hippocampus – a brain region important in learning and memory (Kelso & Brown, 1986; Kopp, 2007). LTP is one of the most commonly studied forms of synaptic plasticity in the hippocampus and has been documented in all three hippocampal pathways: the perforant, the mossy fiber, and the Schaffer collateral pathway (Blokland & Boess, 2008). Since LTP is so prevalent in the hippocampus it is not surprising that this brain region is so strongly linked to learning and memory.

Hippocampal Anatomy and Development

Anatomy

The hippocampus is located within the medial temporal lobe and receives input from the cortex via the perirhinal, the parahippocampal and the entorhinal cortices (Burwell et al., 1995). The perirhinal cortex (which receives input primarily from the ventral visual pathway) and the parahippocampal cortex (which receives input from the parietal lobe) are thought to play an important role in visual-object memory and visuospatial memory, respectively. The perirhinal and parahippocampal cortices relay the information to the entorhinal cortex where it is integrated and then transmitted to the hippocampus. The entorhinal cortex serves as the main input into the hippocampus. The hippocampus also relays information back to the entorhinal cortex, which projects back to the neocortex via the perirhinal and parahippocampal cortices (Burwell et al., 1995).

The hippocampus is comprised of three main regions: the hippocampus proper (made up of CA1, CA2 & CA3 subregions), the dentate gyrus (DG), and the subicular complex (Amaral & Witter, 1989; Martin, 2003). Information is relayed within these hippocampal regions in a

primarily unidirectional manner through excitatory connections. Synaptic innervation occurs through three distinct pathways: the perforant, the mossy fiber, and the Schaffer collateral pathway (Gluck & Meyers, 1997; Goodrich-Hunsaker et al., 2008; Henze et al., 1996; Witter, 2007). In the perforant pathway, information is relayed through projections from the entorhinal cortex to the granule cells in the DG and pyramidal neurons in the CA1 and CA3 (Henze et al., 1996; Witter, 2007). This serves as the main input into the hippocampus. Information is also transmitted through the axons of DG granule cells (mossy fiber pathway), which terminate on pyramidal neurons of the CA3. Axons from CA3 neurons synapse on CA1 pyramidal neurons (Schaffer collateral pathway) as well as project back to both the CA3 region (recurrent collaterals) and the DG (Amaral & Witter, 1989; Henze et al., 1996; Witter, 2007). See Figure 1.1 for a diagram of hippocampal connectivity.

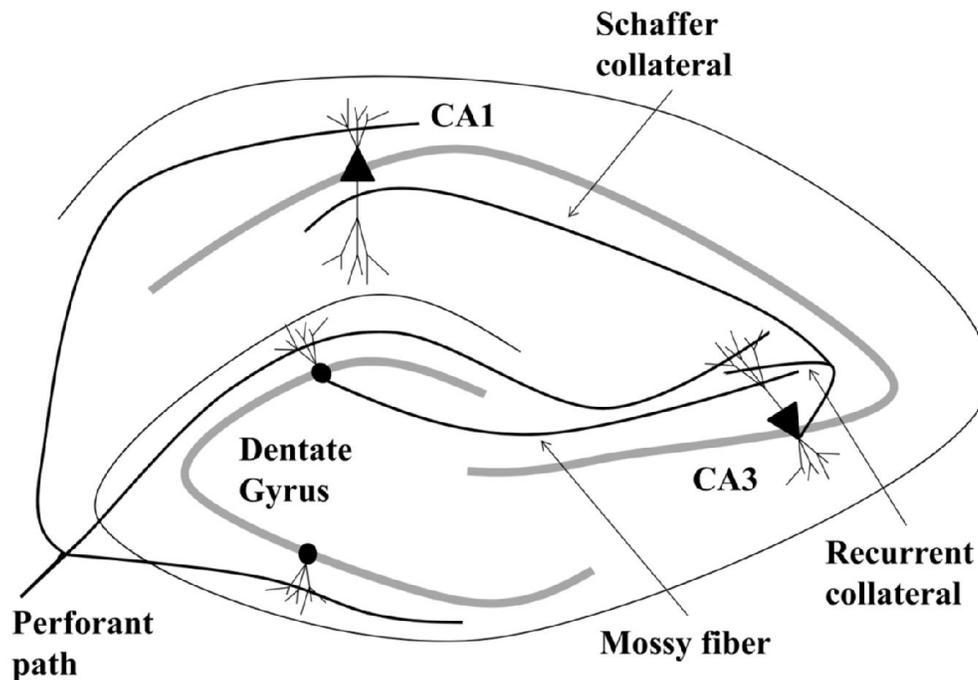


Figure 1.1: Diagram of hippocampal connectivity (adapted from Anastasio, 2010).

The hippocampus proper is subdivided into defined layers according to depth (Anderson et al., 2007). The stratum lacunosum-moleculare is the deepest layer which is innervated by Schaffer collateral fibers from the CA3 to CA1 and perforant path fibers from the entorhinal cortex. The stratum radiatum contains interneurons and receives information via Schaffer collaterals from the CA3 to CA1. The stratum lucidum (SL) is found only in the CA3. Mossy fibers pass through this layer and terminate on the cell bodies located in the stratum pyramidale and on the basal dendrites of pyramidal neurons located in the stratum oriens (SO). Interneurons can also be found in both regions. The alveus is the most superficial layer of the hippocampus proper. Axons from pyramidal neurons pass through this region to the fimbria/fornix which continues on to innervate the hypothalamus and serves as one of the major outputs from the hippocampus (Anderson et al., 2007).

The DG is one of only a few brain regions that are capable of generating new neurons (via progenitor cells) throughout an organism's lifespan (Mizuhashi et al., 2001). It is composed of three layers: granular, molecular, and polymorphic (Gaarskjaer, 1986). The granular layer is comprised of granule cells. The axons of the granule cells form the mossy fibers which innervate pyramidal neurons of the CA3 and interneurons in the polymorphic layer of the DG. The dendritic arbours of the granule cells are located within the molecular layer where they receive information from axons in the entorhinal cortex and the CA3 region. The polymorphic layer is comprised of interneurons which send and receive information to and from the granule cells in the DG (Amaral & Witter, 1989).

The subicular complex is composed of the subiculum, presubiculum and the parasubiculum (Amaral & Witter, 1989). Axons from the CA1 project to all three regions on the subicular complex and to the entorhinal cortex, serving as one of the main outputs from the hippocampus proper.

Development

The rat hippocampus begins to form during the embryonic developmental period with the formation of granule cells between embryonic day 14 and 17 (Gaarskjaer, 1986). Synaptic connections in the DG show dramatic changes from PND4 to 11 (Cotman et al., 1973), whereas CA1 pyramidal neurons undergo a period of intense growth and dendritic expansion from birth to PND7 (Pokorny and Yamamoto, 1981). The axonal terminal fields in the hippocampal CA3 region show a change in their distribution from the SL to the SO beginning on PND18 and ending by PND24 (Holahan et al., 2007). By PND24, the axonal staining patterns to SL and SO of the CA3 and the synaptic density in the DG reflect the connectivity patterns observed in adulthood (Crain et al., 1973; Holahan et al., 2007).

Composition, Structure and Function of Brain Lipids

Lipids are biological molecules found in high concentrations in the brain (Uauy & Mena, 2001). They are important structural components of neurons and glial cells as well as play a critical role in cellular signalling, protein and receptor trafficking and neurotransmission (Ramstedt & Slotte, 2002). Lipids, which can be transferred from mother to fetus via the placenta, are also essential for normal neurodevelopment (Haggerty, 2004; Innis, 2003) and thus, disruptions to lipid homeostasis during development may lead to severe abnormalities in brain structure and function.

Lipid Composition in the Brain

The concentration and composition of lipids in the brain varies between different types of brain matter (O'Brien & Sampson, 1965). Myelin contains the highest concentration of lipids (78-81%) followed by white matter (49-66%) and gray matter (36-40%; O'Brien & Sampson, 1965).

Higher molar percentages of cholesterol, cerebroside, and cerebroside sulfate and lower molar percentages of glycerophosphocholines and glycerophosphoethanolamines are also found in myelin when compared to gray matter (O'Brien & Sampson, 1965). Other lipid classes, such as sphingomyelin (SM), do not vary between myelin and gray matter (O'Brien & Sampson, 1965).

Higher lipid content has also been consistently found in astrocytes when compared to neurons (Norton et al. 1975). Astrocytes have a larger percentage of long-chain unsubstituted fatty acid side chains ($> C18$) on sulfatides (62% vs. 40%) and cerebroside (45% vs. 29%) compared to neurons (Norton et al., 1975). Sphingomyelin fatty acid side chains found in astrocytes also contain a higher concentration of C18:0 chains and a lower concentration of C16:0, C20:0, C22:0, and C24:0 chains compared to neurons (Norton, et al., 1975). Oligodendrocytes (glial cells responsible for the formation of myelin) and axonal lipids contain high concentrations of galactolipids (Norton et al., 1975).

Brain regions are composed of different ratios of myelin to gray matter and glial cells to neurons, and thus, different brain regions will contain of slightly different concentrations of lipids (Chavko et al., 1992). In the rat brain, the medulla oblongata contained the highest concentration of total lipids (111.0 \pm 6.0 mg/g) compared to the hippocampus (72.6 \pm 2.8 mg/g), followed by the cerebellum (62.7 \pm 4.6 mg/g), and the frontal lobes (57.7 \pm 2.1 mg/g; Chavko et al., 1992).

Lipid composition in the brain also varies as a function of age (O'Brien & Sampson, 1965; Soderberg et al., 1990; Svennerholm et al., 1994). O'Brien and colleagues (1965) reported a reduction in lipid content in the white matter of 10 month old infants compared to 6 and 9 year old children, and 55 year old adults. The total concentration of phospholipids and cerebroside decrease and dolichols increase with normal aging (Soderberg et al., 1990; Svennerholm et al.,

1994), while the concentration of gangliosides and isoprenoids, remain constant throughout the lifespan (Soderberg et al., 1990; Svennerholm et al., 1994).

Structure and Functions of Brain Lipids

Lipids play an essential role in brain structure and function (Uauy & Mena, 2001). Although there are many different types of brain lipids, for purpose of this dissertation only phosphatidylcholine (PC), lysophosphatidylcholine (LPC), and SM lipids will be discussed. PC lipids are a subclass of glycerophospholipids composed of a polar phosphocholine head group with two fatty acyl side chains (Holthuis & Levine, 2005). The fatty acyl side chains vary in length and degree of saturation resulting in a large number of possible PC lipids. PC lipids are a major component of cell membranes and are usually found in high concentrations on the extracellular side of the membrane (Holthuis & Levine, 2005). LPC lipids, derived from PC lipids through the removal of one fatty acyl side chain, are also found in cell membranes, but at much lower levels than PC lipids. LPC lipids more commonly function as second messengers that can trigger signaling pathways within the cell (Meyer zu Heringdorf & Jakobs, 2007).

SM lipids belong to a larger category of lipids called sphingolipids. They are composed of a polar phosphocholine head group, a fatty acyl side chain, and an 18-carbon unsaturated amino alcohol called a sphingoid (Ramstedt & Slotte, 2002). Particularly high concentrations of SM lipids are found in the myelin sheath surrounding axons indicating they have an important role in neuronal conduction. SM lipids are also found in cell membranes (along with other sphingolipids and protein receptors) where they form specialized microdomains or platforms called lipid rafts (Pike, 2003; Ramstedt & Slotte, 2002).

Lipid Rafts

Lipid rafts are specialized regions of a cell membrane enriched with cholesterol, and sphingolipids (Pike, 2003; Ramstedt & Slotte, 2002). The high concentration of sphingolipids with saturated acyl chains in lipid rafts allows them to tightly pack together increasing the rigidity of these lipid domains compared to neighboring regions on the cell membrane (Pike 2003; Rajendran & Simons, 2005). Cholesterol is also an essential component of lipid rafts as the structure and function of these rafts is disrupted by the removal of cholesterol (Rajendran & Simons, 2005). Lipid rafts can move within the cell membrane – breaking into smaller rafts or joining with other rafts to form larger ones. Lipid rafts are believed to play an important functional role in many cellular processes including the regulation of membrane fluidity, cell signaling pathways, and protein and receptor trafficking (Pike, 2003; Ramstedt & Slotte, 2002).

Identification and Quantification of Lipids

Lipidomics is an emerging field of research which involves the identification and the quantification of lipid species in biological tissue (Wenk, 2005). Lipidomics research allows investigators to ascertain how different diseases and different environmental experiences, such as exposure to environmental toxicants, alter the lipid metabolome as well as provide valuable insight into possible mechanisms of action underlying these diseases and experiences (Wenk, 2005). High performance liquid chromatography and mass spectrometry are modern research techniques used in analytical chemistry to determine lipid metabolomic profiles of biological tissues.

High-Performance Liquid Chromatography

High-performance liquid chromatography (HPLC) is a technique used to separate the components (analytes) of a chemical mixture (Pulfer & Murphy, 2003). A liquid mixture is loaded into a chromatography column packed with tiny absorbent particles (the stationary phase). Each analyte in the mixture has a slightly different affinity for the absorbent particles based on its physical and chemical properties. When a liquid solvent is forced through the column, the analytes elute at different rates depending of the strength of the interaction between the analyte and the absorbent particles (the mobile phase). Analytes that have stronger affinities with the absorbent particles have longer elution times and vice versa.

Reversed-phase HPLC (also known as hydrophobic HPLC) is a particular type of liquid chromatography used in lipidomics research. The chromatography column is packed with absorbent particles with covalently bonded alkyl chains, such as C₄-bonded silica, resulting in a hydrophobic stationary phase (Pulfer & Murphy, 2003). Lipids are hydrophobic or amphiphilic molecules that bind strongly to a hydrophobic stationary phase. The strength of bond between the lipid analyte and the column increases with the size and degree of saturation of the fatty acid chains on the lipid (Pulfer & Murphy, 2003). Lipids with weaker affinities to the column are more easily removed during the mobile phase and have shorter elution times. The removal of the lipid analytes from the column often uses a gradient elution in which a polar mobile phase gradually becomes more hydrophobic with the addition of organic solvents such as isopropanol (Pulfer & Murphy, 2003). A gradient elution is usually used when analyzing complex lipid mixtures and results in shorter elution times and sharper peaks on the mass spectra (Pulfer & Murphy, 2003).

Mass Spectrometry

Mass spectrometry is a technique which produces a mass spectrum of analytes from a sample (Pitt 2009). There are three parts to a mass spectrometer: an ionization source, a mass analyzer and a detector (Pitt 2009). Molecules must first be ionized before they can be detected by the mass analyzer. Lipid analytes travel through a positively or negatively charged column and are sprayed as a fine mist into the mass analyzer (Pitt 2009). This is referred to as electrospray ionization (ESI) and is the preferred method of ionization of lipid molecules.

There are many types of mass analyzers, but quadrupole mass analyzers are the most common (Pitt 2009). In a quadrupole mass analyzer, each quadrupole is made up of four metal rods that are arranged parallel to one another. The rods opposite one another form a pair – the poles are connected to radio frequency voltages of opposite polarity to trap ions in the centre of the rod pairs (Pitt 2009). The quadrupole acts as a filter by only allowing ions with specific mass to charge ratios (m/z) to pass through. The range of m/z -values that can pass through depends on what voltage is applied (Pitt 2009).

Tandem mass spectrometry uses a triple quadrupole design and can carry out many types of ion scans, including a precursor ion scan (Pitt 2009). In this type of ion scan, the first quadrupole scans all ion masses and allows them to enter the collision quadrupole, where ions fragment upon collision with an inert gas. The third quadrupole selects for ion fragments of a particular m/z -value which are then passed through to the detector generating a mass spectrum (Pitt 2009). The resulting mass spectrum can then be used to identify the components of a mixture, as well as determine their relative abundance in the mixture.

Learning and Memory

Learning and memory are unique cognitive abilities that are essential for many types of behaviours. The ability to learn is possessed by humans and animals alike. It refers to the process by which organisms acquire knowledge and skills through experience (Squire & Kandel, 1999). Newly acquired information is integrated with previously stored information in the brain (i.e., memories). Learning can be inferred through changes in an organism's behaviour as a result of these experiences.

Memory, which can be described as the ability to store, recall and recognize previous experiences, is the persistence of learning. That is, the continuation of behavioural changes as a function of experience. It can be divided into short- and long-term processes. Short-term memory refers to memory for immediate events, which may last for minutes. Long-term memory is a relatively permanent storage of events that may last for days to years (Matlin, 2006). Short-term memory has a finite capacity. George Miller famously reported in 1956 that the capacity of short-term memory was 7 ± 2 pieces of information. Today, estimates of capacity are much lower, ranging from 4 to 5 items (Cowan, 2001). Short-term memory can be further sub-divided into sensory and working memory. Sensory memory refers to an organism's memory for sensory information (i.e., information from one or more of the five senses – vision, audition, taste, touch and smell) from the environment. This type of memory typically degrades very quickly without rehearsal – usually lasting for less than one second (Matlin, 2006). Working memory is a temporary memory store for events actively being processed (Baddeley, 1986). Working memory is comprised of three components: central executive, phonological loop and visuospatial sketchpad. According to Baddeley's model, the central executive is responsible for updating material in storage, shifting attention between various tasks and inhibiting automatic responses, whereas the phonological loop

component refers to the temporary memory store for verbal material. The visuospatial sketchpad component refers to the temporary memory store for visual and spatial information.

Long-term memory can be sub-divided into procedural/nondeclarative, declarative and spatial memories. Nondeclarative memory refers to long-term storage for skills and procedures and is expressed through performance (Matlin, 2006). There are three subtypes of nondeclarative memory: skill learning, priming, and conditioning. Skill learning occurs when an organism learns to perform a challenging task with practice (i.e., learning to read upside down), whereas priming describes when the exposure to an earlier stimulus influences the processing of a later stimulus (i.e., re-using a word that you have heard recently; Matlin, 2006). Conditioning refers to the formation of associations between two or more stimuli, a stimulus and a response or a response and its consequence (Matlin, 2006).

Declarative memory refers to memory for facts and events that can be shown by conscious recollection of information (Matlin, 2006). Declarative memory can be further sub-divided into semantic and episodic memory. Semantic memory describes an organism's generalized memory for facts, independent of time and place, whereas episodic memory is an organism's memory of personal experiences in terms of temporal and spatial context (Matlin, 2006). Spatial memory is often thought of as a form of declarative memory. It can be described as an organism's memory for information about the environment (see section below for more details; Squire & Kandel, 1999). Regardless of the duration of the memory storage or the type of memory stored, the formation and the subsequent recall of a memory follows three stages: (1) acquisition and consolidation in which the information is processed and encoded, (2) retention which consists of the storage of information and (3) retrieval or recall of the stored information in response to a cue.

Spatial Learning and Memory

Spatial memory is a specific form of memory that stores information about the relationship between stimuli in an organism's environment (Squire & Kandel, 1999). It is the process by which sensory information (mostly vision and proprioception – the sense of the body's position in space) from the environment is acquired and integrated to determine the location of an object or oneself in space. Over the years, researchers have uncovered a number of factors that influence spatial information processing, such as stimulus, temporal and movement factors (see White & McDonald, 2002 for review). Distal environmental stimuli (i.e., cues located within the environment far from a point of reference) are important for the processing of spatial information. The relationships between these cues are integrated together to form a spatial representation or 'spatial map' of the environment with respect to a particular reference point. The formation of a spatial map is necessary for an organism's ability to navigate effectively through their environment.

Temporal factors have also been implicated in the acquisition of spatial information (White & McDonald, 2002; White & Ouellet, 1997). In one experiment, researchers reported that the ability to distinguish between two distinct sets of arms in a radial arm maze apparatus (one set that contained food and the other that contained no food) influenced the resulting integration of spatial information. One group of rats was exposed to the food arms of the maze and then exposed to the no-food arms 24 hours later. Each exposure lasted for 30 minutes. The second group of rats was exposed to the food and no-food arms in alternating five minute sessions for 30 minutes. This procedure was repeated 24 hours later. Each group of rats were exposed to the both sets of arms for equal amounts of time. The rats trained in the five minute procedure spent more time searching in the food arms than the no-food arms suggesting that these rats were better able to express their

memory for the location of the food arms. These findings highlight the importance of temporal factors in the acquisition and integration of spatial information.

Movement is a third factor that plays an important role in spatial information processing (White & McDonald, 2002). While self-generated movement in the environment is not necessary to acquire spatial information, the integration of cues from at least two separate locations leads to the formation of a more complete and detailed spatial map of the environment. When allowed to actively explore a maze, rats are more successful in the navigation of that maze, whereas the restriction of movement leads to poorer navigation (White & McDonald, 2002).

Overall, stimulus, temporal and movement factors represent key features in the acquisition of spatial information. When integrated together, these factors provide valuable information about the environment with respect to a particular reference point. Further, the assimilation of these factors allows the organism to learn relationships between different cues in the environment as well as distinguish between ambiguous cues in order to determine the exact location of an object or oneself in space.

Role of the Hippocampus in Spatial Learning and Memory

The hippocampus has long been implicated as a key brain region for spatial learning and memory (Burgess, 2008; Martin et al., 2000). Impaired performance in spatial learning and memory has been well-documented in animals with hippocampal lesions (Logue et al., 1997; Morris et al., 1982) and in animals infused with pharmacological compounds or genetic modifications that interfere with normal hippocampal functioning (Morris et al., 1986; Tsien et al., 1996). The role of the hippocampus in learning and memory was first uncovered in 1957 through the study of patient H.M (Scoville & Milner, 1957). Medial temporal lobe structures, including the

hippocampus, were surgically removed from H.M. in hopes of eliminating seizures originating from those brain regions. The removal of the hippocampus did prevent the seizures but it also interfered with H.M.'s ability to form new declarative memories (e.g., memories for places, objects, people and events). H.M. was still able to recall memories that had been stored several years prior to the surgery – only memories for events from a relatively short-time span prior to surgery and events that took place post-surgery were affected. This suggests the medial temporal lobe, and in particular, the hippocampus, may play an important role in learning and memory processes.

Operant Conditioning

Operant conditioning or instrumental learning is a learning procedure in which an action or behaviour is paired with a consequence. The probability of an organism performing a particular behaviour is dependent on the learned consequences of that same behaviour. Rewarding consequences increase the likelihood of the operant re-occurring (reinforcement), while aversive consequences decrease the probability of the operant re-occurring (punishment; Zellner & Ranaldi, 2010).

There are a number of factors that influence operant conditioning, such as reinforcement/punishment schedules, drive, and incentives. How often the reward/punishment is delivered with respect to behaviour (referred to as the schedule of reinforcement/punishment) affects how quickly an association is formed and the strength of that association (Staddon & Cerutti, 2003). There are two main types of reinforcement/punishment schedules: continuous and partial. In continuous schedules, the behaviour is reinforced or punished every time it is executed

and leads to a high level of responding by the organism (reinforcement) or elimination of the behaviour (punishment).

There are four subtypes of partial schedules: fixed-ratio (FR), fixed-interval (FI), variable-ratio (VR), and variable-interval (VI). In FR and FI schedules, the reward or punishment is delivered after a specified number of responses or a specified amount of time, respectively (Staddon & Cerutti, 2003), whereas in VR and VI schedules, a variable and unpredictable number of responses or amount of time needs to elapse before receiving the reward or punishment, respectively (Roane, 2008). In partial reinforcement/punishment schedules, the organism learns the action-outcome association more slowly than continuous schedules (Staddon & Cerutti, 2003).

Once an operant has been established, the strength of the association can be tested via extinction (Brogden et al., 1938). During extinction, the reward is removed and the behavior no longer results in its presentation. An animal will normally learn that the behavior is no longer predictive of the reward and will stop expressing the behavior (Baldwin & Baldwin, 2001). Once extinction has occurred, animals can be tested to determine if they remember that the behavior no longer produces the reward. Spontaneous recovery is the reemergence of the behavior that was previously extinguished in the absence of the reward (Baldwin & Baldwin, 2001). Re-acquisition of the reward-behavior pairing can also be assessed following extinction to determine how quickly an animal can re-learn this association.

Drive (or motivation) refers to how motivated an organism is to perform a particular behaviour for a reward. For example, rats can be trained to lever press for food rewards. If an animal is food deprived and therefore hungry, it is motivated to perform the behaviour that generates the food reward. Satiated animals do not bar press to the same degree as hungry animals as they are not as motivated by the food reward (Baldwin & Baldwin, 2001).

Motivational drive to receive a reward can be evaluated in operant conditioning tasks using a progressive ratio reinforcement schedule. In a progressive ratio task, the number of behavioral responses required for the reward is incrementally increased after each presentation of that reward (e.g. two lever presses initially produces the reward, then four are required for the next reward, six for next and so on). Progressive ratio is more resistant to extinction than non-rewarded, operant sessions since the reward is still being delivered intermittently (Roane, 2008). This task correlates well with variations in reward and deprivation parameters providing a reliable measure of motivational state (Hodos, 1961, Hodos & Kalman, 1963).

Incentives refer to how attractive a rewarding stimulus is, where strong reward incentives (i.e., presentation of chocolate pellets to hungry animals) quickly facilitate the association between an action and its consequence thereby reinforcing the behaviour. Using “weaker” reward incentives (i.e., the presentation of standard rat chow to hungry animals) delays the formation of an action-outcome association being learned affecting the probability of the behaviour re-occurring (Baldwin & Baldwin, 2001).

Reinforcement/punishment schedules, motivation, and incentives are all important factors in instrumental learning. Together, these factors determine how quickly an organism will learn an association between an action and an outcome, the strength of that association and how resistant the organism is to the extinction of that association.

Role of Dopamine in Operant Conditioning

The dopaminergic pathways in the brain play an important role in reward-based learning and in movement (Squire et al., 2008). There are four dopaminergic pathways found within the central nervous system: the nigrostriatal, mesolimbic, mesocortical and tuberoinfundibular. The

nigrostriatal pathway is essential for the generation of movement. Information is relayed from dopamine neurons located within the substantia nigra pars compacta (SNc) to the striatum (Squire et al., 2008). Individuals with Parkinson's disease show abnormal degeneration of dopamine neurons in the SNc (Squire et al., 2008). The mesocortical and mesolimbic pathways originate in the ventral tegmental area (VTA) where they send their projections to the prefrontal cortex (PFC) and the limbic system, including the nucleus accumbens (Woodward et al., 2006). Both pathways play an important role in reward learning, addiction and schizophrenia (Carlsson et al, 2001; Ikemoto, 2007). Dopamine neurons in the tuberoinfundibular pathway relay information from the arcuate nucleus of the hypothalamus to the median eminence and are important in regulating prolactin secretion from the anterior pituitary gland (Andrews et al., 2001).

Dopamine efflux has been reported to increase during food-based operant extinction procedures (Ahn & Phillips, 2007). Dopamine agonists increase non-rewarded operants while dopamine receptor antagonists suppress the extinction of operants (Duarte et al., 2003; Joel & Doljansky, 2003; Rick et al., 2006). Pre-treatment with D₁- or D₂-like dopamine receptor antagonists has been shown to reduce lever pressing associated with food-based conditioning procedures (Beninger et al., 1987). Dopamine transporter knockout mice, that show elevated levels of dopamine in the synaptic cleft, also show resistance to extinction for food-based operants (Hironaka et al., 2004). Known environmental toxicants such as dicyclohexylphthalate (DCHP), have also been shown to disrupt dopaminergic function and increase hyperactivity in male rats following a single treatment (Ishido et al., 2004). Together, these findings suggest that dopaminergic pathways are highly involved in both reward-based learning and locomotor activity.

Behavioural Measures of Learning and Memory

Morris Water Maze

The Morris water maze was first developed in 1981 by Richard Morris to assess spatial learning in the rat (Morris, 1981, 1984). The maze was initially designed to compare the ability to learn the location of a platform using either proximal or distal cues within the same basic paradigm. Cues are considered proximal when they can be detected by sight, audition or olfaction, whereas distal cues represent cues not directly linked to the target object (i.e., the spatial location of the target was inferred from these cues). At that time, no task was available that could assess both types of cues. The crucial finding from this study was that rats are capable of learning the spatial location of an objection using only distal cues (Morris, 1981).

Water Maze Procedure

Rats were placed in a large pool of water in which an escape platform was hidden just below the surface of the water. Using only distal cues (i.e., cues external to the pool), rats were repeatedly tested from different starting positions on their ability to reach the hidden platform. Normally, rats quickly learn where the platform is located and thus, latency and path length (i.e., path travelled to platform becomes more direct once location is learnt) to reach the hidden platform decreases over time. There are many variations to the standard Morris water maze paradigm, including matching-to-place learning where the platform is located in a different position every day (Whishaw, 1989) and landmark learning where rats use cues located within the pool instead of distal cues to find the platform (Kolb & Walkey, 1987).

Latency to reach the platform was and continues to be the traditional measure of spatial learning for this procedure. However, this may not be the most valid measurement of spatial

learning given that latency is dependent on swimming speed and ability. Further, during training, animals may not be using distal cues to reach the platform (Clapcote & Roder, 2004; Gallagher et al., 1993). It is possible that animals develop an effective searching technique highlighting the need for a more objective measure of spatial learning. To account for this, probe trials (which follow the training session) are used. During a probe trial, the platform is removed and the rat is given a fixed amount time to search the pool. Information, such as the number of phantom platform crossings, average proximity to the target and the amount of time spent in the target annulus and target quadrant are used as measures of spatial learning. In a meta-analysis of 1600 mouse probe trials, researchers evaluated these measures to determine which were the most effective at evaluating spatial learning (Maei et al., 2009). Overall, measures of proximity to the target annulus were more effective than all other measures in assessing spatial learning, emphasizing the importance of including this measure when analyzing water maze data.

Operant Conditioning

A behavioral model that is frequently used to study memory for rewarding events is known as operant conditioning (Skinner, 1974; Thordike, 1992). Operant conditioning, first characterized by Edward Thorndike (1911) and later by B.F. Skinner (1938), is a learning procedure in which the consequences (e.g. a food reward) of a behavior (e.g. pressing a lever) increase or decrease the probability of the behavior being repeated.

Operant Conditioning Procedure

Rats were trained on a reward-based associative learning task using operant conditioning chambers, where the rat learned to press a lever to receive a chocolate pellet. The delivery of the

chocolate pellet was paired with two neutral environmental stimuli: the house light turning off and the lights directly above the lever changing from red to green. These environmental stimuli occurred in all testing sessions including the extinction phase of the task, in which the lever was no longer paired with food delivery. Figure 1.2 shows a schematic of an operant conditioning chamber.

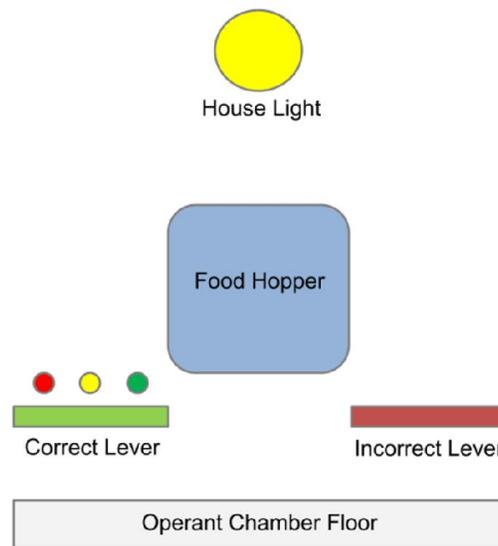


Figure 1.2: Schematic of Operant Chamber. A schematic of the “rat’s view” while facing the cued wall in the operant chamber.

Three reinforcement schedules were used: a fixed-ratio (FR) 2 (where two consecutive lever presses were required for the delivery of a food reward), a progressive ratio (PR) 2 (where two additional lever presses from the previous trial were required to obtain a chocolate pellet) and extinction (where no food reward was given). A FR2 schedule was used during the acquisition and the re-acquisition phases, and was designed for the animals to learn the association between lever pressing and receiving a food reward. The goal of the extinction task was to determine how long

the animal would continue to lever press for food when the reward was no longer paired with this behaviour. Finally, the PR2 schedule was used to determine the length of time to extinction when the number of lever presses before the reward (and time between the instances of reward) is increased.

During the extinction and the PR phases, a normal control animal will learn that the behaviour (lever pressing) and the environmental stimuli (the house light turning off and lights above the lever switching from red to green) are no longer predictive of a reward. Extinction of the lever pressing behaviour will occur more rapidly when no food reward is delivered throughout the entire testing session (i.e., extinction task) than when the number of lever presses for a food reward is increased by increments of two each time (i.e., PR task), since animals in the PR task are still intermittently receiving a reward. The number of lever presses will be recorded every five minutes to evaluate time to extinction in the extinction and PR testing sessions.

Locomotor activity is another behavioral measure that can be obtained from the use of operant chambers. Evaluating locomotion in a reward-based learning environment also allows for conclusions to be drawn regarding the motivation to perform the rewarded behavior (e.g. were high levels of lever pressing during operant training associated with high levels of spontaneous locomotor activity and unintentional lever pressing or were they related to an increased motivational drive to receive the reward). Therefore, when locomotor behavioral measures are used together with extinction and progressive ratio training data, these measures can provide a complete assessment of motivational components of learning and reward.

Phthalates

Phthalates are synthetic chemicals that have been used as plasticizers since the 1920s as a replacement to the more volatile plasticizer camphor (Graham, 1973). It wasn't until the synthesis of polyvinyl chloride (PVC) and the synthesis of di(2-ethylhexyl) phthalate (DEHP) in 1931 and 1933, respectively, that phthalates became widely used (Graham, 1973). Today, the worldwide use of phthalates exceeds three million metric tons annually, with many of these chemicals used in common household products including food storage containers, children's toys, pharmaceuticals, cosmetics, and personal care products (Bizzari et al., 2000; Lyche, et al., 2009). Given the widespread use of plastics in our everyday lives, it is likely that exposure to phthalates is unavoidable.

Physical and Chemical Properties

All phthalates are derived from phthalic anhydride and consist of a benzene ring with a di-ester structure (Graham, 1973). They are prepared through a two-step chemical process. In the first step, which occurs rapidly, phthalic anhydride and alcohol react to form a monoester. In the second step, which occurs more slowly, the monoester is converted into a di-ester through a second alcoholysis reaction. This reaction is reversible and generates H₂O as a by-product (Graham, 1973).

Phthalates are colourless, odourless, oily liquids with low volatility (NICNAS, 2010). The water solubility of phthalates is quite low and is dependent on the molecular weight and the length of the ester side chain (Lyche et al., 2009). As the molecular weight of the phthalate and the length of the side chain increases, water solubility decreases.

Phthalates can be divided into three categories based on the length of the ester side chains: low molecular weight, high molecular weight and transitional or mid-molecular weight (NICNAS, 2010). Low molecular weight phthalates have short, straight side chains of three or less carbon atoms, while transitional phthalates can have side chains that are either straight or branched and contain four to six carbon atoms. 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. Interestingly, transitional phthalates with di-ester side chains in an ortho configuration on the benzene ring (like DEHP) appear to show the greatest toxic potential (Fabjan et al., 2006).

Di(2-ethylhexyl) Phthalate

With approximately three billion kilograms synthesized per annum, DEHP is one of the most commonly used phthalates worldwide (Lyche et al., 2009) and for the purpose of this dissertation, will be the only phthalate discussed in detail. DEHP, also known as bis(2-ethylhexyl) phthalate, Bisofelx 81, Eviplast 80, Octoil, Plantinol DOP and Starfelx DOP, is a branched-chain transitional phthalate with the molecular formula $C_6H_4(C_8H_{17}COO)_2$ and has a molecular weight of 390.57 g/mol (Figure 1.3; Howard & Meyland, 1997). It is produced when the ester functional groups on phthalic acid react with 2-ethylhexanol (Howard & Meyland, 1997). The resulting organic compound is a colourless, odourless, oily liquid with low volatility and low water solubility (Montgomery & Welkomm 1990; Staples et al., 1997; HSDB, 1990). DEHP can be found in both industrial and consumer plastic products including automotive upholstery, perfumes, carpets, insulation, mattress pads, shower curtains, clothing toys, umbrellas, straws, pesticides, medical tubing and medical bags (HCWH, 2002).

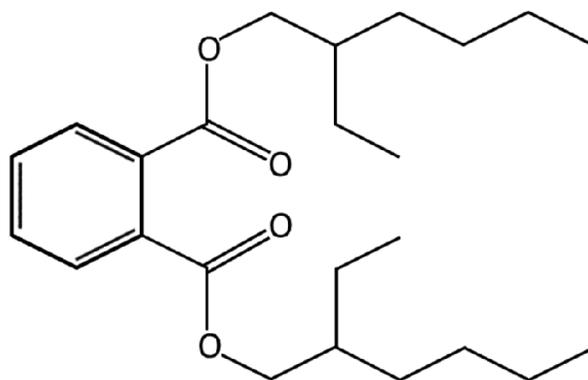


Figure 1.3: Chemical structure of di(2-ethylhexyl) phthalate.

Sources of Exposure

Phthalates are not chemically bound to plastic polymers, and over time approximately 2-50% of phthalates easily migrate out of these products into food, saliva, blood, etc. contaminating those who are in close contact (HCWH, 2002; Heudorf, et al., 2007; Schettler, 2005). Phthalates leach out of plastics and into some matrices more readily than others. For example, phthalates will permeate substances that are lipid soluble more readily than substances that are water soluble (HCWH, 2002). Regardless of the type of matrix, humans are exposed to phthalates in one of four ways: ingestion, inhalation, absorption and intravenous (Latini, 2005; Schettler, 2005).

Ingestion

Ingestion is the most common route of exposure for DEHP, with approximately 90% occurring through oral consumption of food in adults (Clark et al., 2003a, 2003b; Latini, 2005; Wormuth et al., 2006). Exposure to DEHP from dietary sources is much lower in formula-fed and breastfed infants, with 44% and 60% of the estimated intake of DEHP occurring through food intake, respectively (Clark et al., 2003a,b). Plastic food packaging is a major source of DEHP

found in food which can be ingested via the diet (Chen et al., 2008; Houlihan et al., 2002; Kueseng et al., 2007). DEHP easily migrates out of plastic packaging and into the food product. The concentration of DEHP that leaches into food products is dependent on the length of time the plastic packaging is in contact with the food, how the food was prepared, and the lipid solubility of the food.

High levels of DEHP are found in food that has been in contact with plastic food packaging (Chen et al., 2008), such as tempura powder (0.11-68 mg/kg), instant cream soup (0.04–3.1 mg/kg), and fried potato cake (0.05–9.1 mg/kg; WHO 2003). High concentrations of DEHP are found in olive oil (65.8 µg/L) compared to fish oil (37.8 µg/L), and soybean oil (27.3 µg/L; Bagel et al., 2011). Liquids and beverages (excluding oils) with plastic seals have much lower levels of DEHP, such as beer (<0.01-0.04 mg/kg), and orange juice (0.05 mg/kg; WHO, 2003).

In addition, when families reduced their intake of food packaged in plastic, levels of urinary metabolites of DEHP were also reduced (Rudel et al., 2011). Researchers have shown that the concentration of DEHP in a food product is directly associated with the date of packaging, with higher concentrations of DEHP found in food packaged at an earlier date (Kueseng et al., 2007).

The lipid-solubility of the food also determines the amount of DEHP that leaches into it. Since DEHP is highly lipid-soluble, these chemicals permeate lipid-soluble food items very readily with high concentrations of DEHP found in milk (31.4 mg/L), cheese (35 mg/kg), fats and oils (2.4 mg/kg), and poultry (0.9 mg/kg; Clark et al., 2003a; WHO 2003). The primary metabolite of DEHP (mono-2-ethylhexyl phthalate or MEHP) has also been found in breast milk (mean 11 µg/L; range 1.5–1,410 µg/L; Main et al., 2006). Food products with very low lipid-solubility, such as vegetables and cereals, contain much lower concentrations of DEHP, 0.048 mg/kg and 0.05 mg/kg, respectively (Clark et al., 2003a).

The low water solubility of DEHP (0.003 mg/L; Staples et al., 1997) greatly reduces the amount of DEHP that migrates into our drinking water (Clark et al., 2003a). Average levels of DEHP found in drinking water in the USA are less than 1 µg/L, but can range between 0.05-11 µg/L (WHO 2003). In Japan, DEHP levels in tap water range between 1.2-1.8 µg/L (WHO 2003). DEHP concentrations measured in rainwater are much lower and can range between 0.6-3.2 µg/L in Japan and 0.053-0.213 µg/L in the North Pacific (WHO 2003). DEHP concentrations found in the Rhine river in the Netherlands were also low (0.1-0.7 µg/L), but sediments surrounding this river were considerably higher (1-70 mg/kg; WHO 2003).

Ingestion of DEHP can also occur when children chew on or place PVC-plastic toys in their mouths (Lyche et al., 2009). DEHP can easily migrate out of these toys and into saliva (Bouma & Schakel, 2002; Steiner et al., 1998). DEHP (and other phthalates) are added to these toys to soften the plastic. Concentrations of DEHP in PVC-plastic toys have been reported to be as high as 40%, with higher DEHP concentrations resulting in increased DEHP migration into saliva (Lyche et al., 2009). Up until 1985, DEHP was the most commonly used phthalate in children's products when it was replaced with the reputed "less toxic" di-isobutyl phthalate (DINP; Wigle, 2003). In recent years, some governments (e.g., Canada, United States, and European Union) have placed restrictions on the allowable concentrations of phthalates (including DEHP and DINP) in children's products (intended for children under 3 years) to less than 0.1% to minimize children's risk of exposure to phthalates (see "Estimates of human exposure" section for more details).

Phthalates are also found in the coating of pharmaceutical drugs and thus, exposure can occur by ingesting these drugs (Hauser et al., 2004; Schettler, 2005; Lyche et al., 2009). DEHP is

not phthalate commonly found in pharmaceutical drugs, and this source of exposure will not be discussed further.

Inhalation

DEHP has relatively low volatility and does not migrate into the air very easily and thus, a very limited amount of this chemical can be found in the air (Wormuth et al., 2006). However, DEHP binds very readily to particulates in the air (Afshari et al., 2004; Fromme et al., 2004). Individuals can be exposed to DEHP by inhaling these particulates. Levels of DEHP measured in the air near bodies of water (0.4 ng/m³, 1.4 ng/m³, 2 ng/m³, 2.9 ng/m³ of the Gulf of Mexico, the North Pacific, the Great Lakes and the North Atlantic, respectively) are much lower than levels measured in the city (5-132 ng/m³ for New York City, USA; WHO, 2003). DEHP can also be found in indoor air (mainly in house dust) at concentrations that are much higher than outdoor air. The average concentration of DEHP found in house dust of Norwegian homes was 640 µg/g (range 100-1610 µg/g; Oie et al., 1997). Similar levels of DEHP have been found in other countries including Japan and Germany (Becker et al., 2004; Otake et al., 2004). A number of studies have shown strong correlations between the levels of diethyl phthalate (DEP), dibutyl phthalate (DBP), and benzylbutyl phthalate (BBP) measured in house dust and the occupants' urinary metabolites. Strong correlations are not seen when examining DEHP with house dust, indicating that the inhalation of house dust may not be a typical source of exposure to DEHP (Adibi et al., 2003; Becker et al., 2004; Otake et al., 2004).

Absorption

Dermal absorption from personal care products, such as makeup, deodorants, soap, and shampoo is another common source of exposure to phthalates, particularly DEP and DBP (Hubinger & Havery, 2006; Koch et al., 2003; Koo & Lee, 2004). Researchers have identified that DEHP is not a phthalate typically found in these personal care products (Hubinger & Havery, 2006) and thus, this type of exposure will not be discussed further.

Intravenous

Another common route of exposure is through medical procedures, such as blood transfusions and kidney dialysis, with medical devices that contain DEHP (Calafat et al., 2004; Dine et al., 2000; Koch et al., 2006). DEHP is found in medical tubing and blood bags at very high concentrations (20-40%), meaning individuals receiving medical treatment can be exposed to very high levels of DEHP (Lyche et al., 2009). Up to 15% of the total DEHP can leach out of medical products and into bodily fluids where it is distributed throughout the body (Halden, 2010).

Estimates of Exposure

DEHP, as well as primary and secondary metabolites of DEHP, have been detected in amniotic fluid, breast milk, saliva, blood, and urine of humans of all ages (McKee 2004; Calafat et al., 2004; Kato et al., 2004; Silva et al., 2005; Barr et al., 2003; Koch et al., 2005a,b; Preuss et al., 2005). Researchers use one of two methods to estimate human exposure to DEHP. The first method involves estimating DEHP exposure from external sources such as food and dust, whereas the second is based on internal measurements of urinary metabolites of DEHP (Lyche et al., 2009). Levels of DEHP from external sources are estimated from known usage patterns of items that

contain DEHP (i.e., food, toys, etc.) and the length of time individuals spend in environments where exposure may occur (i.e., medical treatments, house dust, etc.). It is necessary to have accurate measurements of DEHP in products and the environment, and to know the ingestion, inhalation, absorption rate of DEHP from products and the environment in order to correctly assess DEHP exposure from external sources (Lyche et al., 2009). Estimates of DEHP exposure made from external sources can be quite complicated as humans are exposed to DEHP from multiple sources and through different routes.

An alternate way to estimate DEHP exposure in humans involves measuring the levels of DEHP metabolites in urine (Lyche et al., 2009). Examining urinary metabolites solves the problem of multiple sources and routes of exposure, therefore ingestion, inhalation, and absorption rates do not need to be calculated. Instead, researchers are required to understand the rates at which DEHP is metabolized in the body and make estimates to determine the exposure dose by measuring primary and secondary metabolites excreted in urine (Lyche et al., 2009). A major flaw with this method is that it assumes individuals metabolize DEHP at a constant rate; however, variables such as gender and age greatly influence DEHP metabolism (Lyche et al., 2009). This method also ignores variability within individuals and assumes people are exposed to DEHP at a constant rate throughout the day. Since DEHP exposure can change over the course of a day, the time the urine sample is taken will directly affect the resulting estimated exposure dose, with samples taken during the evening showing the highest metabolite concentrations compared to samples from the morning or afternoon (Fromme et al., 2007; Aylward et al., 2011).

Legislation

No observable adverse effect levels (NOAELs) are defined as an exposure rate which have no biological consequences to the organism (Faustman & Omenn, 2001). The NOAEL for DEHP is 4.8 mg/kg/day (Wolfe & Layton, 2003; Lyche et al., 2009). Researchers and policy makers use the NOAEL to calculate the tolerable daily intake (TDI) and reference dose (RfD) for DEHP in humans (FDA, 2002). TDI can be defined as “the dose of a compound that is not expected to result in adverse effects following exposure for a defined period” (FDA, 2002, pg. 3), and is usually 100 times less potent than the NOAEL (Faustman & Omenn, 2001; Renwick, 1999). Reference dose (RfD) is the maximum oral dose of a substance at which no adverse effects are observed throughout the lifespan (US EPA, 1999). The United States Environmental Protection Agency has recommended a RfD of 20 µg/kg of body weight for the human population while the European Union has recommended TDIs of 20 µg/kg of body weight and 48 µg/kg of body weight for newborns and adults, respectively (Koch et al., 2006).

Laws that reduced the levels of DEHP and other phthalates found in plastic products used by children under 3 years of age have come into effect in the last decade worldwide. In 1999, the European Union was the first to implement a ban on the use of phthalates (including DEHP) in PVC children’s toys (Greenpeace, 2003). In 2005, phthalates in all children’s toys and child care products were banned in Europe (EU, 2005). In the United States, the American Council on Science and Health ruled in 1999 (despite Europe’s ban) that phthalates posed no risks to human health; as a result legislation that restricted the concentration of phthalates was not implemented (Noble, 1999). Although manufacturers voluntarily removed phthalates from children’s toys in 1999, it wasn’t until 2008 that manufacturers were prohibited from selling children’s toys and child care products with levels of phthalates exceeding 0.1% the United States (1,000 mg/kg;

CPSC, 2010). In June of 2011, Canada also implemented legislation that limited the concentration of phthalates (including DEHP) in children's toys and child care products to a maximum 0.1% or 1000 mg/kg (Health Canada, 2011).

Human Exposure

Ingestion of DEHP for adults consuming products with a high fat content, including milk and cheese, is estimated to be 200 µg per day (WHO, 2003), while inhalation of house dust is estimated to be 0.76 µg per day (Schettler, 2005). Exposure to DEHP through drinking water and air are considered to be negligible (WHO, 2003). Individuals exposed to DEHP in occupational settings or who are undergoing medical treatments are subjected to much higher than average daily exposure levels, with daily exposure rates of 700 µg/kg of body weight and 457 µg/kg of body weight, respectively (Kavlock et al., 2002; Latini, 2005).

Estimations made from measuring primary and secondary metabolites excreted in urine tend to be slightly lower than estimations made from calculating the concentration of DEHP in plastic products and in the environment (Lyche et al., 2009). Recent estimates of human exposure from urinary metabolites suggest that adults are exposed to approximately 3 to 30 µg/kg of body weight per day (Latini, 2005). Another study suggests a mean daily exposure rate of 5.6 µg/kg of body weight for adults, with 95 percentile exposure rates as high as 21 µg/kg of body weight (Koch et al., 2006). Of the 85 participants in this study, only two exceeded the TDI and seven exceeded the RfD. Exposure rates for children were significantly higher with a mean rate of 7.7 µg/kg of body weight, and 95 percentile rate of 25 µg/kg of body weight. Of the 254 children, four exceeded the TDI (for adults) and 27 exceeded the RfD (Koch et al., 2006).

Factors that Affect DEHP Concentration Levels

There are a number of different factors which can affect the concentration of phthalates (including DEHP) present in the human body. For the purpose of the current dissertation, only gender and age will be discussed. Concentrations of phthalates (including DEHP) measured in men and women are different. Two thousand and forty-one urine samples of both men and women were analyzed for primary phthalate metabolites, including MEHP (the primary metabolite for DEHP). Seventy-eight percent of the time women had higher urinary concentrations of MEHP compared to men. Mean concentrations of MEHP were 4.53 $\mu\text{g/g}$ of creatinine (range 4.01-5.11) for women and 3.49 $\mu\text{g/g}$ of creatinine (range 3.06-3.98) for men (CDC, 2009).

Age is another factor that greatly affects the concentration levels of phthalates in our bodies. Although phthalates have been measured in individuals across all developmental stages, there is compelling evidence indicating infants and children have greater levels of these chemicals than adults, specifically DEHP, DBP, BBP and di(n-octyl) phthalate (Koch, et al., 2004; Wormuth, et al., 2006). DEHP has been found in amniotic fluid and can easily cross the placental barrier exposing the fetus (Dostal et al., 1987; Latini et al., 2003). Sensitivity to phthalates decreases with age, with the fetus being the most sensitive to exposure (US EPA, 2009).

Wormuth et al. (2006) calculated participants' daily exposure to eight different phthalates across the lifespan and reported that exposure levels were highest in infants (birth to 12 months) and toddlers (one to three years) when compared to all other age groups. In another study, at least one phthalate metabolite was detected in the urine of all infants, with seven or more detected in the urine of 81% of infants (Sathyanarayana, et al., 2008). The urinary concentration of phthalate metabolites in these infants was related to the mother's use of infant care products, such as infant lotion and shampoo, whereby higher urinary concentrations were correlated with higher infant

product use. The concentration of DEHP metabolites detected in the urine of individuals undergoing medical treatment is also dependent on age. The estimated exposure of neonates is much higher than adults receiving similar medical treatments, including blood transfusions (22.6 and 8.5 mg/kg/d, respectively) and parenteral nutrition (2.5 and 0.13 mg/kg/d, respectively; Calafat et al., 2004; Green et al., 2005; Weuve et al., 2006).

It has been estimated that exposure to phthalates is twice as high in children as adults, with 40 % of children (age two to six) showing higher urinary concentrations of phthalate metabolites than the maximum concentration measured in adults (Koch et al., 2004; 2006). In another study, children age 6 and up had higher concentrations of 11 of the 12 phthalate metabolites tested in their urine when compared to adults (CDC, 2009). This heightened exposure to phthalates in infants and children, which may be attributable to their higher metabolic rate (i.e., they breathe more oxygen, drink more fluids and eat more food per kilogram of body weight than adults), is especially concerning given that key organ systems, such as the brain, undergo rapid development during these times.

Pharmacokinetics

Pharmacokinetics refers to the process by which the body absorbs, distributes, metabolizes and excretes a drug or toxin (Benet & Zia-Amirhosseini, 1995). When examining DEHP, this process is highly dependent on a wide variety of factors including age, gender, species, and route of administration.

Route of Administration

The route of administration of DEHP is an important factor that mediates the concentration of DEHP and its metabolites in the body. Pollack et al., (1985) examined how different routes of administration affected DEHP distribution and metabolism in rats. A single administration of DEHP via intra-arterial (i.a.) and intraperitoneal (i.p.) injections led to significantly lower circulating MEHP levels relative to DEHP levels when compared to oral exposure (Pollack et al., 1985). High levels of MEHP following oral exposure can be attributed to the metabolism of DEHP to MEHP in the gut. When given orally, 80% of DEHP is metabolized into MEHP, whereas only 1% of DEHP was broken down into MEHP after i.a. or i.p. administration (Pollack et al., 1985). Multiple dosing of DEHP for 7 days did not lead to the bioaccumulation of circulating levels of DEHP or MEHP; although there were still marked differences in the ratio of MEHP to DEHP between oral and i.p. exposure routes, indicating that the biological effects of DEHP are highly dependent on route of administration (Pollack et al., 1985).

Absorption and Distribution

In rodents, DEHP and MEHP undergo rapid absorption via the gastrointestinal tract upon ingestion (Pollack et al., 1985; Rhodes et al., 1986). The concentration of DEHP in the gastrointestinal tract peaked immediately after exposure and then quickly declines (Ikeda et al., 1980). DEHP is also absorbed into the bloodstream, and concentrations of DEHP in blood peaked after 3 hours (Pollack et al., 1985). DEHP is highly lipophilic as indicated by levels of DEHP metabolites that were four and six times higher in adipose tissue and liver than any other tissue (CPSC, 2010). DEHP does not bioaccumulate in tissues, even with repeat dosing (Pollack et al., 1985), and after four days only 0.1% of DEHP metabolites are found in tissue (Lake et al., 1975).

The age of the animal and time of exposure is also important in determining absorption. Young animals (postnatal day 25) showed higher concentrations of DEHP metabolites than animals 40 and 61 days old (Sjoberg et al., 1985). DEHP is also quickly absorbed in humans following oral exposure, with approximately 67% of the DEHP dose excreted after a 24 hour time period (Koch et al., 2006). The bioavailability of DEHP following oral exposure is 30-50% and 37.7-46.5% in rats and humans, respectively (ECB, 2008).

Metabolism and Excretion

As mentioned, once DEHP enters the body it is rapidly metabolized in the gastrointestinal tract by gut lipases, such as esterase, hydrolases and pancreatic lipases (Frederiksen et al., 2007; Kluwe, 1982). When compared to adults, infants have much lower levels of these gut lipases and thus, they may not be capable of metabolizing and excreting phthalates as efficiently (Lyche et al., 2009). DEHP undergoes a two part metabolism (Frederiksen et al., 2007). In the initial phase DEHP is hydrolyzed into its monoester, MEHP, which is then excreted in urine or hydrolyzed into one of four major secondary metabolites: mono(2-ethyl-5-hydroxylhexyl) phthalate (5OH-MEHP), mono(2-ethyl-5-oxohexyl) phthalate (5oxo-MEHP), mono(2-ethyl-5-carboxylpentyl) phthalate (5cx-MEPP) and mono[2-(carboxylmethyl)hexyl] phthalate (2cx-MMHP; Koch et al., 2006). The half-lives of the secondary metabolites are significantly longer than DEHP and MEHP. When secondary metabolites are used as biomarkers to estimate DEHP exposure, the estimates are considerably higher than estimates made using circulating levels of MEHP or DEHP (Fromme et al., 2007; Koch et al., 2006).

During the second phase of metabolism, secondary metabolites are either excreted in urine or conjugated into glucuronic acid and then excreted in urine (Frederiksen et al., 2007; Peck &

Albro, 1982). Approximately 80 to 90% of secondary metabolites are conjugated into glucuronic acid in adults (Peck & Albro, 1982). Children under three months may be more vulnerable to phthalate exposure because the glucuronidation pathway has not yet developed (Cresteil, 1998) and thus, they may not have the same capacity to metabolize and eliminate phthalates from their system (Lyche et al., 2009).

Toxic Potential

The health impact of phthalates is related to the length of the ester side chain. Only transitional phthalates with ester side chains in the “ortho” position, like DEHP, have been identified as having toxic potential in humans (Fabjan et al., 2006). It is possible that other phthalates may present some health risk to the human population; however, research on these phthalates is quite limited. According to the Federal Hazardous Substance Act (FHSA), DEHP is labelled a chronic toxicant with no acute toxicity (CPSC, 2010). Chronic exposure can be defined as an exposure period longer than 365 days, while acute exposure is an exposure period of less than 14 days (CPSC, 2010). The CPSC (2010) considers DEHP to be a known animal and possible human reproductive and developmental toxicant (CPSC, 2010).

Reproductive and Developmental Toxicity

Recent animal toxicity studies have indicated that exposure to DEHP early in life results in severe disorders in developing male reproductive organs, including defects in the external genitalia, undescended testes, and testicular lesions (see Lyche et al., 2009; Swan, 2008 for review). Male lab animals also show a permanent feminization of the reproductive system, including the retention of nipples and decreased anogenital distance (the distance between the anus

and the genitals; Foster et al., 2006). Reduced anogenital distance has also been observed in human studies, with an inverse relationship between maternal phthalate metabolite levels and anogenital distance in male infants (Swan et al., 2005).

High maternal levels of phthalate metabolites (particularly MEHP) are correlated with shortened gestational periods and an increase in preterm birth (Meeker et al., 2009; Whyatt et al., 2009). This is concerning given that infants born prematurely or early (37-38 weeks instead of 39-40 weeks) have a greater risk of learning disabilities, and increased risk of respiratory and cerebrovascular health issues later in life (Kirkegaard et al., 2006; Koupil et al., 2005). Low birth weight has also been linked to high levels of phthalates including DEHP (Zhang et al., 2009). Levels of MEHP measured in the cord blood of babies with low birth weights (5.5 lbs. or less) were increased two-fold compared to normal-weight babies (Zhang et al., 2009). In animals the association is less clear, with some researchers reporting increased birth weights in male rats following DEHP exposure (Tanaka, 2002; 2003), while others have shown a 15% decrease in birth weight following exposure (Gray et al., 2000).

The age at which rats reach puberty was delayed (46.3 ± 0.1 days postpartum) following exposure to high doses of DEHP (750 mg/kg) compared to normal controls (41.5 ± 0.1 days postpartum; Ge et al., 2007). At low doses of DEHP (10 mg/kg), the onset of puberty in rats was accelerated (39.7 ± 0.1 days postpartum; Ge et al., 2007). Noreiga et al. confirmed in a 2009 study that high DEHP exposure (300 mg/kg and 900 mg/kg) did lead to delayed puberty in rats. In contrast, low doses of DEHP (10 mg/kg and 100 mg/kg) did not affect the onset of puberty (Noreiga et al., 2009). In humans the onset of puberty appears to be unaffected by DEHP early in life (Rais-Bahrami et al., 2004). Male and female participants (age 14 to 16) who were exposed to

high levels of DEHP as neonates from medical procedures showed no alterations in pubertal maturity (Rais-Bahrami et al., 2004).

Carcinogenicity

Rodents have an increased incidence of hepatocellular tumors following long-term, high doses of DEHP (David et al., 1999; Hayashi et al., 1994; Lake et al., 1987). The mechanism by which DEHP mediates hepatocarcinogenic effects is not relevant to humans (CPSC, 2010) and thus, the International Agency for Research on Cancer (IARC) downgrade its classification in 2000 to “cannot be classified as to its carcinogenicity to humans” (IARC, 2000).

Endocrine Disruption

The endocrine system is important for maintaining a number of homeostatic mechanisms such as reproduction, metabolism, and other regulatory functions (Crisp et al., 1998). It is comprised of a number of glands (including the pituitary and adrenal glands) which release hormones directly into the bloodstream. Hormones are slow acting, but can have long-lasting effects on both growth and development (Crisp et al., 1998).

Endocrine disrupting chemicals are natural or synthetic compounds that mimic or interfere with the biosynthesis, metabolism or action of endogenous hormones and thus, interfere with homeostatic maintenance (Crisp et al., 1998). DEHP has been described as a synthetic toxicant with endocrine disrupting characteristics. Although the mechanism of action underlying DEHP toxicity is not yet understood, it is likely that DEHP’s action as an endocrine disrupting chemical may at least in part contribute to the neurodevelopmental and behavioural changes observed in

animals and humans. In particular, the effects of DEHP exposure on estrogen and androgen signalling will be discussed.

Estrogenic Effects. There is strong evidence to support the idea that DEHP may lead to alterations of estrogen signalling pathways. *In vitro*, exposure to DEHP (but not MEHP) led to weak estrogenic activity via human estrogen receptor (ER) α but not human estrogen receptor ER β (Takeuchi et al., 2005). In the presence of 17 β -estradiol, weak anti-estrogenic effects have been reported in ER α but not ER β (Takeuchi et al., 2005). Researchers have also demonstrated significant down-regulation of the progesterone receptor (an estrogen target) in males and females, and down-regulation of CYP17a1 (a gene important in the synthesis of steroid hormones) in females at low doses of DEHP, suggesting low levels of serum estradiol levels in DEHP exposed animals (Pocar et al., 2012).

Decreased serum levels of estradiol and progesterone have been reported in female rats and in cultured female rat granulosa cells following exposure to DEHP and MEHP (Davis et al., 1994a; 1994b; Lovekamp-Swan & Davis, 2001; Svechnikova et al., 2007). Estradiol and progesterone levels in male rodents were not evaluated in these studies. The administration of DEHP did not induced estrogenic effects in ovariectomized rats (Zacharewski et al., 1998). The suppression of estradiol production in female rat granulosa cells by MEHP may be related to the suppression of aromatase transcripts and the reduced availability of aromatase enzyme (Davis et al., 1994b; Lovekamp-Swan & Davis, 2001). The down-regulation of CYP19a1 has also been shown following DEHP treatment in male and female mice (Pocar et al, 2012).

Pocar et al. (2012) hypothesized that low levels of estrogen may have a negative effect on the hypothalamic-pituitary-gonadal axis and thus, can affect the secretion of gonadotropins, such as luteinizing hormone (LH) and follicle-stimulating hormone (FSH). LH is important in

testosterone synthesis, and LH mRNA levels are up-regulated and LH receptors are down-regulated in DEHP-exposed males and females (Pocar et al., 2012). FSH is important in promoting normal gonadal function, and in normal controls, FSH release elevates intracellular levels of cAMP – a protein important in steroidogenesis (Heindel & Chapin, 1989). Following treatment with MEHP, the ability of FSH to elevate cAMP was reduced by 40% (Heindel & Chapin, 1989). Researchers also found that FSH mRNA was up-regulated in males (but not females) after DEHP treatment, and the FSH receptor was down-regulated in both males and females (Pocar et al., 2012).

Androgenic Effects. Androgens, such as testosterone, are critical for the masculinization of the brain. The anti-androgenic properties of DEHP do not appear to involve the androgen receptor (AR). DEHP does not act as an AR antagonist (Gray et al., 1994; Kelce et al., 1994), and neither DEHP nor MEHP show an affinity for AR (Parks et al., 2000; Takeuchi et al., 2005). Instead, the anti-androgen effects of DEHP may be related to the disruption of Sertoli and Leydig cell function during sensitive developmental periods. Treatment with DEHP during early developmental periods led to a 35 % decrease in the number of Sertoli cells (Dostal et al., 1988) and disrupted Leydig cell function (Jones et al., 1993; Parks et al., 2000). Leydig cells are essential for the synthesis of testosterone, whereas Sertoli cells play a critical role in brain masculinization and defeminisation by converting testosterone into estradiol via the aromatase enzyme. A reduction in the number or in the functioning of Leydig and Sertoli cells can lead to altered testosterone, and estradiol levels, which may have critical consequences for the masculinization of the brain.

A number of studies have reported decreased serum testosterone levels in animals following treatment with DEHP (Akingbemi et al., 2001; Borch et al., 2004; 2006; Ge et al., 2007; Noreiga et al., 2009; Parks et al., 2000). In one study, a 60-85% reduction in testosterone

production was reported following DEHP treatment, such that testosterone levels in males were reduced to levels seen in females (Parks et al., 2000). There is a slight discrepancy in the literature at what dose DEHP decreases testosterone production, with some studies reporting reductions at low (10 mg/kg) and high doses (200 mg/kg; Akingbemi et al., 2001; Borch et al., 2004; 2006; Ge et al., 2007), while others have demonstrated effects at only high doses (300 and 900 mg/kg; Noreiga et al., 2009). Additionally, it appears the timing of DEHP exposure is very important in determining how testosterone production will be affected. Akingbemi et al., (2001) have shown that maternal exposure to DEHP reduced testosterone production in male offspring at postnatal days 21 and 35, but at postnatal day 90 the testosterone production was equivalent to male controls. Postnatal treatment with DEHP for 14 days also reduced testosterone levels in males, but when animals were exposed for 28 days the testosterone levels increased (Akingbemi et al., 2001).

The production of testosterone in Leydig cells is regulated by LH – a hormone released from the anterior pituitary gland. DEHP treatment can alter LH levels and thus, can indirectly affect testosterone production (Akingbemi et al., 2001). Timing also appears to be central in how LH levels will be altered following DEHP treatment. Maternal and acute postnatal (14 days) exposure to DEHP reduced LH levels in males, while longer postnatal exposure periods of 28 days led to increased LH levels in males (Akingbemi et al., 2001). In a more recent study, mRNA levels of LH were significantly up-regulated and LH receptors were down-regulated at low doses of DEHP following maternal and postnatal exposure in males and females (Pocar et al., 2012).

Neurotoxicity

Experiments that investigated the effects of phthalate exposure on brain development are limited, and will be discussed in the subsequent chapters. While the number of research studies

examining the neurotoxic effects of phthalates has been increasing in recent years, there are still a lot of unanswered questions regarding their toxic potential. Thorough investigations that evaluate the effects of phthalate exposure on neurobiology will be critical in understanding the neural and molecular mechanisms which contribute to phthalate-induced changes in the developing brain.

Synthesis

The above overview of neuroplasticity, lipids, learning and memory, and phthalates provides a background for understanding the studies outlined in the subsequent chapters. These studies explored the effects of acute postnatal exposure to phthalates on neurodevelopmental and behavioural correlates in juvenile and young adult rats. All studies shared a common goal: to determine the toxic potential of phthalate treatment during a sensitive period of development, along with assessing possible vulnerabilities to phthalate exposure within each gender.

Chapter II

Acute Postnatal Phthalate Exposure Disrupts Hippocampal Development in Juvenile Male Rats

The brain is an incredibly plastic organ that can be modified by environmental experience in developing and mature organisms (Citri & Malenka, 2008). The developing brain has an increased capacity for modification by the environment given that rapid structural and functional changes are taking place at this time. Fully developed neurocircuitry maintains the capacity to be altered by environmental experiences; however, these adjustments are often more restricted (Greenough et al., 1987; Shonkoff et al., 2009).

There are two competing perspectives regarding the impact of plasticity on developing organisms (Anderson et al., 2011). From one perspective, early developmental plasticity in the brain is viewed as beneficial and is associated with the successful adaptation to adverse environmental experiences. That is, the developing brain is more flexible and thus, is capable of recovering from insults more successfully than the mature brain (Anderson et al., 2011). There have been documented medical cases of children sustaining severe brain injuries, including the removal of one cerebral hemisphere, but exhibiting no permanent deficits in cognitive functioning (Ballantyne et al., 2008; Dennis & Whitaker, 1976; Smith & Sugar, 1975).

The opposing perspective regards plasticity in the immature brain as a vulnerability (Anderson et al., 2011). Developing organisms are viewed as more susceptible to environmental insults given the increased risk of disruption to developmental processes in the brain. This phenomenon has also been documented in the literature, with reports of children showing poorer

outcomes than adults following brain injury (Anderson & Moore, 1995; Hessen et al. 2007). This chapter explored the effects of acute exposure to the environmental toxicant, phthalate, in developing and young adult rats from both early plasticity and early vulnerability perspectives.

Phthalates have been measured in individuals across all developmental stages with compelling evidence showing that infants and children have a greater exposure to these chemicals than adults (Koch, et al., 2004; Wormuth, et al., 2006). Wormuth et al. (2006) calculated daily exposure to eight different phthalates across the lifespan and reported that exposure levels were highest in infants (birth to 12 months) and toddlers (one to three years) when compared to all other age groups. In another study, at least one phthalate metabolite was detected in the urine of all infants, with seven or more detected in the urine of 81% of infants (Sathyanarayana, et al., 2008). It has been estimated that exposure to phthalates is twice as high in children as adults, with 40 % of children (age two to six) showing higher urinary concentrations of phthalate metabolites than the maximum concentration measured in adults (Koch et al., 2004).

Animal toxicity studies have indicated that exposure to phthalates early in life results in severe disorders in the developing male reproductive system, including defects in the external genitalia, undescended testes and testicular lesions (see Lyche et al., 2009; Swan, 2008 for review); however, much less is known about the effects of phthalates on brain. Adult male and female rats, and adult male mice that were exposed to chronic oral doses of DEHP showed significant increases in brain weight at high doses (rats: 789-938.5 mg/kg/day; male mice: 1266.1 mg/kg/day). Only male mice showed a decrease in brain weight at chronic low doses of DEHP (19.2 mg/kg/day). The brain weight of female mice was unaffected by DEHP exposure (David et al., 2000a; 2000b). In contrast, Rhodes et al., (1986) observed no differences in brain weight in phthalate-exposed and control marmoset monkeys, suggesting that changes in brain weight may be restricted to rodents.

A number of alterations in the physiology and chemistry of the brain have also been reported in rats following DEHP exposure. Adult rats exposed to DEHP or DEHP metabolites showed significantly reduced activity in membrane Na^+/K^+ -ATPase that coincided with neuronal degeneration (Dhanya et al., 2003; 2004). Na^+/K^+ -ATPase is an enzyme important for cation transportation (also referred to as the sodium-potassium pump) and when inhibited, can lead to neuronal cell death (Xiao et al., 2002). Dhanya et al. hypothesized that the neuronal degeneration observed in these studies may be mediated by disruptions to ion homeostasis (such as increased intracellular levels of Ca^{+2}) caused by the inhibition of Na^+/K^+ -ATPase activity. In another study, researchers showed that intracellular levels of Ca^{+2} are affected upon DEHP exposure. Ca^{+2} levels in neurosecretory and pheochromocytoma cells increased quite rapidly; within minutes of DEHP administration (Tully et al., 2000).

Perinatal exposure to DEHP at low doses (0.135 mg/kg) also suppressed aromatase enzyme activity in the hypothalamic/preoptic area in one-day-old male rat pups, whereas higher doses (1.215 and 5 mg/kg) had no effect (Andrade, et al., 2006). In one-day old females, DEHP exposure did not affect aromatase activity, suggesting phthalate exposure may differentially affect the brains of developing males and females. At PND22, females showed an increase in aromatase activity at all phthalate doses, but in males only one of the lower doses (0.405 mg/kg) led to an increase in activity (Andrade et al., 2006). The aromatase enzyme is critical for the masculinization of the brain via the conversion of testosterone into estradiol in the brain. Thus, reductions in the activity of this enzyme may have drastic consequences for brain masculinization during neurodevelopment, resulting in a more feminized version of the brain.

A number of other important processes in the brain undergo widespread changes in early, postnatal stages of life, including extensive connectivity-based changes in the hippocampus

(Holahan et al., 2007). Mossy fiber projections to the CA3 hippocampal region develop rapidly between PND18 and PND21 in Long Evans rats. By PND24, the axonal staining patterns to the SO of the CA3 reflect connectivity patterns observed in adulthood (Holahan et al., 2007). These striking changes in hippocampal connectivity correspond with the emergence of enhanced performance on spatial learning and memory tasks between PND19 and PND20, indicating a sensitive developmental period for hippocampal connectivity and cognitive function (Keeley et al., 2010). Exposure to adverse environmental experiences, such as phthalates, during this sensitive period of hippocampal development may pose a greater risk of disruption to normal hippocampal connectivity than environmental insults occurring after hippocampal development is complete.

Objectives and Hypotheses

The purpose of the current study was to examine the effect of acute phthalate exposure on hippocampal morphological outcomes in developing rats during a period of significant hippocampal development (PND16 to PND22), and in young adult rats long after hippocampal development was complete (PND68 to PND74). It was hypothesized that postnatal phthalate exposure during a sensitive developmental period would produce more extensive disruptions in hippocampal neurobiology, with the possibility of differential effects between genders.

Materials and Method

Animals

Four untimed pregnant female Long Evans rats (approximately 13 days gestation) were purchased from Charles River Laboratories (St. Constant, Québec). The pregnant females were singly-housed in polycarbonate 48 x 26 x 20 cm³ cages within a temperature controlled

environment. The day the pups were born was recorded as PND0. Pups (n = 20 males; n = 20 females) were weaned on PND22 and group-housed, with males and females in separate cages. All rats were on a 12 hour light-dark cycle (lights on at 8:00 a.m.) with *ad libitum* access to food (Purina rat chow) and tap water. All experiments were conducted at Carleton University and approved by the Institutional Animal Care Committee as per guidelines established by the Canadian Council on Animal Care.

DEHP Injections

DEHP (Sigma-Aldrich; St. Louis, MO, USA) or vehicle (saline) was injected i.p. daily into awake rat pups from PND16 to PND22 inclusive (n = 5 males; n = 5 females) or into adult rats from PND68 to PND74 inclusive (n = 5 males; n = 5 females). Each rat was injected in the late morning (between 10:30 and 11:00 AM) and was returned to their home cage following the injection. Rats were randomly assigned to treatment (DEHP) and control (vehicle) groups counterbalanced across all litters. The 10 mg/kg DEHP solution was prepared using DEHP (1000 mg/kg) and 0.9 % NaCl (physiological saline) immediately before each injection. This dose was chosen based on previous reproductive toxicology findings that doses as low as 10 mg/kg lead to decreased testosterone production (Akingbemi et al., 2004). Rats (n = 5 juvenile males; n = 5 juvenile females; n = 5 adult males; n = 5 adult females) receiving vehicle injections were injected with 0.9 % NaCl.

Tissue Processing

Rats were euthanized 4 days after the final DEHP injection. Rats injected postnatally were euthanized on PND26 (n = 10 males; n = 10 females) and rats injected as young adults were

euthanized on PND78 (n = 10 males; n = 10 females) with an overdose of Dorminal (0.2 mL of 50 mg/kg). Figure 2.1 shows a timeline of the experimental procedures. Brains were extracted and postfixed overnight in 4 % paraformaldehyde/ 0.01 M phosphate buffer. Brains were then cryoprotected in 30 % sucrose/ 0.01 M phosphate buffer solution (PBS) and stored at 4 °C until sectioning. Brains were sectioned into 60 µm coronal sections using a Leica CM1900 cryostat. Sections were taken from the dorsal hippocampus and stored in a 0.1 % sodium azide solution in PBS at 4 °C.

Synaptophysin Immunofluorescent Labeling

Sections from the dorsal hippocampus for each group were chosen and labeled for synaptophysin (to mark presynaptic terminals). Sections were washed in 0.1%/Triton-X/ 0.01 M PBS (T-PBS), pH 7.4, for 15 minutes then transferred to a blocking solution (1x animal-free blocker (AFB) in T-PBS; Vector Laboratories, Burlingame, CA, USA) for 60 minutes to prevent non-specific binding. Tissue was incubated in a rabbit polyclonal primary antibody directed against synaptophysin (1:2500; Millipore; Billerica, MA, USA) overnight in AFB/T-PBS at room temperature. Sections were washed the following day for 15 minutes in T-PBS and incubated for 2 hours at room temperature with a fluorescently-labeled (Alexa Fluor 594) goat anti-rabbit secondary antibody (1:500; Invitrogen; Burlington, ON, Canada) in AFB/T-PBS. Sections were rinsed for 15 minutes in PBS, mounted onto glass slides, and coverslipped using Fluormount (Sigma-Aldrich) mounting medium. Slides were stored at 4 °C in slide boxes to protect from light exposure.

Immunohistochemistry

Dorsal hippocampus sections were stained for doublecortin (DCX) and calbindin-D28K. DCX is a microtubule-associated protein expressed by neuronal precursor cells and immature neurons (Rao and Shetty, 2004) and calbindin is a calcium-binding protein that plays a critical role in synaptic communication and calcium regulation in the brain (Kojetin et al, 2006).

Sections were washed three times in T-PBS for 5 minutes per wash then incubated in 0.3% hydrogen peroxide/ T-PBS for 30 min to remove endogenous peroxidase activity. Sections were washed three more times in T-PBS for 5 minutes per wash, transferred into AFB/ T-PBS for 60 minutes, then incubated overnight at room temperature in goat polyclonal anti-doublecortin primary antibody (1:200; Santa Cruz Biotechnologies; Santa Cruz, CA, USA) in AFB/ T-PBS. The same procedure was followed for calbindin staining using 0.1 %/Triton-X/ 0.01 M tris-buffered saline (T-TBS). Tissue was incubated in goat polyclonal anti-calbindin primary antibody (1:200; Santa Cruz Biotechnologies; Santa Cruz, CA, USA) in AFB/ T-TBS overnight.

The following day for both stains, tissue was washed three times in T-PBS for 5 minutes per wash then incubated for 2 hours with a biotinylated donkey anti-goat secondary antibody (1:100; Vector Laboratories) in AFB/ T-PBS. Sections were washed three times for 5 minutes each wash in T-PBS then incubated in an avidin-biotin complex (ABC; Vector Laboratories) in T-PBS for 2 hours. Sections were rinsed three times in PBS for 5 minutes each and staining was visualized with a 0.5 %, 3,3'-diaminobenzene (Sigma-Aldrich) solution. Juvenile tissue sections were enhanced with 1 % cobalt chloride and 1 % nickel ammonium sulfate. Adult tissue sections were only visualized with a 0.5 %, 3,3'-diaminobenzene solution. Sections were washed in PBS for five minutes then mounted onto glass slides.

Adult tissue sections were not counterstained. Juvenile DCX sections were counterstained with pyronin Y (Sigma-Aldrich) the following day. Slides with tissue sections were rinsed with distilled water for 2 minutes then submerged in a pyronin Y solution for 2 minutes. Slides were immersed in distilled water for 2 minutes to remove excess stain then dehydrated in 70 %, 95 % and 100 % ethanol for 1, 5 and 10 minutes, respectively. Slides were transferred to Clearene for a minimum of 15 minutes before being coverslipped with Permount (Sigma-Aldrich) hardset mounting medium.

Cresyl Violet Staining

Sections containing the dorsal hippocampus were float mounted on microscope slides. Each slide with mounted sections was submerged in the following series of solutions for 2 minutes each: 100 %, 95 % and 70 % ethanol. Sections were rinsed in distilled water to remove excess ethanol and placed in 1 % cresyl violet solution in distilled water for 3 minutes. Sections were rinsed in distilled water to remove excess stain and immersed in a 0.8 % acetic acid solution in distilled water until fiber tracks became unstained (about 3-5 minutes). They were then placed in 70 %, 95 % and 100 % ethanol for 2 minutes each. Sections were placed in Clearene solution for a minimum of 15 min before they were coverslipped with Permount mounting medium.

Immunofluorescent Quantification

Fluorescent images of the CA3 region were captured at 10 x magnification using a Retiga-2000R camera and an Olympus BX61 microscope. Coronal sections were sampled from the dorsal hippocampus of each rat and averaged across each brain. Approximate bregma levels were -3.14 to -3.30. Area measurements for each region of interest were taken as averages from both coronal

levels. The areas of the SL and the SO were estimated by outlining the synaptophysin-positive region. Synaptophysin-positive staining was defined as having an intensity value twice that of the background as measured on the CA3 stratum radiatum region. Comparisons between groups were made using the ratio of the area in SO to that in SL to account for size variations between individual rats. An experimenter who was blind to group assignment carried out all analyses.

Immunohistochemical Quantification

Images of the DG granule cell layer and CA1 and CA3 pyramidal cell layer were captured at 20 x magnification using an Olympus U-CMAD3 camera and an Olympus BX51 microscope with a motorized stage. Coronal sections were sampled from the hippocampus of each rat and averaged across each brain. Approximate bregma levels were (-4.16 to -4.30) for DCX, (-4.30 to -4.52) for calbindin, and (-3.60 to -3.80) for cresyl violet. The optical fractionator method (West et al., 1991) was used to estimate the total number of neurons. Each hippocampal region was outlined using the tracing function in the Stereo Investigator software at 10 x magnification. Counting parameters were set to a counting frame of 60 x 60 μm with 25 desired counting sites randomly distributed throughout the hippocampal region of interest, and a dissector depth of 10 μm centered between top and bottom guard zones. Neurons were counted at 60 x magnification (oil immersion) when the top of the cell body of stained neurons was in focus within the counting frame and the dissector depth. An experimenter who was blind to group assignment carried out all analyses.

The optical fractionator method generated two estimates of the total number of DCX-positive in the DG, total number of cells in the DG, CA1 and CA3 and the total number of calbindin-positive cells in the DG: the estimated total by mean measured section thickness and the

estimated total by number weighted section thickness neurons. Analyses were conducted using the estimate of total neurons by number weighted section thickness as it was a more accurate reflection of the total number of neurons. Final quantification values were represented as the total number of neurons per 10,000 μm^2 to adjust for slight variances between each rat.

Statistical Analyses

Independent-samples *t*-tests were conducted to compare differences in synaptophysin, DCX, cresyl violet and calbindin staining between vehicle- and DEHP-treated males and between vehicle- and DEHP-treated females at PND26 and PND78.

Results

DCX, Calbindin, and Cresyl Violet Staining in the DG

Figures 2.2A show representative images of DCX staining in the DG at PND26. There was no significant effect of treatment on the number on DCX positive neurons in the DG in females at PND26 (Figure 2.2B; $p > 0.05$) or at PND78 (Figure 2.2C; $p > 0.05$), or in males at PND78 (Figure 2.2C; $p > 0.05$). As shown in Figure 2.2B, males exposed to DEHP showed a significant reduction in DCX positive neurons in the DG when compared to vehicle-treated males at PND26 ($t(8) = 2.48, p < 0.05$). Figure 2.3A shows representative images calbindin-positive straining in the DG at PND26, and Figure 2.4A show representative images cresyl violet straining in the DG at PND26 and PND78. No differences were found between any groups in the number of calbindin-positive granule cells at PND26 (Figure 2.3B; $p > 0.05$) or in DG cell density at PND26 (Figure 2.4B; $p > 0.05$) or PND78 (Figure 2.4C; $p > 0.05$).

Synaptophysin and Cresyl Violet Staining in the CA3

Synaptophysin staining was delineated and measured in the CA3 SL and SO (microns squared; Figure 2.5). The ratio of staining in the SO to the SL (SO:SL) and area of staining (μm^2) in the SO and the SL were the basis of between group comparisons, where larger areas of SO staining signified increased axonal innervation to this region and larger ratios indicated more staining in the SO. No differences were observed between vehicle- and DEHP-treated females in the SO:SL ratio at PND26 or PND78 (Figure 2.5B and 2.5C; $p > 0.05$) or the area of SL and SO staining (Figure 2.5F and 2.5G; $p > 0.05$) at PND26 or PND78. In males, compared to vehicle-injected controls, DEHP treatment significantly reduced the SO:SL ratio at PND26 ($t(8) = 3.48$, $p < 0.01$; Figure 2.5B) and PND78 ($t(8) = 5.98$, $p < 0.001$; Figure 2.8C), and the area of staining in the SO at PND26 ($t(8) = 2.77$, $p < 0.05$; Figure 2.5D) and PND78 ($t(8) = 5.46$, $p < 0.001$; Figure 2.5E). There was no difference in the area of staining in the SL between vehicle- and DEHP-treated males at PND26 (Figure 2.5D; $p > 0.05$) or PND78 (Figure 2.5E; $p > 0.05$).

Figures 2.6A show representative images cresyl violet staining in the CA3 at PND26 and PND78. There were no significant differences in CA3 cell density in the female groups at PND26 (Figure 2.6B; $p > 0.05$) or PND78 (Figure 2.6C; $p > 0.05$) or in males at PND78 (Figure 2.6C; $p > 0.05$). The total number of cells was significantly reduced in males exposed to DEHP compared to vehicle-treated males at PND26 ($t(8) = 3.57$, $p < 0.01$; Figure 2.6B).

Cresyl Violet Staining in the CA1

Figures 2.7A show representative images cresyl violet staining in the CA1 at PND26 and PND78. No differences were found in the male or female groups at PND26 (Figure 2.7B; $p > 0.05$) or PND78 (Figure 2.7C; $p > 0.05$).

Discussion

The present study was the first of its kind to demonstrate that acute exposure to phthalates (DEHP) disrupted hippocampal connectivity in the rat. Phthalate-induced changes in the hippocampus were evaluated following DEHP treatment during a sensitive period of hippocampal development (PND16 to PND22), and during young adulthood (PND68 to PND74) long after hippocampal development was complete. Untreated male and female rats showed extensive synaptophysin-positive labeling in the CA3 region in the SO during postnatal development (PND26) and young adulthood (PND78), indicating significant axonal innervation to this hippocampal layer. When male rats were treated with DEHP either postnatally or as young adults, there was a reduction in axonal staining in the SO.

The hippocampal axonal terminal fields in CA3 undergo a period of widespread connectivity-based changes between PND18 and PND21 until stabilizing at PND24, showing elevated staining in the SO over this period of time (Holahan et al., 2007; Keeley et al., 2010). Postnatal exposure to DEHP between PND16 and PND22 likely disrupted the formation of normal hippocampal connections to the CA3 region in male rats resulting in reduced axonal innervation of the SO. The more interesting finding was that DEHP treatment during young adulthood also interfered with axonal innervation to the CA3 SO region in male rats. These rats were exposed to DEHP between PND68 and PND74, long after axonal innervation to the CA3 SO region was complete. The reduction in synaptophysin-positive labeling in the CA3 SO region in this group indicates that extensive remodelling and retraction of axonal projections to the SO had occurred following DEHP treatment. These findings suggest that hippocampal connectivity in the CA3 SO region may be particularly sensitive to environmental insults throughout the lifespan as acute

DEHP exposure interfered with proper CA3 SO connectivity not only during development, but long after development was complete.

Postnatal DEHP treatment in male rats also led to a reduction in cell density in the stratum pyramidale (SP) in the CA3 region compare to male controls. No differences in CA3 cell density were observed in male rats exposed to DEHP during young adulthood, indicating that while DEHP treatment interfered with connectivity in the CA3 region in adult rats, the disruptions to the CA3 region were more severe when rats were exposed to DEHP postnatally. A reduction in the number of DCX-positive cells (immature neurons) in the DG was also observed in male rats treated with DEHP during postnatal development, but not in male rats treated with DEHP during young adulthood. This reduction in developing male rats was specific to immature neurons in the DG – calbindin-positive granule cells (mature neurons) and overall cell density in the DG were not affected by DEHP exposure. Together, these findings reveal widespread interruptions in hippocampal neurobiology in male rats treated with DEHP during a sensitive period of postnatal hippocampal development. DEHP exposure during young adulthood resulted in much more limited disruptions to hippocampal connectivity, which was restricted to the CA3 hippocampal region. These findings support the theory that there is a period of increased sensitivity to environmental insults, like phthalates, in the hippocampus of developing male rats.

The increased vulnerability of male rats to DEHP treatment during postnatal development may be related to immature development of metabolic pathways in the gastrointestinal tract. Once DEHP enters the body it is rapidly metabolized into its monoester, MEHP, in the gastrointestinal tract by gut lipases (Frederiksen et al., 2007; Kluwe, 1982). MEHP is excreted in urine or hydrolyzed into one of four major secondary metabolites: 5OH-MEHP, 5oxo-MEHP, 5cx-MEPP, and 2cx-MMHP (Koch et al., 2006). The secondary metabolites are either excreted or conjugated

into glucuronic acid and then excreted (Frederiksen et al., 2007; Peck & Albro, 1982). Approximately 80 to 90% of DEHP secondary metabolites are conjugated into glucuronic acid in adults and then excreted (Peck & Albro, 1982). The glucuronidation pathway in children under three months is not fully developed, placing them at risk for higher levels of circulating DEHP and its metabolites in the body (Creteil, 1998) In addition, when compared to adults, infants have much lower levels of gut lipases necessary for metabolizing DEHP and thus, may not have the same capacity to metabolize and eliminate phthalates from their system (Lyche et al., 2009). Experimental studies which measure levels of gut lipases and DEHP metabolites are required to elucidate the role of DEHP metabolism in the increased sensitivity of developing organisms to DEHP exposure.

The CA1 hippocampal region did not show the same degree of damage from DEHP exposure; however, DEHP may still retard developmental processes in this region. Developing rats in the present study were treated with DEHP during an important window for CA3 development and thus, this region showed considerable sensitivity to postnatal DEHP exposure. Sensitive developmental processes for CA1 (and DG) connectivity occur earlier than the sensitive time period for CA3 development. Synaptic connections in the DG show dramatic changes from PND4 to PND11 (Cotman et al., 1973), whereas CA1 pyramidal neurons undergo a period of intense growth and dendritic expansion from birth to PND7 (Pokorny and Yamamoto, 1981). Quite likely, if rats were exposed to DEHP during these sensitive periods of DG and CA1 development, developmental abnormalities in these hippocampal regions would become apparent. Future investigations evaluating DEHP-induced alterations in hippocampal neurobiology following exposure at different developmental time points are necessary to determine whether the CA3 region has a unique sensitivity to DEHP treatment.

The most interesting finding in the present study was that male rats appeared to be more vulnerable to the effects of DEHP exposure than female rats. Unlike male rats, no differences in axonal distribution or cell density in the CA3 (as well as DG and CA1) were found between vehicle- and DEHP-treated females following the administration of DEHP postnatally or during young adulthood. This increased sensitivity to DEHP in male rats may be explained partly by its disruption of aromatase enzyme activity. The conversion of androgens, such as testosterone, into estradiol by aromatase is critical for the masculinization of the brain (McEwen et al., 1977; Naftolin et al., 1975). Researchers have shown that perinatal DEHP treatment suppressed aromatase enzyme activity in the brains of male rats (Andrade et al., 2006) and down-regulated CYP19a1 (the gene which encodes the aromatase enzyme) in the testes of mice (Pocar et al., 2012). Serum testosterone levels were also reduced following DEHP treatment in male rats (Akingbemi et al., 2001; Borch et al., 2004; 2006; Ge et al., 2007; Jones et al., 1993; Noreiga et al., 2009; Parks et al., 2000). These findings suggest a possible role for DEHP in the disruption of brain masculinization via the suppression of aromatase activity and/or reductions in serum levels of testosterone. Since testosterone and aromatase activity are critical for the masculinization of the brain, it is not surprising that males were considerably more sensitive to DEHP exposure than females.

Conclusion

Data from the current study showed for the first time that systemic treatment with 10 mg/kg phthalate during postnatal development (PND16-PND22) disrupted hippocampal neurobiology in the male rat. The CA3 hippocampal region was particularly sensitive to postnatal phthalate exposure, with male rats displaying reduced axonal innervation to the CA3 SO region and a

decrease in cell density in the CA3 SP region. A reduction in immature neurons in the DG was also observed in male rats treated postnatally with phthalates, indicating extensive disruptions to hippocampal neurobiology in this group. Male rats exposed to phthalates during young adulthood (PND68 to PND74) showed disruptions in CA3 hippocampal connectivity but these effects were limited to reductions in axonal innervation to the CA3 SO region. Given that more widespread alterations in hippocampal neurobiology were observed in male rats following postnatal phthalate treatment, these findings suggest that the hippocampus of developing male rats may be more susceptible to the neurotoxic effects of phthalates. No differences were observed in females exposed to DEHP during postnatal development or young adulthood indicating that males have a gender-specific vulnerability to phthalate exposure. These findings recommend more extensive animal studies on phthalate exposure during the vulnerable postnatal developmental period when rapid structural and functional changes are taking place.

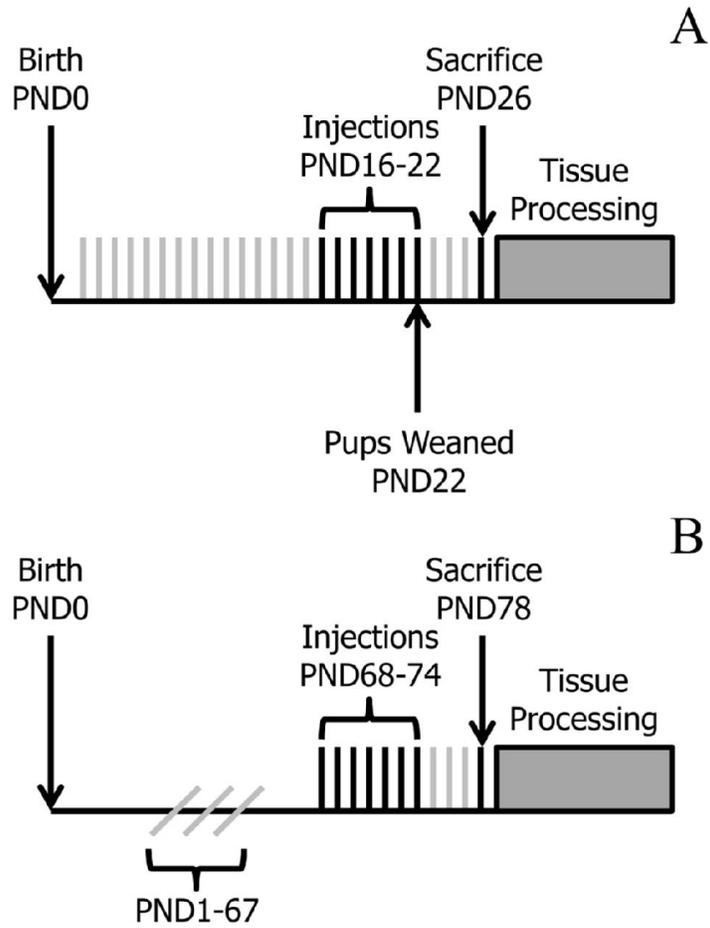


Figure 2.1: Timeline of experimental procedures. A timeline of di(2-ethylhexyl) phthalate treatment in (A) juvenile and (B) young adult rats. PND: Postnatal day; Injections: Injections of 10 mg/kg di(2-ethylhexyl) phthalate or vehicle.

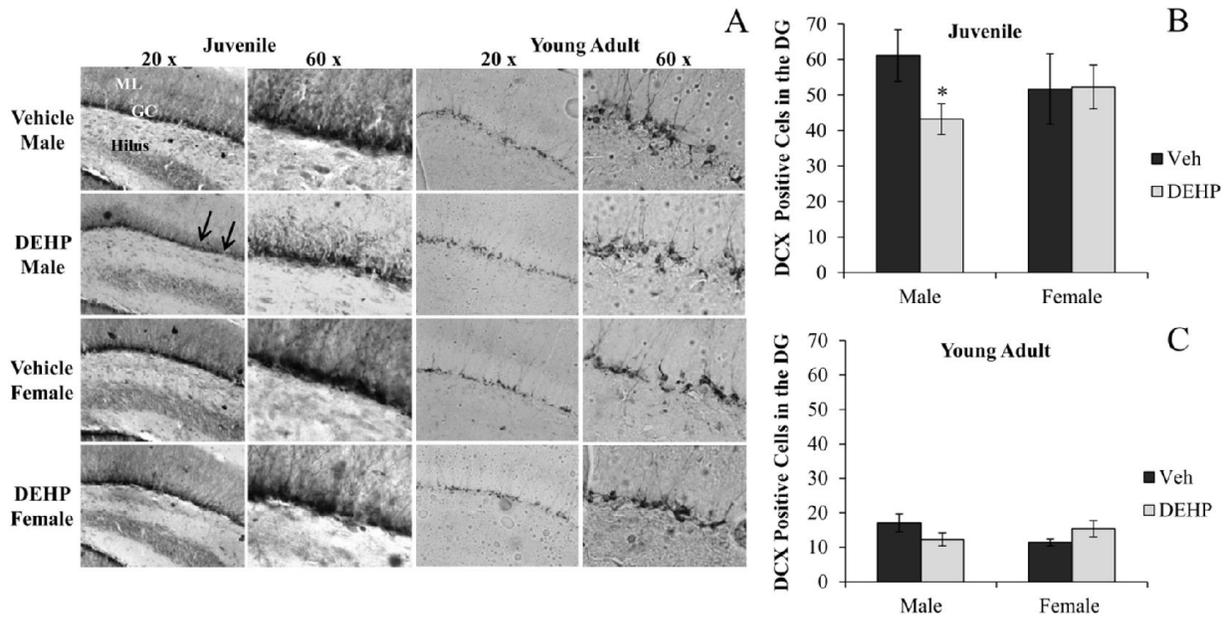


Figure 2.2: Doublecortin staining in the dentate gyrus. (A) Representative photomicrographs (20 x and 60 x) of doublecortin (DCX) staining in the dentate gyrus (DG). Quantification of the number of DCX-positive cells in the DG granule cell layer (GC) in male and female rats exposed to 10 mg/kg di(2-ethylhexyl) phthalate (DEHP) during (B) postnatal development and (C) young adulthood compared to controls (Veh). Male rats exposed to DEHP during postnatal development showed a significant reduction in DCX positive cells in the GC compared to controls indicated by downward arrows in image. There were no differences detected in the total number of DCX-positive cells in the DG of male rats treated with DEHP as young adults or female rats treated with DEHP as juveniles or young adults compared to controls. ML: molecular layer; * = $p < 0.05$. Error bars represent standard error of the mean.

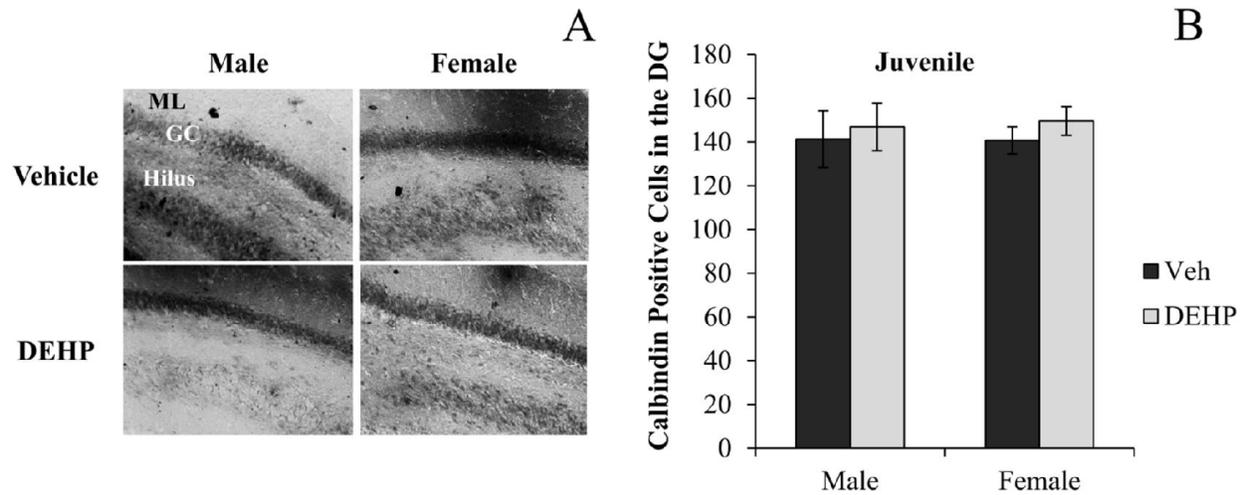


Figure 2.3: Calbindin staining in the dentate gyrus. (A) Representative photomicrographs (20 x) of calbindin staining in the dentate gyrus (DG). Quantification of the number of calbindin-positive cells in the DG granule cell layer (GC) in male and female rats exposed to 10 mg/kg di(2-ethylhexyl) phthalate (DEHP) during (B) postnatal development compared to controls (Veh). There were no differences detected in the total number of cells in the DG in male or female rats exposed to DEHP compared to controls. ML: molecular layer. Error bars represent standard error of the mean.

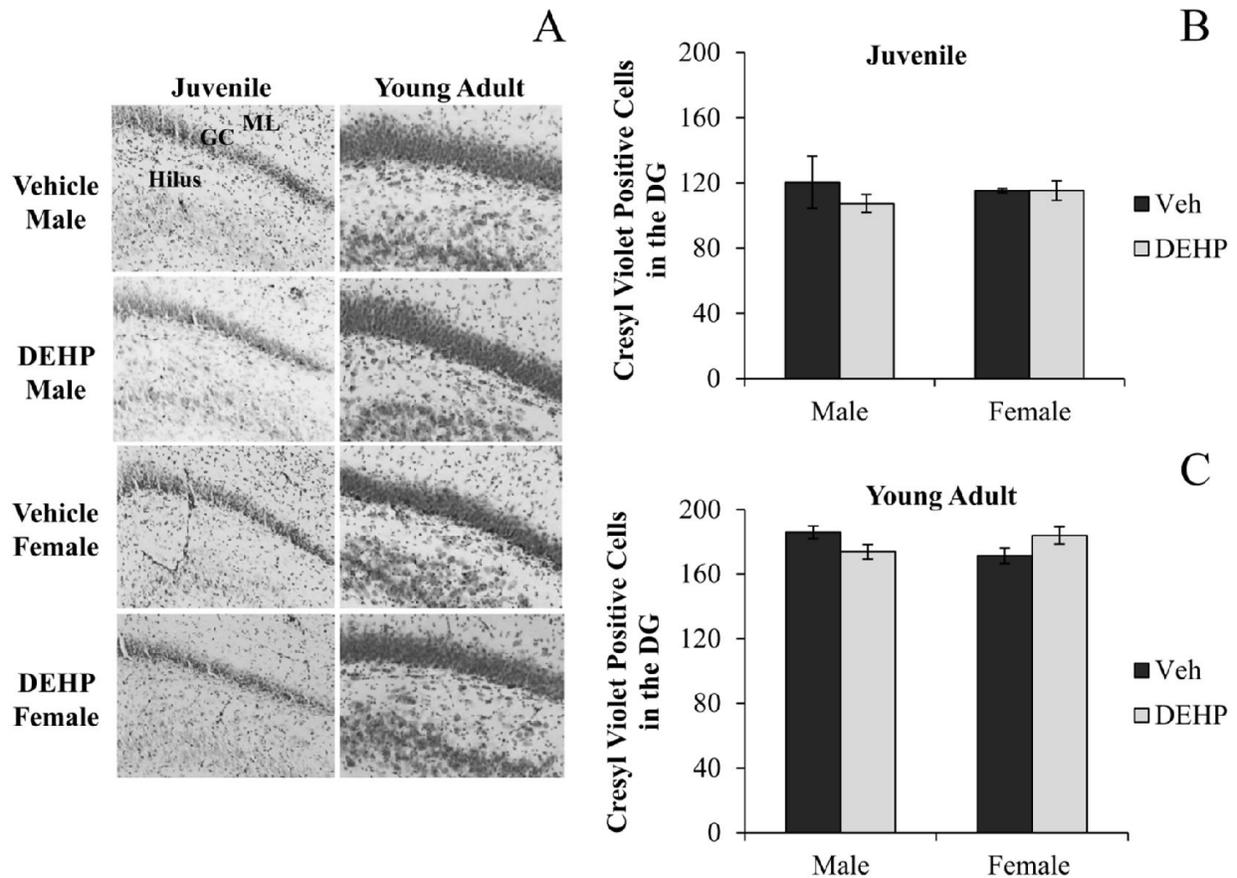


Figure 2.4: Cresyl violet staining in the dentate gyrus. (A) Representative photomicrographs (20 x) of cresyl violet staining in the dentate gyrus (DG). Quantification of the number of cell bodies in the DG granule cell layer (GC) in male and female rats exposed to 10 mg/kg di(2-ethylhexyl) phthalate (DEHP) during (B) postnatal development and (C) young adulthood compared to controls (Veh). There were no differences detected in the total number of cells in DG GC in male or female rats treated with DEHP as juveniles or young adults compared to controls. ML: molecular layer. Error bars represent standard error of the mean.

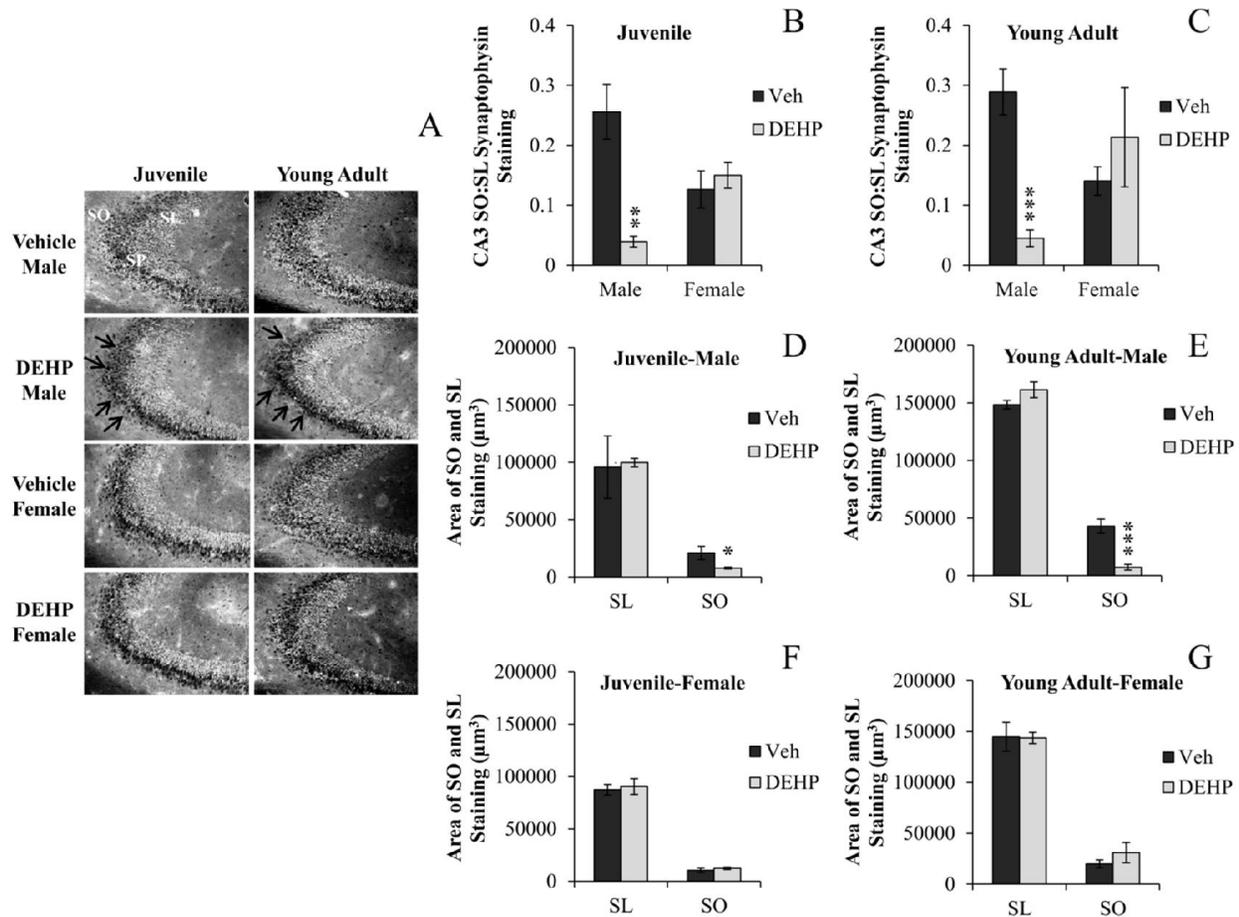


Figure 2.5: Synaptophysin staining in the CA3 region. (A) Representative photomicrographs of synaptophysin staining in the CA3 region (20 x). The ratio of synaptophysin staining in the SO to the SL quantified for male and female rats exposed to 10 mg/kg di(2-ethylhexyl) phthalate (DEHP) during (B) postnatal development and (C) young adulthood compared to controls (Veh). The area of synaptophysin staining (μm^2) in the stratum oriens (SO) and the stratum lucidum (SL) of the CA3 region quantified for male and female rats exposed to DEHP during postnatal development (D & F) and young adulthood (E & G). Male rats exposed to DEHP during postnatal development and young adulthood showed a significant reduction in the SO:SL ratio compared to controls indicated by downward arrows in image. There were no differences detected in the CA3 SO:SL ratio of female rats treated with DEHP as juveniles or young adults compared to controls. SP: stratum pyramidale; * = $p < 0.05$; ** = $p < 0.01$; *** = $p < 0.001$. Error bars represent standard error of the mean.

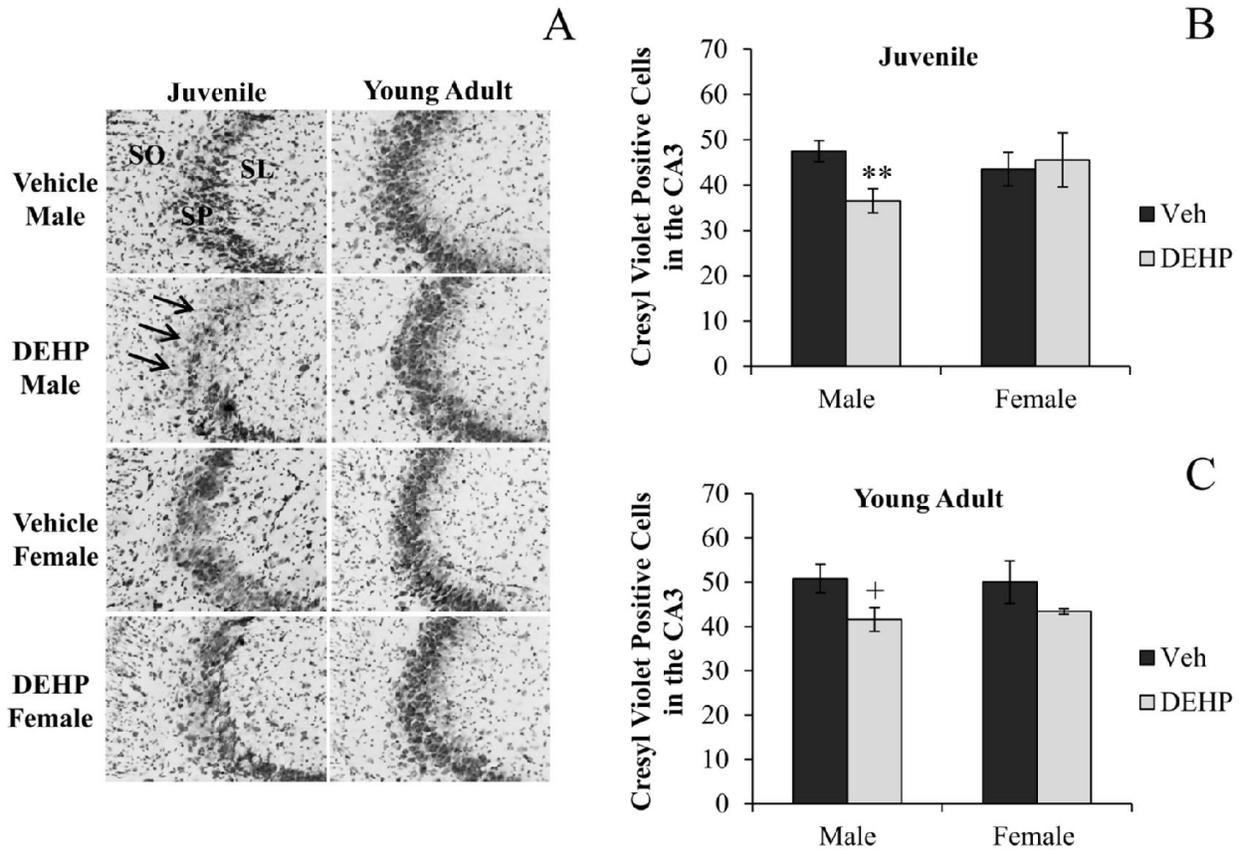


Figure 2.6: Cresyl violet staining in the CA3 region. (A) Representative photomicrographs (20 x) of cresyl violet staining in the CA3 region. Quantification of the number of cell bodies in the CA3 stratum pyramidale (SP) in male and female rats exposed to 10 mg/kg di(2-ethylhexyl) phthalate (DEHP) during (B) postnatal development and (C) young adulthood compared to controls (Veh). The total number of cells in CA3 SP was significantly reduced in male rats exposed to DEHP during postnatal development compared to controls. There were no differences detected in CA3 cell density in male rats treated with DEHP as young adults or female rats treated with DEHP as juveniles or young adults compared to controls. SO: stratum oriens; SL: stratum lucidum; * = $p < 0.05$. Error bars represent standard error of the mean.

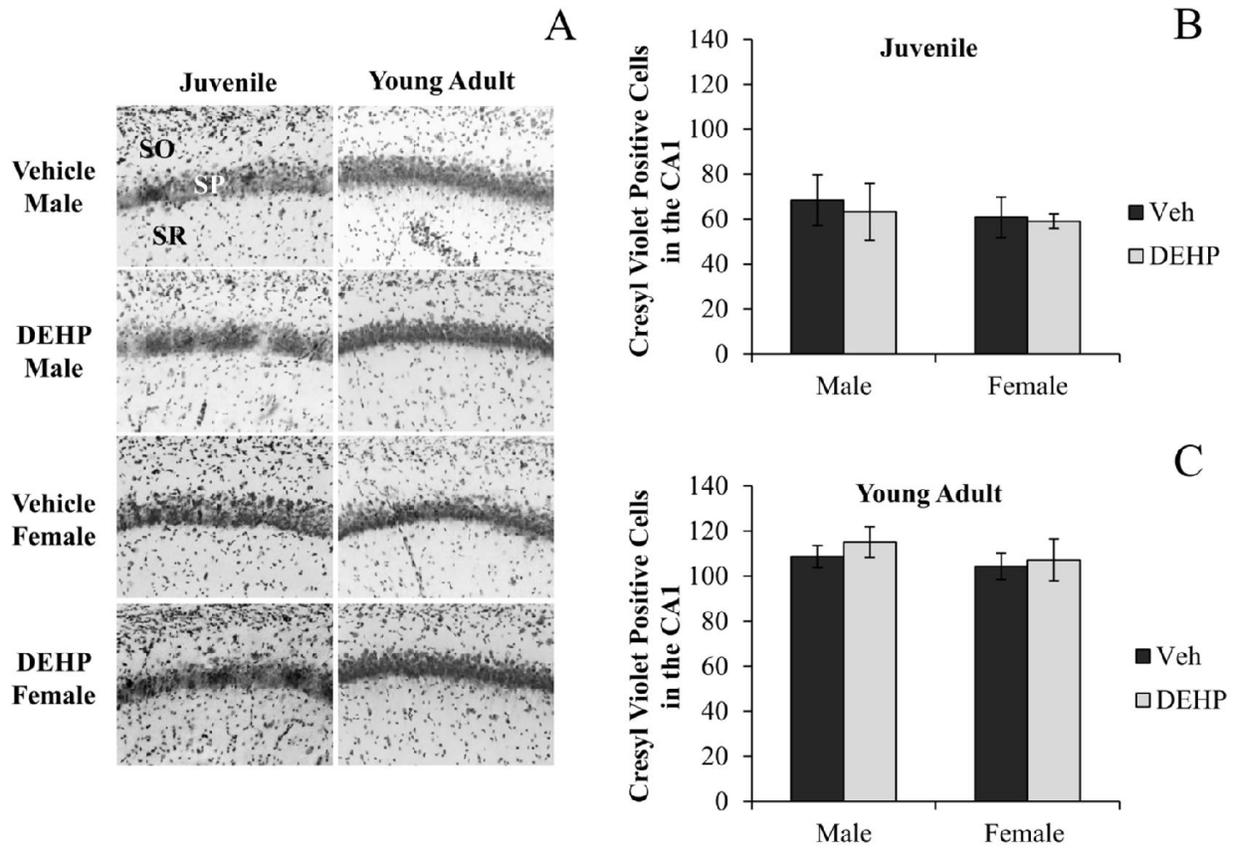


Figure 2.7: Cresyl violet staining in the CA1 region. (A) Representative photomicrographs (20 x) of cresyl violet staining in the CA1 region. Quantification of the number of cell bodies in the CA1 stratum pyramidale (SP) in male and female rats exposed to 10 mg/kg di(2-ethylhexyl) phthalate (DEHP) during (B) postnatal development and (C) young adulthood compared to controls (Veh). There were no differences detected in the total number of cells in CA1 SP in male or female rats exposed to DEHP compared to controls. SO: stratum oriens; SR: stratum radiatum. Error bars represent standard error of the mean.

Chapter III

Reduced Hippocampal Dendritic Spine Density and BDNF Expression in Juvenile Male Rats Following Acute Postnatal Exposure to Phthalates

In Chapter II, extensive abnormalities in hippocampal neurobiology following DEHP treatment during postnatal development (days 16-22) in male rats were found. Acute DEHP treatment of the same length in adult rats did not have as severe of an effect on hippocampal neurocircuitry providing support for 1) a sensitive period of hippocampal development between PND16-PND22 in rats, and 2) the idea that developing organisms may have a unique vulnerability to phthalate exposure. This chapter will expand on the experiment presented in Chapter II by evaluating the consequences of acute postnatal phthalate exposure on neuroconnectivity in the hippocampus of male and female developing rats.

Experimental investigations exploring structural-based alterations in hippocampal connectivity in the rat following early developmental phthalate treatment are limited. Systemic treatment with 10 mg/kg di(2-ethylhexyl) phthalate (DEHP) during postnatal development led to a marked reduction in synaptophysin staining (a protein critically involved in synapse formation and maintenance) in the CA3 stratum oriens dorsal hippocampal region of male rats, indicating decreased axonal innervation to this region (Chapter II). A reduction in synaptophysin protein expression, along with disruptions in synaptic ultrastructure (such as loss of synapses and a wider synaptic gap), were observed in the hippocampus of male and female rats following perinatal

dibutyl phthalate (DBP) exposure (Li et al., 2013a). These studies indicate early developmental exposure to phthalates may interfere with proper synaptic development in the dorsal hippocampus. The formation and maintenance of synaptic connections in developing organisms is critical for proper dendritic arborisation, and the failure to form these connections during development can disrupt dendritic outgrowth (Frotscher et al., 2000; McAllister, 2000; Tailby et al., 2005).

Connectivity-based changes in the brain are likely mediated through coordinated increments in growth factors and decrements in anti-growth factors. Brain-derived neurotrophic factor (BDNF) is a growth factor which plays an important role in neuronal and synaptic development, including the stimulation of dendritic and axonal growth in hippocampal neurons (Danzer et al., 2002; Horch, 2004; Lowenstein & Arsenault, 2006). The exogenous application of BDNF increased axonal growth in cultured dentate granule cells (Qiao et al., 2001). Conversely, decreased hippocampal BDNF *in vitro* and *in vivo* may directly reduce dendritic spine outgrowth in hippocampal neurons (Kellner et al., 2014; Zagrebelsky & Korte, 2014). The effect of early developmental phthalate exposure on BDNF expression in the brain has not been thoroughly investigated. Only two studies have examined the relationship between phthalate treatment and BDNF levels, and have produced contradictory findings. Perinatal treatment with 500 mg/kg of DBP decreased BDNF expression in the hippocampus (Li et al., 2013a). Conversely, perinatal treatment with a higher dose of DBP (675 mg/kg) increased levels of hippocampal BDNF expression (Li et al., 2010). These studies suggest a possible role for phthalates in regulating hippocampal BDNF expression.

BDNF also provides support to neurons to promote their survival (Han et al., 2000). The protective effect of BDNF against cell apoptosis is largely through the suppression of caspase-3 activity (Han et al., 2000). *In vitro* treatment with low doses of DEHP enhanced caspase-3 protein

expression and reduced the viability of Neuro-2a, MVLN, and AGS cell lines (Lin et al., 2011; 2013; Mankidy et al., 2013). Exposure to mono(2-ethylhexyl) phthalate (MEHP; the primary metabolite of DEHP) *in vitro* also reduced U937 cell viability, increased caspase-3 and BAX (a pro-apoptotic protein) expression, and decreased Bcl-2 (an anti-apoptotic protein) expression (Yokoyama et al., 2003). MEHP treatment has also been found to initiate apoptosis in the testes of rats and mice via increased caspase-3 expression, oxidative stress, and Fas-signalling activation (Dalgaard et al., 2001; Kasahara et al., 2002; Richburg et al., 1999; 2000).

In the brain, postnatal exposure to low doses of DEHP has been shown to reduce the density of mature and immature dorsal hippocampal neurons in the male rat, suggesting heightened cell apoptosis in the hippocampus (Chapter II). Perinatal DBP exposure stimulated caspase-3 activity in rats and significantly reduced the density of mature hippocampal neurons (Li et al., 2013a). Other apoptotic markers, including TUNEL and Annexin V-propidium iodide, were also up-regulated in the hippocampus; further supporting the idea of enhanced cell apoptosis following early developmental exposure to phthalates (Li et al., 2013a).

Objectives and Hypotheses

The primary goal of this work was to further characterize the impact of postnatal phthalate exposure on dorsal hippocampal development in juvenile rats. Hippocampal dendritic morphology (branching, spine density) and mRNA expression of BDNF and caspase-3 were evaluated in male and female juvenile rats following repeated postnatal exposure to 10 mg/kg DEHP. It was expected that DEHP exposure would reduce dendritic branching and spine density, and increase BDNF and caspase-3 mRNA expression in the hippocampus. A secondary goal was to evaluate DEHP mediated effects across gender. Previous work has suggested that male rats may be more

vulnerable to early developmental DEHP exposure (Chapter II) and thus, it was expected that these alterations in hippocampal dendritic morphology and mRNA expression would be more pronounced in males than in females.

Materials and Method

Animals

Four untimed pregnant female Long Evans rats (approximately 13 days gestation) were purchased from Charles River Laboratories (St. Constant, Québec). The pregnant females were singly-housed in polycarbonate 48 x 26 x 20 cm³ cages within a temperature controlled environment. The day the pups were born was recorded as PND0. Pups (n = 20 males; n = 20 females) were weaned on PND22 and group-housed, with males and females in separate cages. All rats were on a 12 hour light-dark cycle (lights on at 8:00 a.m.) with *ad libitum* access to food (Purina rat chow) and tap water. All experiments were conducted at Carleton University and approved by the Institutional Animal Care Committee as per guidelines established by the Canadian Council on Animal Care.

DEHP Injections

DEHP (Sigma-Aldrich; St. Louis, MO, USA) or vehicle (corn oil) was injected intraperitoneal (i.p.) into awake rats daily from PND16 to PND22 inclusive (n = 10 males; n = 10 females). Each rat was injected in the late morning (between 10:30 and 11:00 AM) and was returned to their home cage with their mother following the injection. Rats were randomly assigned to treatment (DEHP) and control (vehicle) groups counterbalanced across all litters. The 10 mg/kg DEHP solution was prepared fresh using DEHP (1000 mg/kg) and corn oil immediately before

each injection. This dose was chosen based on previous neurotoxicology findings showing abnormal hippocampal development in male Long Evans rats following acute postnatal treatment with 10 mg/kg DEHP (Chapter II). Rats (n = 10 males; n = 10 females) receiving vehicle injections were injected with corn oil.

Golgi-Cox Impregnation Method

Tissue Processing. Rats (n = 5 male DEHP; n = 5 female DEHP; n = 5 vehicle male; n = 5 vehicle female) were euthanized on PND26. Rats were euthanized via transcardial perfusion with 0.9 % physiological saline following overdose of Dorminal (0.2 ml of 50 mg/kg). Brains were extracted and prepared for Golgi impregnation using the Golgi-Cox technique. Immediately after extraction, brains were steeped in potassium dichromate, mercuric chloride, and potassium chromate solution (Golgi fix solution) for 4 days. Following this period, brains were washed 3 times in distilled H₂O (DH₂O): 4 hours, 3 hours and overnight, respectively. Brains were then cryoprotected in a graduated sucrose sequence: 10 % sucrose for 8 hours, 20 % sucrose overnight and 30 % sucrose for a minimum of 4 days. Dorsal hippocampal sections (200 µm) were obtained using a Vibratome and mounted onto gelatinized slides. The mounted sections were placed in a humidified, dark plastic box for 24 hours. Each slide with mounted sections was rinsed in DH₂O for 1 minute and submerged in 28 % Ammonium Hydroxide for 40 minutes. Sections were rinsed in DH₂O for 1 min and submerged in Kodak film fix A (diluted 1:1 with DH₂O) for 40 minutes. They were then rinsed two more times in DH₂O for 1 minute each before being immersed in 50 %, 70 % and 95 % ethanol for 1 min each. From this point on, all ethanol and Clearene solutions were desiccated with type 3A molecular sieve, 1/16” pellets. Sections were immersed three times in 100% ethanol for 5 minutes each then submerged in a 33 % ethanol, 33 % Clearene and 33 %

chloroform solution for 10 minutes. Sections were immersed two times in a Clearene solution for 15 minutes each before the slides were coverslipped with Permount mounting medium. The slides were then placed in a desiccated box for 4 days before morphological analysis.

Morphological analysis. Individual neurons were reconstructed at 100 x magnification on an Olympus BX51 microscope using Neurolucida software (MBF Bioscience Inc., Williston, VT). Neurons were selected at random from the dorsal hippocampal for each hippocampal region: DG, CA1 and CA3. Approximate bregma level -3.14 to -3.60 mm. A total of 3-5 neurons were traced for each hippocampal region per rat and were averaged across each region per rat. Neurons that were reconstructed were consistently impregnated and did not overlap with neighbouring cells. For all neurons, the cell body, apical and basal dendrites, and visible dendritic spines were reconstructed. Measurements recorded from each neuronal tracing included cell body area, the number of dendritic branch points, and dendritic spine density for both basal and apical dendrites. An experimenter who was blind to group assignment carried out all analyses.

Reverse Transcription-Quantitative Polymerase Chain Reaction (qPCR) Analysis

A second group of rats (n = 5 male DEHP; n = 5 female DEHP; n = 5 vehicle male; n = 5 vehicle female) were euthanized on PND26 by overdose (Dorminal; 0.2 ml of 50mg/kg). Brains were extracted and the dorsal hippocampus was collected from each rat. Tissue was placed in tubes on dry ice and stored at -80°C for later quantification of mRNA expression of genes of interest: BDNF and caspase-3, and a housekeeping gene: GAPDH.

Tissue was homogenized in Trizol and RNA was extracted as per instructions from commercially available PureLink RNA Micro Scale Kit (Thermo Fisher Scientific Inc., Catalog #: 12183016). The RNA was then reverse-transcribed into cDNA using SuperScript II reverse

transcriptase (Invitrogen, Burlington, ON, Canada). The resulting cDNA samples were analyzed via qPCR using a SYBR green detection protocol (Bio-Rad, CA, USA) and a MyiQ2 RT-PCR Detection System (Bio-Rad, CA, USA). All primer pairs generated 100-200 base pair amplicons and had a minimum efficiency of 90 %. The cycle threshold (Ct) of the housekeeping gene GAPDH was subtracted from the Ct of each gene of interest (Δ Ct) to normalize BDNF and caspase-3 expression levels. Δ Ct values were converted to mRNA fold changes using the $2^{-\Delta\Delta Ct}$ method (Livak & Schmittgen, 2001; Schmittgen & Livak, 2008). Primer sequences used: BDNF forward: GGACATATCCATGACCAGAAAGAAA, reverse: GCAACAAACCACAACATTATCGAG; caspase-3 forward: ATTGAGACAGACAGTGGAAC, reverse: GAGGAATAGTAACCGGGTG; GAPDH forward: GCCATCAACGACCCCTTCAT, reverse: CCGCCTGCTTCACCACCTTC.

Statistical Analyses

Morphological data from reconstructed neurons were analyzed using fixed factor analysis of variance (ANOVA) with gender (male and female) and treatment (DEHP and vehicle) as the fixed factors. Independent variables included cell body area, dendritic branching, and dendritic spine density in the DG, CA1, and CA3. Reverse transcription qPCR data were analyzed using fixed factor ANOVAs with gender (male and female) and treatment (DEHP and vehicle) as the fixed factors. P-values of less than 0.05 were considered statistically significant. Simple main effect follow-up analyses and pairwise comparisons were conducted using a Bonferroni correction to control from Type I error.

Results

No Effect of DEHP Treatment on Cell Body Size or Dendritic Branching

The effect of early developmental exposure to DEHP on cell body area and dendritic branching in the DG, CA1, and CA3 regions of the dorsal hippocampus was evaluated in male and female juvenile rats. No differences were observed in the total area of the cell bodies located in the DG, CA1 or CA3 between DEHP- and vehicle-treated rats of either gender ($p > 0.05$). The total number of branches on apical and basal dendrites of CA3 neurons, on apical and basal dendrites of CA1 neurons, and on basal dendrites of DG granule cells following DEHP exposure were not significantly different from vehicle controls in male or female rats ($p > 0.05$).

DEHP Exposure Reduced Dendritic Spine Density on CA3 Neurons in Male Rats

Figure 1A and 2A shows representative photomicrographs of spine density on apical and basal dendrites of CA3 neurons, respectively. Analyses revealed significant gender x treatment interactions of spine density on both apical ($F(1,16) = 5.90, p < 0.05$; Figure 1B) and basal dendrites ($F(1,16) = 11.03, p < 0.01$; Figure 2B). Simple main effects and pairwise comparisons revealed reductions in CA3 apical and basal spine density between DEHP-treated male rats and male controls ($F(1,16) = 9.14, p < 0.01$; $F(1,16) = 15.46, p < 0.001$; respectively). DEHP treatment did not alter dendritic spine density in apical or basal dendrites of CA3 neurons in female rats ($F(1,16) = 0.17, p = 0.69$; $F(1,16) = 0.59, p = 0.46$; respectively). Figures 3A, 4A, and 5A show representative photomicrographs of spine density on apical and basal dendrites of CA1 neurons, and on basal dendrites on DG granule cells, respectively. No differences in spine density on basal or apical dendrites of CA1 neurons or basal dendrites on DG granule cells were found

between DEHP-treated male and female rats when compared to controls of the same gender ($p > 0.05$; Figure 3B; 4B; 5B).

DEHP Exposure Reduced Hippocampal BDNF mRNA Expression in Males

There was a main effect of DEHP treatment ($F(1,16) = 4.90, p < 0.05$), but no effect of gender on BDNF mRNA expression in the hippocampus of juvenile rats (Figure 6). This reduction in BDNF transcripts was restricted to male rats, with a 53.2% reduction in BDNF mRNA expression in DEHP-treated male rats compared to male controls ($F(1,16) = 7.25, p < 0.05$). No alterations in BDNF mRNA expression were found between DEHP-treated and control female rats, indicating male rats may be more sensitive to postnatal DEHP exposure ($F(1,16) = 0.19, p = 0.67$). No differences were observed in casapase-3 mRNA expression between DEHP-treated male and female rats compared to controls of the same gender ($p > 0.05$; Figure 7).

Discussion

The present study evaluated alterations in dorsal hippocampal dendritic morphology, and BDNF and caspase-3 mRNA expression following exposure to a low dose of DEHP during early postnatal development in male and female rats. DEHP exposure did not alter cell body size or the number of dendritic branches on DG, CA1 or CA3 neurons in male or female rats. Structural changes in dendritic complexity were specific to reductions in spine density on CA3 apical and basal dendrites in DEHP-treated male rats compared to controls. No differences in spine density were observed in male rats in the DG or the CA1, and no differences in spine density were observed in any dorsal hippocampal region in female rats.

DEHP-induced reductions in CA3 spine density in male rats may have profound consequences for hippocampal connectivity. Synaptic innervation of the CA3 dorsal hippocampal region occurs through three distinct pathways: the perforant pathway, the mossy fiber pathway and the recurrent collaterals within the CA3 itself (Henze et al., 1996; Witter, 2007). In the perforant pathway, information is relayed through projections from the entorhinal cortex to distal CA3 apical dendrites located in the stratum lacunosum-moleculare hippocampal layer. Mossy fiber synaptic inputs (from DG granule cells) terminate on more proximal CA3 apical dendrites in the stratum lucidum, whereas recurrent collaterals from CA3 neurons project back to the CA3 region, synapsing on basal dendrites in the stratum oriens, and mid-apical dendrites in the stratum radiatum (Henze et al., 1996; Witter, 2007). In the present study, a reduction in spine density was observed throughout the entire CA3 dendritic tree, including CA3 basal dendrites and proximal, mid, and distal CA3 apical dendrites following DEHP treatment in male rats. These findings suggest that early developmental exposure to DEHP may lead to a widespread reduction in synaptic connections to the CA3 from multiple brain regions in male rats, including the DG, the entorhinal cortex, and the CA3.

Structural changes at synapses, including dendritic spine formation, have a direct influence on synaptic function (Edwards, 1995) and thus, it is conceivable that this reduction in dendritic spine density in DEHP-treated male rats may also lead to a corresponding decrease in synaptic efficacy. In a recent experiment, the effect of perinatal DBP exposure on synaptic function of rat CA1 neurons was investigated (Li et al., 2013a). DBP treatment reduced the slope and the amplitude of field excitatory postsynaptic potentials in CA1 neurons, indicating a decrease in synaptic function (Li et al., 2013a). Electrophysiological experiments evaluating synaptic function in DG, CA1 and CA3 hippocampal neurons following DEHP exposure are recommended. It is

expected that a similar decline in synaptic efficacy would be observed in the CA3 due to the decrease in spine density.

The reduction in spine density on CA3 neurons in male DEHP-treated rats parallels our previous findings indicating that postnatal exposure to DEHP decreased axonal innervation in the dorsal CA3 distal stratum oriens of male rats (Chapter II). Abnormal hippocampal synaptic ultrastructure, including the loss of synapses, was also found following perinatal DBP treatment in male and female rats (Li et al., 2013a). These findings highlight a pattern of abnormal hippocampal connectivity and development following phthalate treatment, establishing a link between early developmental exposure to phthalates and considerable reduction in hippocampal synaptic complexity.

No differences in dendritic spine density were observed in the DG or CA1 of DEHP-treated male or female rats compared to their respective controls. This finding is partially consistent with previous studies that revealed significant changes in hippocampal morphology in the DG and CA3, but not the CA1 (Chapter II). The rats in the present study were exposed to DEHP between PND16-22, a period of significant dorsal CA3 hippocampal development (Holahan et al., 2007). The peak developmental time period for the dorsal CA1 hippocampal region begins at birth and continues until PND7 (Pokorny & Yamamoto, 1981). For the DG, the peak developmental time period is from PND4 to PND11 (Cotman et al., 1973). Since the CA1 and DG region had likely already fully developed at the time of DEHP exposure (PND16-22), perhaps this region was less sensitive to DEHP exposure and no significant alterations in hippocampal dendritic morphology were observed.

Dorsal hippocampal BDNF mRNA expression was also down-regulated by approximately 50% in male rats exposed to DEHP (10mg/kg). No differences in BDNF expression were observed

in female rats. The down-regulation of hippocampal BDNF mRNA expression in DEHP-treated male rats may represent a molecular mechanism mediating the reduction in dendritic spine density in DG and CA3 neurons. BDNF is important for dendritic outgrowth and the formation of new synaptic connections (Danzer et al., 2002; Horch, 2004; Lowenstein & Arsenault, 1996). These findings highlight the importance of investigating the probable connection between DEHP-induced down-regulation of BDNF (and other genes involved in dendritic/axonal growth) and structural-based alterations in hippocampal morphology.

BDNF also plays a critical role in regulating cell apoptosis via caspase-3 suppression (Han et al., 2000). DEHP-treatment did not change expression levels of hippocampal caspase-3 mRNA expression in male or female rats despite the significant down-regulation of hippocampal BDNF expression. This finding was unexpected as previous studies had reported phthalate-induced reductions in hippocampal cell density and the up-regulation of caspase-3 activity (Chapter II; Li et al., 2013a). In previous work from our lab using the same DEHP exposure procedure, decreased dorsal hippocampal cell densities were reported in DEHP-treated male rats on PND26 (Chapter II). It is possible that caspase-3 activity may have been up-regulated during the DEHP treatment days (PND16-22) leading to reduced CA3 hippocampal cell density. Once DEHP treatment ended on PND22, caspase-3 activity would have returned to baseline levels and thus, no differences in caspase-3 mRNA expression would have been detected when assessed on PND26.

This hypothesis is consistent with a previous study that showed increased caspase-3 expression only on days during which phthalate treatment occurred (Li et al., 2013a). Increased hippocampal caspase-3 expression (along with reductions in hippocampal cell density) at PND5 and PND21 were observed following exposure to DBP from gestational day 7 to PND21 in male and female rats (Li et al., 2013a). When caspase-3 expression was measured 5 weeks after the last

day of phthalate treatment, no differences in caspase-3 expression were present suggesting heightened caspase-3 activity may occur only in conjunction with simultaneous phthalate treatment. While it appears caspase-3 activity may return to normal levels once phthalate treatment is terminated, the long-term neurodevelopmental and behavioural consequences of phthalate-induced alterations in neurochemical signalling pathways, including caspase-3 and BDNF, are unknown and require further investigation.

Reductions in hippocampal dendritic spine density and BDNF mRNA expression were gender-specific. No differences in DG or CA3 dendritic spine densities or in BDNF mRNA expression were observed between DEHP- and vehicle-treated female rats, suggesting male rats may be particularly vulnerable to early developmental DEHP exposure. This finding is consistent with a previous study from our laboratory revealing alterations in dorsal hippocampal morphology following postnatal DEHP exposure in male, but not female rats (Chapter II). The vulnerability of male rats to DEHP exposure may involve the anti-androgenic properties of DEHP. Low doses of DEHP have been shown to suppress aromatase enzyme activity and reduce serum testosterone levels (similar to levels seen in females) in male rats (Akingbemi et al., 2001; Andrade et al., 2006; Borch et al., 2004; 2006; Ge et al., 2007; Noriega et al., 2009; Parks et al., 2000). DEHP and MEHP treatment in female rats has been associated with decreased levels of estradiol and progesterone (Davis et al., 1994ab; Lovekamp & Davis, 2001; Svechnikova et al., 2007).

Gonadal hormones are critically involved in the organization and maintenance of synaptic connections in the brain (Cooke & Woolley et al., 2004; Kovacs et al., 2003; Leranthe et al., 2003; 2004). There is evidence for sexual dimorphism in the regulation of spine synaptic density on dorsal CA1 pyramidal neurons in adult rats. There is an activational effect of estrogen and progesterone on CA1 spine synaptic density in female rats (Gould et al., 1990; Leranthe et al., 2000;

2002; Woolley & McEwen, 1992), but not male rats (Leranth et al., 2004). There is also an activational effect of androgens (testosterone and dihydrotestosterone) on CA1 spine synaptic density in both male and female adult rats (Kovacs et al., 2003; Leranth et al., 2003; 2004). Spine density in cultured CA3 pyramidal neurons in adult male rats is decreased following the application of estradiol (Tsurugizawa et al., 2005), but increased in the presence of testosterone or dihydrotestosterone (Hatanaka et al., 2009).

Estrogen and testosterone also appear to have a neuroprotective effect on cell survival. The application of estrogen to cultured hippocampal neurons from rat pups (gestational day 18) increased Bcl-xL (an anti-apoptotic protein) expression and reduced beta-amyloid-induced apoptosis (Pike, 1999). *In vivo*, estrogen deprivation (by ovariectomy) in adult female rats potentiated apoptosis in DG granule cells when compared to controls (Liu et al., 2001). Treatment with exogenous 17 β -estradiol reversed apoptosis in ovariectomized rats indicating low physiological levels of estrogens increases the susceptibility of DG granule cells to cell death (Liu et al., 2001). There was no added benefit of elevated 17 β -estradiol treatment in rats with normal physiological levels of estrogen (Liu et al., 2001).

The application of testosterone or dihydrotestosterone in cultured hippocampal neurons from rat pups (gestational day 17-19) attenuates beta-amyloid-induced apoptosis (Nguyen et al., 2005; Pike, 2001). The neuroprotective effects of testosterone and dihydrotestosterone can be blocked by preventing the phosphorylation of pro-apoptotic protein BAD (Nguyen et al., 2005). Conversely, elevated levels of testosterone (above normal physiological levels) promoted apoptosis in cultured human neuroblastoma cells while elevated levels of 17 β -estradiol had no effect (Estrada et al., 2006).

Together these findings suggest that abnormal levels of gonadal hormones may have profound consequences for spine growth and cell survival. Given the endocrine disruption properties of DEHP, it is possible that altered levels of these gonadal hormones may be contributing to reductions in CA3 synaptic connectivity and spine growth in male rats. This may also explain the gender-specific detriment of DEHP on the hippocampus of male rats, and suggest that altered testosterone and/or aromatase enzyme activity may be involved in DEHP-induced hippocampal dysfunction. Further investigation is required to determine whether these changes in hippocampal neurobiology in DEHP-treated male rats can be attributed to low levels of testosterone and/or the suppression of aromatase activity. Circulating levels of gonadal hormones in female rats will also need to be assessed to uncover possible explanations as to why DEHP treatment had no effect on female hippocampal neuroconnectivity.

Conclusion

The present results revealed that exposure to DEHP between PND16-PND22 in male rats reduced spine density on basal and apical dendrites of CA3 neurons compared to vehicle-treated males. Hippocampal BDNF mRNA expression was also down-regulated in male rats exposed to DEHP and may represent a molecular mechanism underlying the reduction in dendritic spine density. No differences in hippocampal spine density or hippocampal BDNF mRNA expression were observed in female rats treated with DEHP compared to controls, suggesting male rats may be more vulnerable to early developmental DEHP exposure. DEHP treatment did not affect hippocampal caspase-3 mRNA expression in male or female rats. These data provide initial evidence between early developmental exposure to DEHP and neurodevelopmental deficits in

male rats, as well as highlight the importance of elucidating molecular mechanisms of DEHP-induced neurodevelopmental dysfunction.

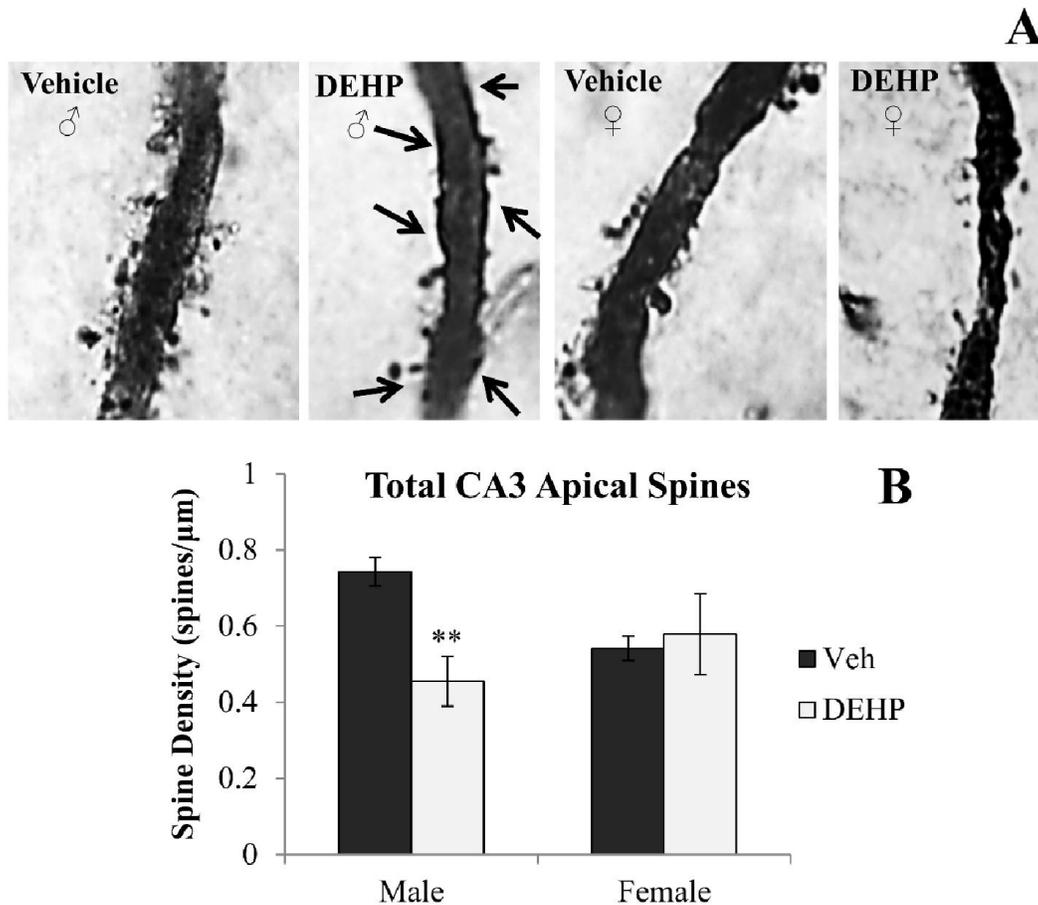


Figure 3.1: Spine density on apical dendrites of CA3 neurons. (A) Representative photomicrographs (100 x) of spine density on CA3 apical dendritic branches in the stratum lucidum. Quantification of the total number of apical dendritic spines per μm (B) in male and female rats exposed to phthalate (DEHP) compared to controls (Veh) of the same gender. Male rats exposed to DEHP showed a significant reduction in spine density on CA3 apical dendrites compared to male controls. Arrows in the DEHP-male image show regions of reduced dendritic spine density. There was no significant effect of DEHP treatment on CA3 apical spine density in female rats compared to controls. DEHP: di(2-ethylhexyl) phthalate; ** = $p < 0.01$. Error bars represent standard error of the mean.

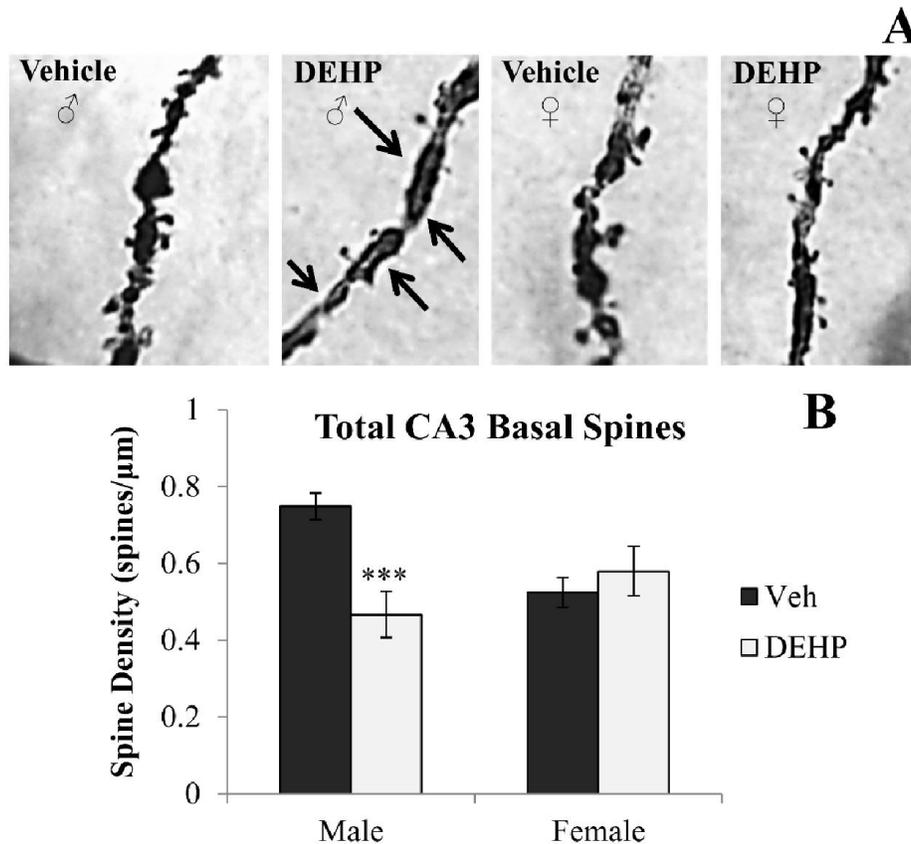


Figure 3.2: Spine density on basal dendrites of CA3 neurons. (A) Representative photomicrographs (100 x) of spine density on CA3 basal dendrites in the stratum oriens. Quantification of the total number of basal dendritic spines per μm (B) in male and female rats exposed to phthalate (DEHP) compared to controls (Veh) of the same gender. Male rats exposed to DEHP showed a significant reduction in spine density on CA3 basal dendrites compared to controls. Arrows in the DEHP-male image show regions of reduced dendritic spine density. There was no significant effect of DEHP treatment on CA3 basal spine density in female rats compared to controls. DEHP: di(2-ethylhexyl) phthalate; *** = $p < 0.001$. Error bars represent standard error of the mean.

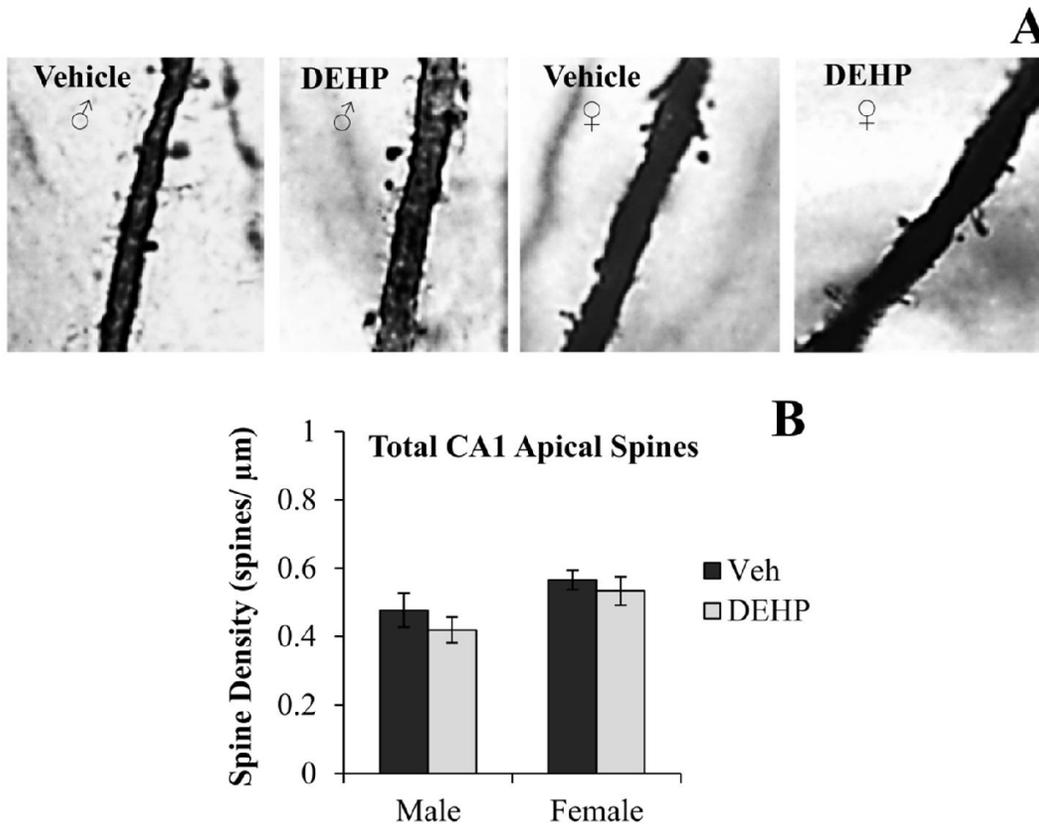


Figure 3.3: Spine density on apical dendrites of CA1 neurons. (A) Representative photomicrographs (100 x) of spine density on CA1 apical dendrites in the stratum radiatum. Quantification of the total number of apical dendritic spines per μm (B) in male and female rats exposed to phthalate (DEHP) compared to controls (Veh) of the same gender. There were no differences in CA1 apical dendritic spine density in male or female rats exposed to DEHP compared to controls. DEHP: di(2-ethylhexyl) phthalate. Error bars represent standard error of the mean.

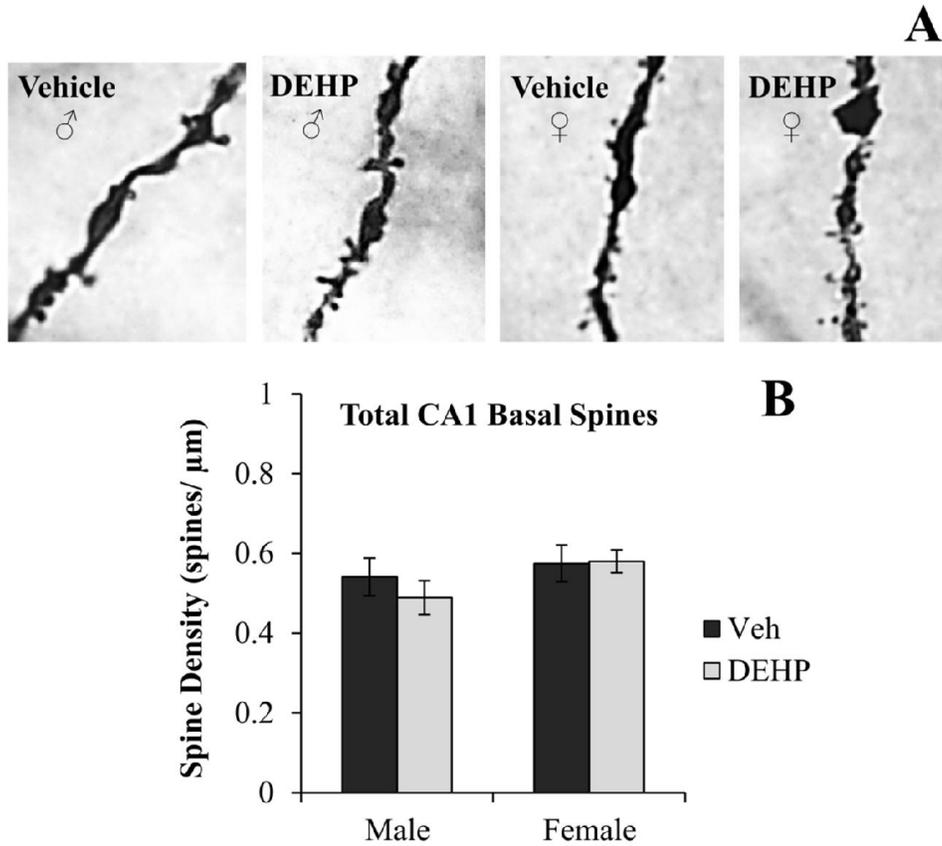


Figure 3.4: Spine density on basal dendrites of CA1 neurons. (A) Representative photomicrographs (100 x) of spine density on CA1 basal dendrites in the stratum oriens. Quantification of the total number of basal dendritic spines per μm (B) in male and female rats exposed to phthalate (DEHP) compared to controls (Veh) of the same gender. There were no differences in CA1 basal dendritic spine density in male or female rats exposed to DEHP compared to controls. DEHP: di(2-ethylhexyl) phthalate. Error bars represent standard error of the mean.

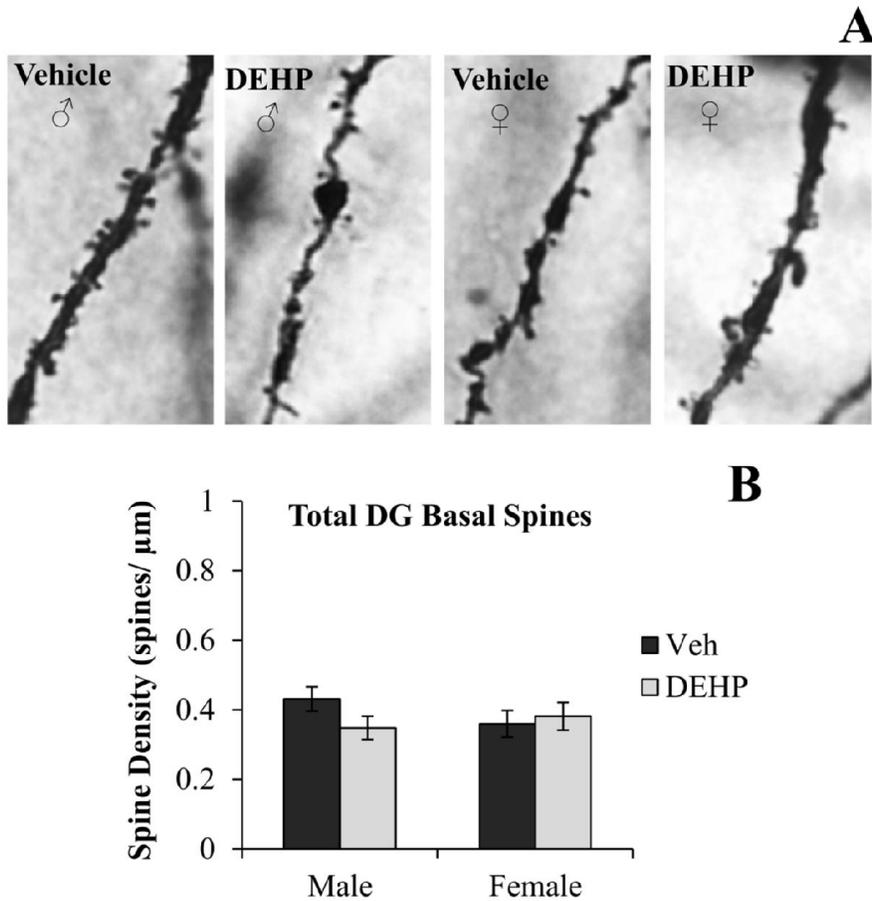


Figure 3.5: Spine density on basal dendrites of dentate gyrus granule cells. (A) Representative photomicrographs (100 x) of spine density on basal dendrites in the dentate gyrus (DG). Quantification of the number of basal dendritic spines per μm (B) in male and female rats exposed to phthalate (DEHP) compared to controls (Veh) of the same gender. There were no differences in DG basal dendritic spine density in male or female rats exposed to DEHP compared to controls. DEHP: di(2-ethylhexyl) phthalate. Error bars represent standard error of the mean.

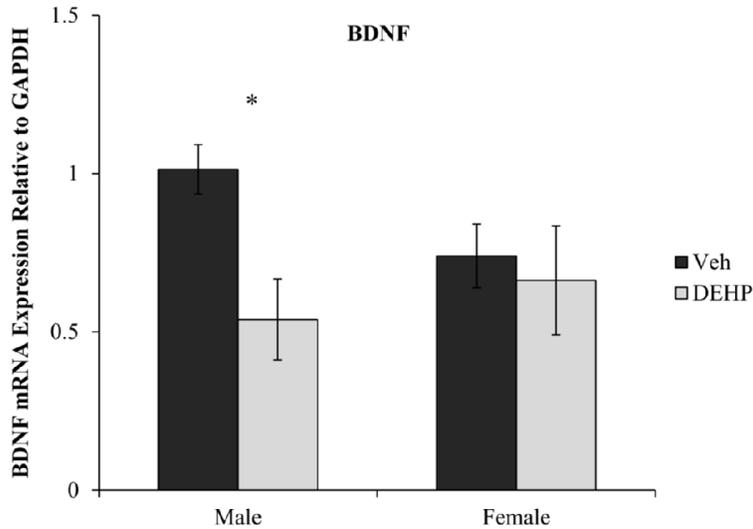


Figure 3.6: BDNF mRNA expression the dorsal hippocampus. Quantification of BDNF mRNA expression relative to housekeeping gene (GAPDH) in male and female rats exposed to phthalate (DEHP) compared to controls (Veh) of the same gender. Male rats exposed to DEHP showed a significant reduction in BDNF mRNA expression compared to controls. There was no significant effect of DEHP treatment on BDNF mRNA expression in female rats compared to controls. DEHP: di(2-ethylhexyl) phthalate; * = $p < 0.05$. Error bars represent standard error of the mean.

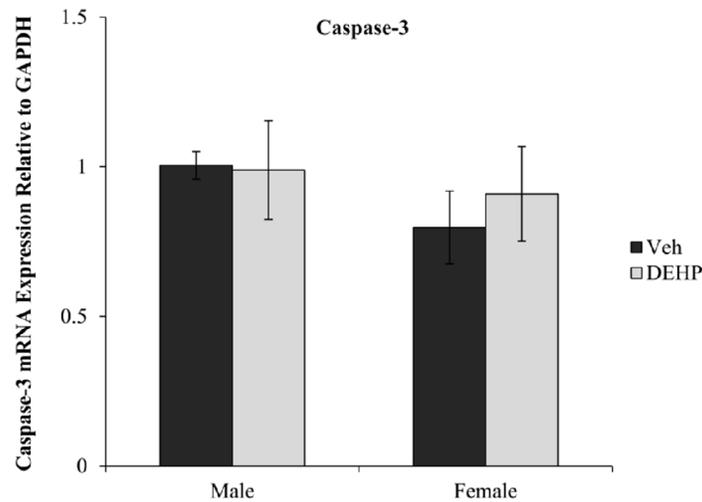


Figure 3.7: Caspase-3 mRNA expression the dorsal hippocampus. Quantification of caspase-3 mRNA expression relative to housekeeping gene (GAPDH) in male and female rats exposed to phthalate (DEHP) compared to controls (Veh) of the same gender. There were no differences in caspase-3 mRNA expression in male or female rats exposed to DEHP compared to controls. DEHP: di(2-ethylhexyl) phthalate. Error bars represent standard error of the mean.

Chapter IV

The Up-regulation of Hippocampal Lipids in Juvenile Female Rats Following Acute Postnatal Phthalate Treatment

In Chapters II and III we showed that postnatal exposure to DEHP interfered with normal hippocampal development selectively in male rats, included a reduction in the density of mature and immature hippocampal neurons, reduced CA3 dendritic spine density and axonal innervation, and decreased expression of hippocampal BDNF transcripts. The hippocampus of female rats was unaffected by postnatal exposure to DEHP. This chapter expanded on the findings discussed in Chapter II and III by examining the impact of early developmental DEHP exposure on hippocampal lipid profiles in developing rats.

Lipids are biological molecules essential for normal neurodevelopment and are involved in a wide variety of cellular processes, including cellular signalling, protein and receptor trafficking, neurogenesis, and myelination (Innis, 2003; Ramstedt & Slotte, 2002; Uauy & Mena, 2001). Slight changes in the abundance of certain lipid species in the brain may drastically alter normal neurodevelopment via membrane stability, cell signalling, and cell survival. Altered lipid composition in the brain has been reported following exposure to the environmental toxicant DEHP. A reduction in squalene, C₂₇ and C₃₀ sterols, free cholesterol esters, and sphingomyelin (SM) were detected in fetal rat brains following *in utero* exposure to DEHP (Bell et al., 1979; Xu et al., 2007). DEHP treatment also decreased the concentration of mono- and polyunsaturated fatty acids in fetal rat brains, and reduced the concentration of docosahexaenoic acid (DHA) in cholesterol esters, diacylglycerols, phosphatidylserines, lysophosphatidylcholines (LPC), and SM

(Xu et al., 2007). This study also found reduced concentrations of arachidonic acid (AA) in cholesterol esters and LPC lipids in fetal rat brains from DEHP-treated dams (Xu et al., 2007).

DEHP-induced changes in lipid composition have been more widely studied outside of the brain, particularly in the liver and gonadal organs (Bell et al., 1976; 1978ab; 1979; 1980; Mitchell et al. 1985; 1986; Yanagita et al., 1978). Phospholipid and free fatty acid content in the liver, as well as triglyceride (TG) content in the liver and kidneys were reduced in adult male rats exposed to dietary DEHP (Bell et al., 1976). Young male rats fed a DEHP diet had increased levels of hepatic phosphatidylethanolamine (PE), and decreased levels of hepatic phosphatidylcholine (PC) and TG that were detectable 1 day after DEHP exposure (Yanagita et al., 1978). In this study, DEHP treatment increased the concentration of AA, stearic, and oleic fatty acids in hepatic PC and TG. They also reported a decrease in the concentration of palmitic acid and DHA in hepatic PE, and a decrease in linoleic fatty acids in hepatic TG (Yanagita et al., 1978). Chronic maternal exposure to DEHP also decreased C₂₇ and C₃₀ sterols and the sterol precursor squalene in the liver of fetal rats (Bell et al., 1978a). These same lipids were also reduced in the liver and testes of adult male rats (Bell et al., 1979; Bell, 1980), and in the adrenal glands of male and female adult rats (Bell, 1980). A reduction in the concentration of plasma phospholipids, TG, and cholesterol was also reported in male rats following postnatal DEHP exposure (Bell et al., 1978ab; Yanagita et al., 1978).

Chronic dietary treatment with DEHP led to an accumulation of TG and of lipid-loaded lysosomes (or lipid droplets) in the livers of male and female adult rats (Mitchell et al. 1985). *In vitro* treatment with MEHP increased the presence of lipid droplets in rat hepatocytes and MA-10 Leydig cells suggesting increased lipid synthesis in these cells (Dees et al., 2001; Mitchell et al. 1986). Recent experiments have identified over 50 genes involved in lipid metabolism that were

up-regulated in rat embryo and human fetal gonad cultures treated with MEHP, including liver X receptor alpha (LXR α), sterol regulatory element-binding protein (SREBP) 1c, and SREBP2 (Muczynski et al., 2012; Robinson et al., 2012). MEHP exposure was shown to up-regulate the expression of LXR α which subsequently enhanced the expression of SREBP1c and SREBP2 – transcription factors important in regulating phospholipid, TG, and cholesterol synthesis. The up-regulation of SREBP1c and SREBP2 may represent a mechanism for increased lipid synthesis in MEHP-treated cell cultures (Muczynski et al., 2012).

Abnormal lipid synthesis may also be mediated by abnormal activation of peroxisome proliferator-activated receptors (PPAR) by DEHP and its metabolites (Xu et al., 2005; 2006). PPAR receptors play an important role in the regulation of lipid/essential fatty acid transporters and enzymes, as well as in the maintenance of lipid/essential fatty acid homeostasis (Lemberger et al., 1996). DEHP and its metabolites (MEHP and 2-ethylhexanoic acid) can bind readily to PPAR receptors– acting as an agonist and increasing the uptake, transport, and accumulation of essential fatty acids (Sharma et al., 1988; Xu et al., 2005; 2006).

Objectives and Hypotheses

The present study examined the effect of postnatal (days 16 – 22) DEHP exposure on male and female rat hippocampal development with the primary goal of establishing whether DEHP treatment altered the lipid profile in the hippocampus. It was hypothesized that postnatal DEHP exposure would decrease the composition of LPC, PC and SM in the hippocampus of male rats. No differences in lipid composition were expected between DEHP-treated female rats and female controls.

Materials and Method

Animals

Two untimed pregnant female Long Evans rats (approximately 13 days gestation) were purchased from Charles River Laboratories (St. Constant, Québec) for this experiment. The pregnant females were singly-housed in polycarbonate 48 x 26 x 20 cm³ cages within a temperature-controlled environment. The day the pups were born was recorded as postnatal day (PND) 0. Pups (n = 6 males; n = 6 females) were weaned on PND22 and group-housed, with males and females in separate cages. All rats were on a 12 hour light-dark cycle (lights on at 8:00 a.m.) with *ad libitum* access to food (Purina rat chow) and tap water. All experiments were conducted at Carleton University and approved by the Institutional Animal Care Committee as per guidelines established by the Canadian Council on Animal Care.

DEHP Injections

DEHP (Sigma-Aldrich; St. Louis, MO, USA) or vehicle (corn oil) was injected i.p. daily into awake rat pups from PND16 to PND22 inclusive (n = 3 males; n = 3 females). Each rat was injected in the late morning (between 10:30 and 11:00 AM) and was returned to their home cage with their mother following the injection. Rats were randomly assigned to treatment (DEHP) and control (vehicle) groups counterbalanced across all litters. The 10 mg/kg DEHP solution was prepared fresh using DEHP (1000 mg/kg) and corn oil immediately before each injection. This dose was chosen based on previous neurotoxicology findings showing abnormal hippocampal development in male Long Evans rats following acute postnatal treatment with 10 mg/kg DEHP (Chapter II and III). Rats (n = 3 males; n = 3 females) receiving vehicle injections were injected with corn oil.

Tissue Processing and Lipid Extraction

Rats were euthanized on PND26 by transcardial perfusion with physiological saline and brains were extracted, flash frozen in 100 % ethanol on dry ice and stored at -80 °C until use. The lipids were extracted using a modified Bligh and Dyer extraction protocol (Bligh & Dyer, 1959; Bonin et al., 2004). The entire hippocampus was dissected from all brains, transferred into a glass tube and kept on ice. Hippocampi were homogenized in 4.0mL of acidified methanol (2% acetic acid in methanol). Next, 3.2 mL of 0.1 M sodium acetate, 41.3 µL of 10 µM C13:0 LPC (an internal standard), and 3.8 mL of chloroform were added to the homogenate and centrifuged for 2 minutes at 4°C, 2000 RPM in a Sorvall ST 16R Centrifuge (Thermo Scientific). The chloroform layer (bottom phase) was retained and transferred to another glass tube. Another 2.0 mL of chloroform was added to the homogenate, inverted three times to gently mix the immiscible solvent layers, centrifuged for 2 minutes at 4 °C, 2000 RPM and the chloroform layer was retained. This was repeated two more times. The extracted chloroform layers were evaporated under a constant stream of nitrogen gas and the lipids were re-suspended in 300 µL of absolute ethanol. The sample was incubated at 30 °C for 10 minutes until the lipids were dissolved and then centrifuged for 1 minute at 4 °C, 2000 RPM to remove any remaining particles. The sample was transferred into an amber glass vial and stored at -20 °C (under nitrogen gas) until mass analysis. All samples were analyzed within 48 hours of the extraction.

High Performance Liquid Chromatography and Mass Spectrometry

Chromatography columns composed of fused silica with an inner diameter of 200 µm (Polymicro Technologies, AZ, USA) were cut to a length of 15 cm and then dipped in a 3:1 solution of Kasil 1678 potassium silicate and formamide (Promega, WI, USA) forming a frit on one end.

Twenty-four hours later, the fritted columns were cut to 10 cm and packed with 5cm of ReproSil-Pur C₄ size 5 µm stationary phase (Dr. Maisch GmbH, Germany) in acetone using a nitrogen pressure vessel. A PicoFrit Emitter (New Objective MA, USA) was packed with 1 cm of ReproSil-Pur C₄ in acetone and cut to a length of 5 cm.

Each lipid sample was analyzed in triplicate and was prepared immediately before each run (1 µL of sample, 5 µL of absolute ethanol, and 34 µL of deionized water). Samples were loaded into an UltiMate 3000 autosampler (Dionex, CA, USA) maintained at 4 °C. An UltiMate 3000 pump loaded 20 µL of the sample onto the C₄ chromatography column. All mobile phases were prepared using HPLC grade solvents: (A) 30 % methanol in 10 mM ammonium acetate, (B) isopropanol with 10 mM ammonium acetate and (D) hexane. Each lipid mass analysis was 60 minutes in length, and began with a 100 % A mobile phase. Four minutes later, solvent B was gradually increased until the mobile phase became 100 % B at the 45.5 minute mark where it remained at 100 % B for another 1 minute. At the 46.6 minute mark solvent D gradually increased until the mobile phase became 100 % D where it remained until the end of the run. Immediately after each lipid sample was analyzed a 140 minute hexane wash was run (90 % D and 10 % B). At the 130 minute mark, the mobile phase switched to 100 % B and then at 137 minutes switched to 100 % A. A 40 minute blank was run immediately following the wash cycle to check for carry over. The mobile phase began with 100 % A before gradually switching to 100 % B beginning at the 4 minute mark and ending at 28 minutes. The mobile phase remained at 100 % B for another minute before returning to 100 % A until the end of the run.

Chromatographically separated lipids were directly analysed using an AB Sciex QTRAP 4000 ESI-MS/MS Hybrid Triple Quadrupole/Linear Ion Trap (AB Sciex, Framingham, MA). The QTRAP was run in positive ion mode with the ESI nanospray voltage set at 3 kV, curtain gas at

20 and declustering potential at 25 V. A precursor ion scan and an enhanced mass spectrum (EMS) were conducted using a mass range of 250-1500 Da. The precursor ion scan was set to analyze precursors of m/z 184 with collision energy of 40.0 eV and collision cell exit potential of 10 V.

Data Analyses

Mass spectra were analyzed using Analyst Software version 1.5.1 (AB Sciex, Framingham, MA). The precursor m/z 184 scans conducted over the 60 minute gradient were manually analyzed to generate a list of PC and SM m/z -values. The m/z -values (± 0.2 Da) were entered into MultiQuant 2.1.1 (AB Sciex, Framingham, MA). All desired peaks were integrated and an output containing a variety of parameters, such as peak area and retention time, was generated. The peak area of each lipid was divided by the peak area of the internal standard C13:0 LPC (m/z 454) and then again by the total weight of the hippocampus to adjust for slight variances within each sample run and between samples, respectively. The peak areas of each PC and SM species were average between replicated analyses and standard deviations were calculated. The LIPID MAPS MS Prediction Tool (Lipidomics Gateway) and VaLID: Visualization and Phospholipid Identification prediction database were used to determine the classification of all lipid masses identified in the precursor m/z 184 scan (Blanchard et al., 2013).

Statistical Analyses

Statistical significance was determined using independent-samples t-tests to compare the peak areas of each identified lipid following exposure to vehicle or DEHP in male and female rats. Each gender was assessed separately: DEHP-treated males verses vehicle-treated males and DEHP-treated females verses vehicle-treated females.

Results

Each lipid sample ($n = 3/\text{group}$) was analyzed in triplicate for a total of 36 analyses. Eighty-seven lipid species were identified through manual inspection of the extracted ion chromatograms produced by the precursor m/z 184 ion scan: 15 were classified as LPC, 52 were classified as PC, and 20 were classified as SM. Figure 4.1 shows a heat map of all identified lipid masses common to every group (DEHP-treated and untreated, male and female rats) clustered into 3 categories based on their lipid classes (LPC, PC, and SM). The m/z -values (± 0.2 Da) of each identified lipid species were entered into MultiQuant 2.1.1 software program which automatically determined the chromatographic peak area of each species for each of the 36 analyses. In order to correct for small systematic variations between each run, the peak area of each lipid species was divided by the peak area of the internal standard (LPC C13:0; m/z 454) from the same run. The resulting ratios were defined as the normalized amounts of each lipid. This ratio was then divided by the weight (in grams) of the hippocampus for the respective sample in order to obtain a standardized ratio of the normalized amount of lipid per gram of hippocampus for each sample. Tables 1 and 2 list the predicted LPC, PC, and SM lipids and their changes in relative abundance per gram of hippocampus following DEHP treatment for males and females compared to controls of the same gender. LIPID MAPS MS Prediction Tool (Lipidomics Gateway) and VaLID lipid prediction software (Blanchard et al., 2013) were used to predict the most likely classification for each lipid mass identified in the precursor m/z 184 scan.

There was no significant effect of DEHP treatment on the relative concentration of total lipids, LPC, PC, or SM, in the hippocampus of male rats ($p > 0.05$; Figure 4.2A). DEHP-treated female rats showed a significant increase in total hippocampal lipids ($t(4) = 2.70, p < 0.05$), as well

as total SM ($t(4) = 2.89, p < 0.05$), and total PC ($t(4) = 2.62, p < 0.06$) lipids (Figure 4.2B). No differences were found in total LPC lipids ($p > 0.05$) in female rats (Figure 4.2B).

When each identified lipid species was analyzed separately, there was a significant increase in the relative amount of a single LPC lipid (m/z 468.3; $t(4) = 3.66; p < 0.05$) in DEHP-treated males compared to controls (Figure 4.3). No effect of DEHP treatment was observed on the relative abundance of any LPC lipid in females ($p > 0.05$; Figure 4.3).

No changes in the relative abundance of PC or SM lipids were observed in the hippocampus of male rats ($p > 0.05$; Figures 4.4A, 4.4C, and 4.5A). There was a significant increase in the relative amount of 10 PC lipids in DEHP-treated female rats compared to controls (Figure 4.4B and 4.4D): m/z 732.6, ($t(4) = 2.71; p < 0.05$); m/z 758.6, ($t(4) = 2.95; p < 0.05$); m/z 760.6, ($t(4) = 2.63; p < 0.06$); m/z 764.6, ($t(4) = 2.62; p < 0.06$); m/z 782.6, ($t(4) = 3.18; p < 0.05$); m/z 788.7, ($t(4) = 2.68; p < 0.06$); m/z 806.6, ($t(4) = 4.17; p < 0.05$); m/z 808.6, ($t(4) = 3.33; p < 0.05$); m/z 834.6, ($t(4) = 2.69; p < 0.06$); m/z 838.6, ($t(4) = 2.85; p < 0.05$). All 10 PC lipids contained unsaturated fatty acid side chains with at least 1 double bond; 6 PC species contained 4 or more double bonds. Total carbons of both fatty acid side chains ranged from 32 to 40.

DEHP-treated female rats also showed an increase in the relative abundance of 6 SM lipids compared to controls (Figure 4.5B): m/z 731.6, ($t(4) = 2.83; p < 0.05$); m/z 733.6, ($t(4) = 3.17; p < 0.05$); m/z 761.7, ($t(4) = 2.60; p < 0.06$); m/z 787.7, ($t(4) = 2.85; p < 0.05$); m/z 789.7, ($t(4) = 2.59; p < 0.06$); m/z 807.6, ($t(4) = 4.21; p < 0.05$). Of the 6 SM lipids, three contained a sphingosine base with fatty acid side chains ranging from 18 to 24 carbons; only 1 of the 3 fatty acid side chains was unsaturated. The remaining three SM lipids contained a sphinganine base with saturated fatty acid side chains ranging from 18 to 22 carbons.

Discussion

This experiment investigated the impact of acute postnatal exposure to DEHP on hippocampal lipid composition in male and female juvenile rats. Three categories of lipids (LPC, PC, and SM) were identified and their relative abundances in the hippocampus were quantified via HPLC separation and precursor ion scanning for m/z 184. DEHP treatment during a postnatal time period (PND16-PND22) resulted in the up-regulation of overall lipid content in the hippocampus of female rats compared to controls. No changes in overall lipid content were found in the hippocampus of male rats.

This increase in hippocampal lipids in DEHP-treated female rats is consistent with previous reports. Elevated lipid content and the up-regulation of genes involved in lipid metabolism were reported in isolated rat hepatocytes, MA-10 Leydig cells, rat whole embryo cultures (gestational day 10), and human fetal testes and ovaries following treatment with DEHP or MEHP (Dees et al., 2001; Mitchell et al. 1985; 1986; Muczynski et al., 2012; Robinson et al., 2012). The up-regulation of hippocampal lipids in DEHP-treated female rats may be mediated by the activation of PPARs by DEHP and its metabolites. PPARs are important regulators of lipid homeostasis (Lemberger et al., 1996) that can be found throughout the brain, particularly in the hippocampus (Cullingford et al., 1998; Michalik et al., 2002; Moreno et al., 2004). The activation of PPARs by DEHP has been shown to increase the uptake, transport, and accumulation of essential fatty acids (Sharma et al., 1988; Xu et al., 2005; 2006).

Postnatal DEHP treatment had no effect on total LPC content in male or female rats. The individual analysis of each identified LPC lipid revealed a significant two-fold increase in the relative abundance of a single LPC (m/z 468.3) in DEHP-treated male rats. This LPC contained a

single fatty acid side chain with 14 carbons and no double bonds. There were no significant differences in individual LPC lipid species in DEHP-treated female rats compared to controls.

Postnatal DEHP treatment also increased total hippocampal PC and SM lipid content in female rats. These findings were inconsistent with a previous study. Xu and colleagues (2007) reported DEHP-induced reductions in SM lipids and no changes in PC lipids in fetal rat brains. Methodological variations including different brain regions (hippocampus versus whole brain), different ages of the rats (juvenile versus fetal), different routes and times of administration (postnatal i.p. versus *in utero* exposure), and widely different doses used (10mg/kg versus 1500 mg/kg) between the present and previous studies may account for the discrepancy in experimental results.

Analyzing each lipid species independently identified 10 PC and 6 SM lipids in DEHP-treated female rats with a significant two-fold or higher increase in relative abundance. All 10 PC lipids contained unsaturated fatty acid side chains with at least 1 double bond and total number of carbons ranging from 32 to 40. Identifying PC lipid species is difficult as many fatty acid combinations can be made with the same number of carbons – especially for larger lipids. The most probable conformations for each PC lipid (as predicted by VaLID prediction software; Blanchard et al., 2013) are listed in Table 1. Five of the six SM lipids contained saturated fatty acid side chains with a total number of carbons ranging from 18 to 22. The remaining SM lipid had an unsaturated fatty acid side chain with 24 carbons and 4 double bonds. The most probable conformations for each SM lipid (as predicted by LIPID MAPS MS Prediction Tool; Lipidomics Gateway) are listed in Table 2. No differences in hippocampal PC or SM lipids were observed between DEHP-treated and vehicle-treated male rats.

The increase in PC and SM lipids in DEHP-treated female rats was unexpected as previous work showed a gender-selective detrimental effect of postnatal DEHP administration on hippocampal development in male rats (Chapters II and III). Developmental exposure to DEHP disrupted CA3 hippocampal connectivity (as shown by reductions in CA3 axonal innervation and dendritic spine density), and reduced the density of immature neurons in the DG and mature neurons in the CA3 of male, but not female rats. Perhaps the up-regulation of PC and SM hippocampal lipids in DEHP-treated female rats protect the female rat hippocampus from damage by early developmental DEHP exposure.

The neuroprotective effects of lipids have been well documented in the literature (see Bazan, 2005 and Chin & Bazan for review). Previous studies indicated that the down-regulation of sphingolipids (which include SM lipids) with C18-C24 fatty acid side chains were linked to the degeneration of cerebellar Purkinje and granule cells in the cerebellum, and the accumulation of lipofuscin and ubiquitin in the CA3 hippocampal region of mice (Imgrund et al., 2009; Zhao et al., 2011). These studies suggest that a decrease in the abundance of sphingolipids (which include SM lipids), particularly those with C18-C24 fatty acid side chains, may be detrimental to neuronal health. Therefore, the up-regulation of SM lipids with C18-C24 fatty acid side chains in the hippocampus of DEHP-treated female rats may protect against DEHP-induced reductions in hippocampal connectivity and cell density.

Conversely, the up-regulation LPC and PC lipids contribute to pathways leading to apoptosis (Meyer zu Heringdorf & Jakobs, 2007; Hochreiter-Hofford & Ravichandran, 2013; Kakisaka et al., 2012; Sun et al., 2009). *In vitro* treatment with LPC increased apoptotic morphology and Fas-signaling, and reduced cell viability of H19-7 hippocampal progenitor cells (Sun et al., 2009). In the present study, the up-regulation of a pro-apoptotic LPC lipid was observed

in developing male rats treated with DEHP. Elevated levels of this LPC lipid in DEHP-treated male rats may underlie their increased vulnerability to reductions in hippocampal cell density. This suggests a possible mechanistic role for LPC lipids in mediating DEHP-induced neurotoxicity in male rats.

Interestingly, unsaturated fatty acid-containing PC lipids were up-regulated in the hippocampus of female rats following postnatal DEHP treatment. The carbon-carbon double bonds in unsaturated PC lipids amplify the oxidization potential thereby increasing the vulnerability to apoptotic cell death (Esposti, 2002; Cantrel et al., 2009). The overexpression of anti-apoptotic protein bcl-2 in HL-60 cells increased saturated and decreased unsaturated fatty acids in phospholipids (which include PCs) providing protection against oxidation and promoting cell survival (Cantrel et al., 2009). It is possible that the combined effects of elevated PC and SM hippocampal lipids in DEHP-treated female rats may have a downstream protective effect on hippocampal neurons by promoting their survival. The up-regulation of anti-apoptotic promoting SM lipids may suppress the effects of elevated pro-apoptotic promoting PC lipids, protecting the hippocampus from DEHP-induced apoptosis in female rats. In DEHP-treated male rats there was no increase in anti-apoptotic SM lipids, but there were elevated levels of a single pro-apoptotic LPC rendering the hippocampus vulnerable to apoptotic cell death.

The presence of specific polyunsaturated fatty acids in lipids promotes neuronal survival by attenuating apoptosis (Bazan, 2005; Hoggies et al., 2003; Strokin et al., 2006; Wang et al., 2003; 2006). Increased concentrations of DHA in brain phospholipids play a critical role in hippocampal cell survival, protecting against hypoxia/ischemia-induced apoptosis (Strokin et al., 2006). Pretreatment with DHA protected cultured rat hippocampal tissue from glutamate cytotoxicity (Wang et al., 2003). Similarly, a derivative of DHA, neuroprotectin D1, promoted neuronal

survival after oxidative stress via the up-regulation anti-apoptotic proteins (Bcl-2 and Bcl-xL), the down-regulation pro-apoptotic proteins (BAX and BAD), and the suppression of caspase-3 activity (Bazan, 2005). Perinatal treatment with DHA also attenuated the neurodegeneration of cholinergic neurons and their axons following excitotoxic brain damage in juvenile rats (Hogyes et al., 2003). Pretreatment with AA reduced oxidative stress-induced neuronal cell death in cultured rat hippocampal tissue (Wang et al., 2006). The neuroprotective effect of DHA and AA is regulated by the activation of PPAR receptors (Adkins & Kelley, 2010; Wang et al., 2006). Given that DEHP and its metabolites can bind readily to PPAR receptors, these findings indicate a possible role for DHA and AA in modulating the effects of DEHP.

A previous study has shown *in utero* exposure to DEHP reduced the concentration of polyunsaturated fatty acids DHA and AA in membrane lipids (including LPC and SM lipids) in fetal rat brains (Xu et al., 2007). Decreased concentrations of DHA and increased concentrations of AA were also reported in liver of DEHP-treated male rats (Yanagita et al., 1978). In future studies it would be of interest to evaluate the effects of DEHP on fatty acid composition in the hippocampus to determine if changes in fatty acids act as potential mediators of hippocampal toxicity in DEHP-treated male rats, and hippocampal protection in DEHP-treated female rats.

Conclusion

Systemic treatment with 10 mg/kg DEHP between postnatal day 16 and 22 led to elevated levels of phosphatidylcholine and sphingomyelin in the hippocampus of female rats. There was no effect of DEHP exposure on the overall abundance of phosphatidylcholine or sphingomyelin in male rats, or of lysophosphatidylcholine in male or female rats. Individual analyses of each identified lipid species revealed 10 phosphatidylcholine and 6 sphingomyelin lipids in DEHP-

treated females, and a single lysophosphatidylcholine in DEHP-treated males with a two-fold or higher increase in relative abundance. These results are congruent with previous work that found that postnatal exposure to DEHP had a near-selective detrimental effect on hippocampal development in male, but not female rats. The up-regulation of hippocampal lipids may serve a neuroprotective role in DEHP-treated female rats and may underlie the resistance of the female rat hippocampus to modification by DEHP. These data represent a novel assessment of the toxic potential of phthalates in developing organisms and highlight the importance of evaluating the impact that chemicals commonly found in household products have on neurodevelopment.

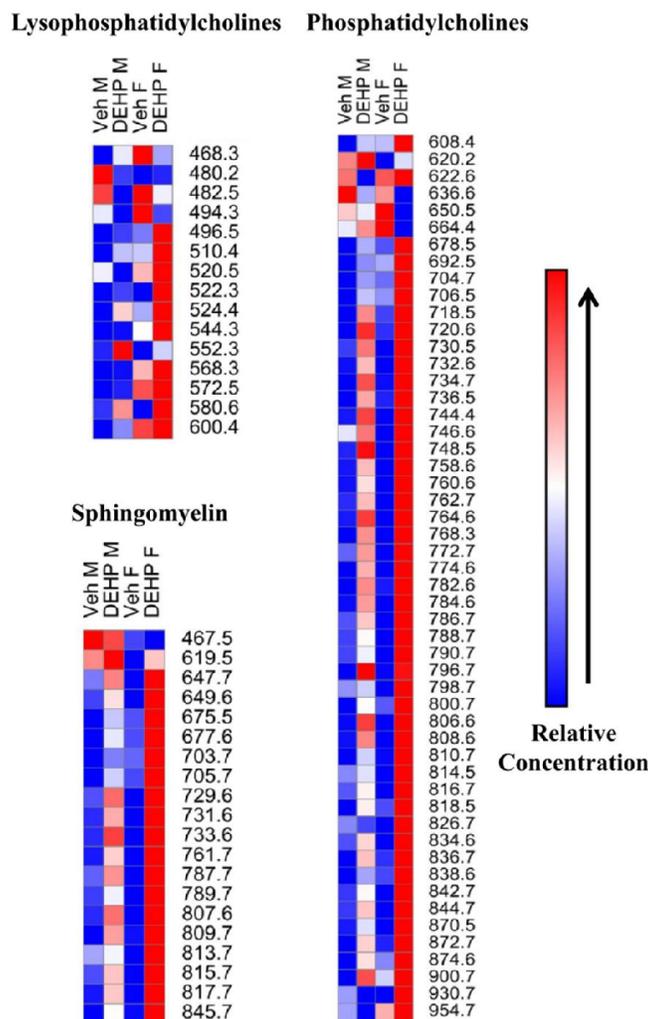


Figure 4.1: Heat map visualization of identified lipid masses. The heat maps represent 87 lipid masses identified in the precursor m/z 184 scan that are clustered into 3 categories based on their lipid classes (lysophosphatidylcholine, and phosphatidylcholine, and sphingomyelin). All lipid masses were common to each treatment condition (vehicle male, DEHP male, vehicle female, DEHP female) and are shown at the top of each heat map. The m/z of the identified lipids is shown to the right each heat map. Each column represents an independent treatment condition and each row corresponds to a single lipid mass. The heat map color scale denotes to the relative concentration of each lipid mass relative to the minimum and maximum of that lipid for all groups and is shown on the right-hand side of the figure. Dark blue indicates the lowest concentrations, dark red indicates the highest concentrations, and white indicates intermediate concentrations. DEHP treatment produced distinct lipid patterns in the hippocampus of male and female rats, with DEHP-treated female rats showing the highest relative concentration of almost all identified lipid masses in each lipid category. DEHP: di(2-ethylhexyl) phthalate; Veh: vehicle M: male; F: female. The heat maps were generated using GENE-E freeware (<http://www.broadinstitute.org/cancer/software/GENE-E/index.html>).

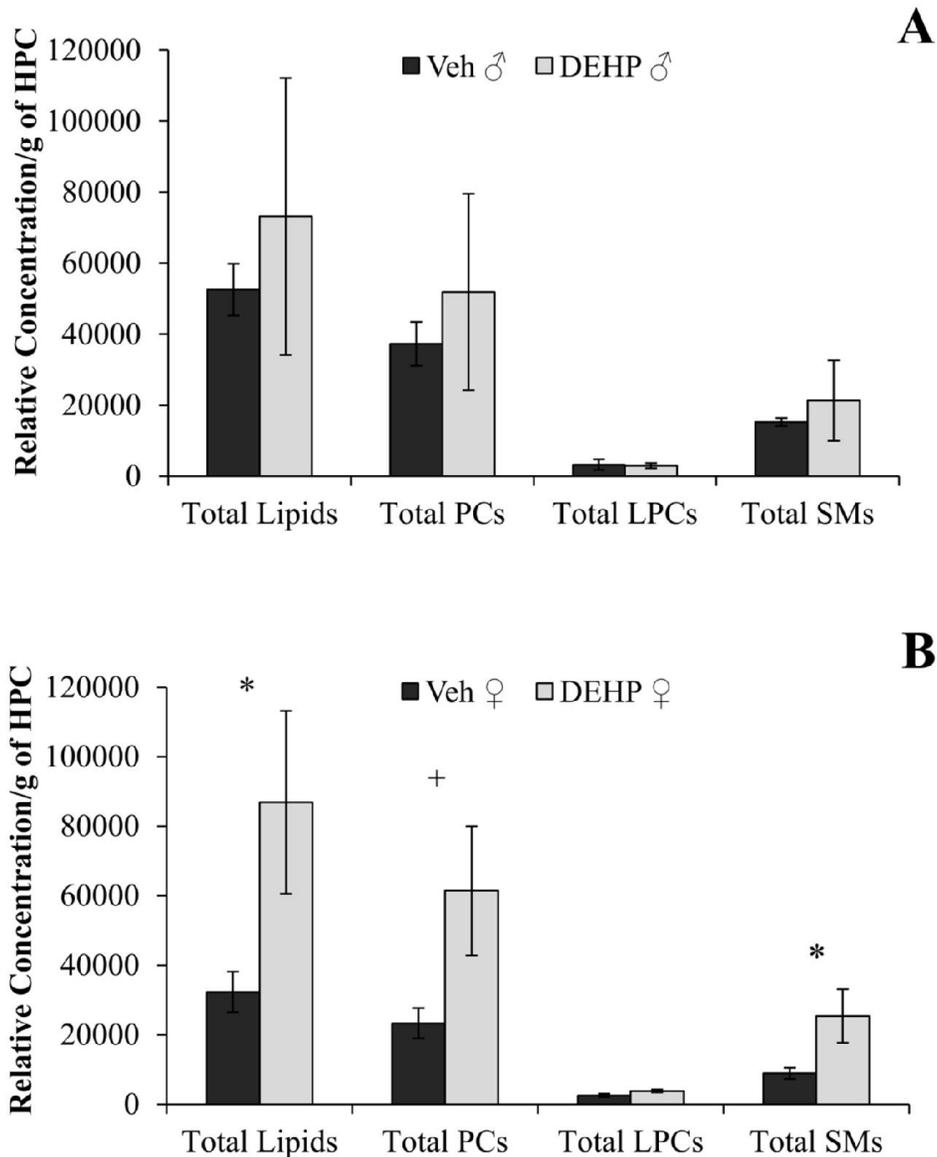


Figure 4.2: Total lipid content in the hippocampus. The relative concentrations of total lipids, lysophosphatidylcholine (LPC), phosphatidylcholine (PC), and sphingomyelin (SM) per gram of hippocampus (HPC) between male (A) and female (B) rats treated with phthalate (DEHP) compared to controls (Veh) of the same gender. Total lipid content, PC and SM lipids were up-regulated in female rats treated with DEHP compared to controls. There was no significant effect of DEHP treatment on total lipid content, PC or SM lipids in male rats compared to controls. DEHP: di(2-ethylhexyl) phthalate; * = $p < .05$; + = $p < .06$. Error bars represent standard error of the mean.

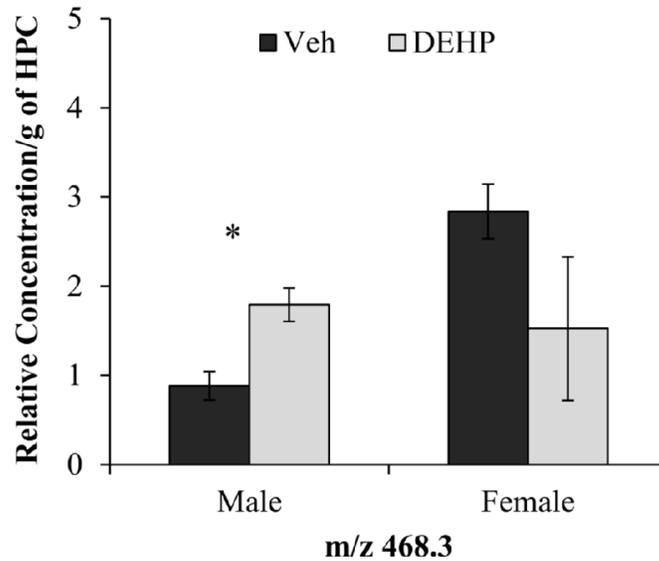


Figure 4.3: Lysophosphatidylcholine lipids in the hippocampus. The relative concentrations of lysophosphatidylcholine (m/z 468.3) per gram of hippocampus (HPC) between male (A) and female (B) rats treated with phthalate (DEHP) compared to controls (Veh) of the same gender. DEHP-treated male rats showed an increase in the relative concentration of one lipid mass (m/z 468.3) compared to controls. There was no significant effect of DEHP treatment on the relative concentration of LPC lipids in female rats compared to controls. DEHP: di(2-ethylhexyl) phthalate; * = $p < .05$. Error bars represent standard error of the mean.

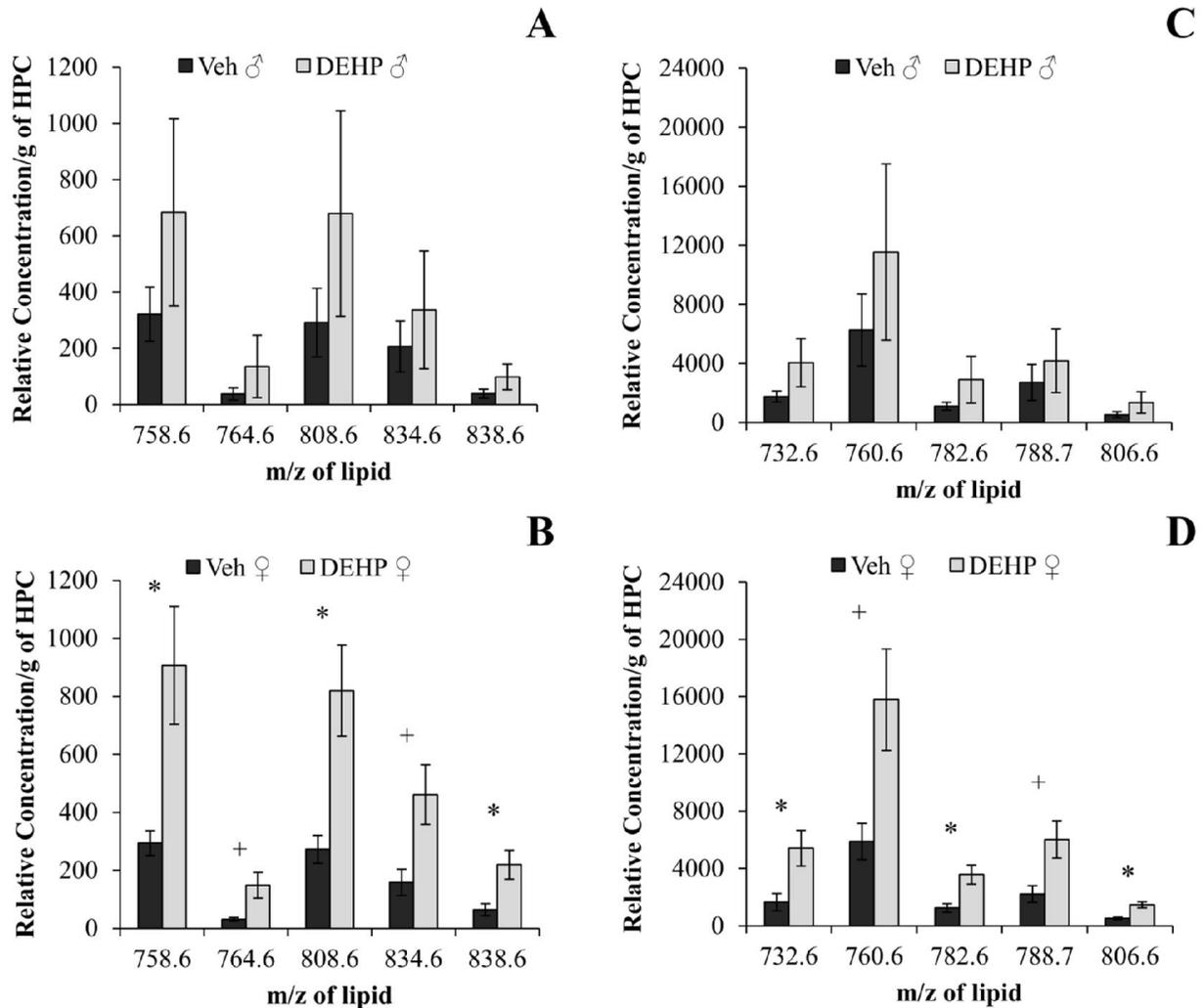


Figure 4.4: Phosphatidylcholine lipids in the hippocampus. The relative concentrations of lower intensity (A-B) and higher intensity (C-D) phosphatidylcholine lipids per gram of hippocampus (HPC) between male and female rats treated with phthalate (DEHP) compared to controls (Veh) of the same gender. DEHP-treated female rats showed an increase in the relative concentration of 10 PC lipids compared to controls. There was no significant effect of DEHP treatment on the relative concentration of PC lipids in male rats compared to controls. DEHP: di(2-ethylhexyl) phthalate; * = $p < .05$; + = $p < .06$. Error bars represent standard error of the mean.

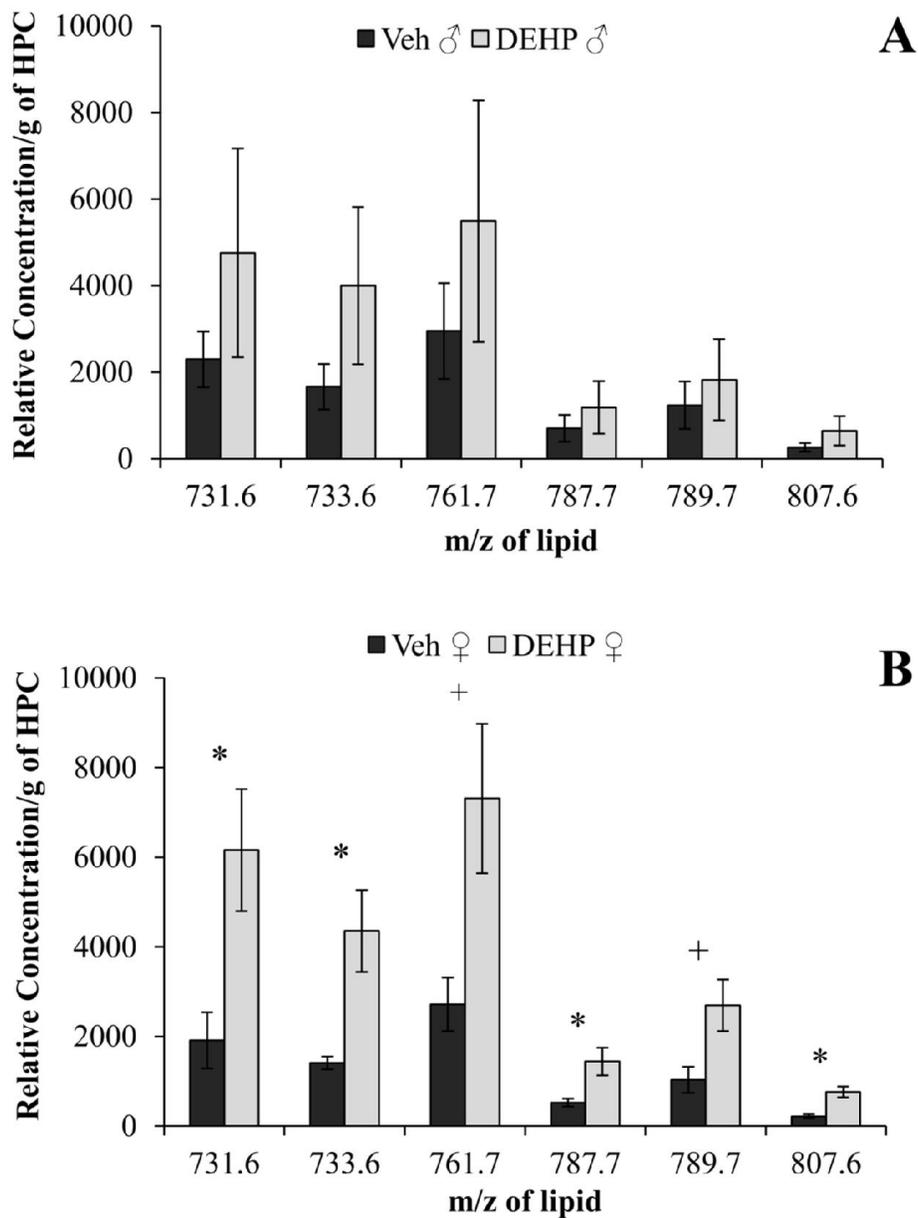


Figure 4.5: Sphingomyelin lipids in the hippocampus. The relative concentrations of sphingomyelin (SM) lipids per gram of hippocampus (HPC) between male (A) and female (B) rats treated with phthalate (DEHP) compared to controls (Veh) of the same gender. DEHP-treated female rats showed an increase in the relative concentration of 6 SM lipids compared to controls. There was no significant effect of DEHP treatment on the relative concentration of SM lipids in male rats compared to controls. DEHP: di(2-ethylhexyl) phthalate; * = $p < .05$; + = $p < .06$. Error bars represent standard error of the mean.

Table 1. Fold change in lysophosphatidylcholine and phosphatidylcholine lipids in the hippocampus. Fold change in the relative concentrations of lysophosphatidylcholine (LPC) and phosphatidylcholine (PC) lipids per gram of hippocampus in di (2-ethylhexyl) phthalate (DEHP) treated male and female juvenile rats compared to controls of the same gender.

m/z	Total Carbons: Double Bonds	Predicted Lipid Species***	Fold Change	
			Males	Females
468.3	(14:0)	LPC (14:0)	2.03 ↑ DEHP ^b	--- ^a
732.6	(32:1)	PC (16:1/16:0)	---	3.29 ↑ DEHP ^b
758.6	(34:2)	PC (18:1/16:1)	---	3.08 ↑ DEHP ^b
760.6	(34:1)	PC (18:1/16:0)	---	2.68 ↑ DEHP ^c
764.6	(35:6)	PC (18:4/17:2)	---	4.74 ↑ DEHP ^c
782.6	(36:4)	PC (18:2/18:2)	---	2.87 ↑ DEHP ^b
788.7	(36:1)	PC (18:1/18:0)	---	2.72 ↑ DEHP ^c
806.6	(38:6)	PC (20:3/18:3)	---	2.82 ↑ DEHP ^b
808.6	(38:5)	PC (20:3/18:2)	---	3.00 ↑ DEHP ^b
834.6	(40:6)	PC (20:3/20:3)	---	2.91 ↑ DEHP ^c
838.6	(40:4)	PC (20:3/20:1)	---	3.40 ↑ DEHP ^b
Total LPC			---	---
Total PC			---	2.63 ↑ DEHP ^c
Total Lipids (LPC, PC and SM)			---	2.69 ↑ DEHP ^b

^a No change; ^b $p < .05$; ^c $p < .06$

***As predicted by VaLID lipid prediction software (Blanchard et al., 2013)

Table 2. Fold change in sphingomyelin lipids in the hippocampus. Fold change in the relative concentrations of sphingomyelin (SM) lipids per gram of hippocampus in di (2-ethylhexyl) phthalate (DEHP) treated male and female juvenile rats compared to controls of the same gender.

m/z	Long-chain Base	N-Acyl	Predicted Lipid Species***	Fold Change	
				Males	Females
731.6	Sphingosine	(18:0)	SM (d18:1/18:0)	--- ^a	3.22 ↑ DEHP ^b
733.6	Sphinganine	(18:0)	SM (d18:0/18:0)	---	3.08 ↑ DEHP ^b
761.7	Sphinganine	(20:0)	SM (d18:0/20:0)	---	2.69 ↑ DEHP ^c
787.7	Sphingosine	(22:0)	SM (d18:1/22:0)	---	2.76 ↑ DEHP ^b
789.7	Sphinganine	(22:0)	SM (d18:0/22:0)	---	2.61 ↑ DEHP ^c
807.6	Sphingosine	(24:4)	SM (d18:1/24:4)	---	3.47 ↑ DEHP ^b
Total SM				---	2.83 ↑ DEHP ^b

^a No change; ^b $p < .05$; ^c $p < .06$

*** As predicted by LIPID MAPS MS Prediction Tool (Lipidomics Gateway)

Chapter V

Spatial Memory Impairments in Female Rats Following Acute Postnatal Exposure to Phthalates

The effect of phthalate exposure on spatial function has not been thoroughly examined in animals or in humans. Animal studies have produced conflicting results in regards to tasks involving spatial learning and memory. Some researchers have reported altered behavioural performance on spatial tasks following phthalate treatment; while others reported no effects (Boberg et al., 2011; Li et al., 2009; 2010; 2013; Tanaka 2002; 2005). In juvenile mice, chronic low dose exposure to DEHP in the diet (0.01, 0.03, 0.09 %) did not affect performance on a water version of the T-maze in either gender (Tanaka 2002; 2005). Conversely, perinatal exposure to low doses of DBP in the diet (30-55 mg/kg) impaired spatial learning performance in juvenile male rats (Li et al., 2009). At high doses, exposure to DBP produced conflicting behavioural results (Li et al., 2009; 2010; 2013a). Researchers reported impaired spatial memory acquisition and retention when performing a water maze task following perinatal exposure to 800-1250 mg/kg DBP (in the diet) in male juvenile rats (Li et al., 2009) and 500 mg/kg DBP (intra-gastric) in male and female juvenile rats (Li et al., 2013a). Interestingly, perinatal exposure to 675 mg/kg DBP via gavage improved the acquisition and the retention of a spatial memory in a water maze task in juvenile male rats (Li et al., 2010).

Similarly, an increase in performance on a spatial water maze task was observed in juvenile female rats treated perinatally with high doses of DINP (900 mg/kg; Boberg et al., 2011). This increase in spatial performance was task-specific as no improvements in spatial function were

observed in DINP-treated females on a second spatial learning task, the radial arm maze. DINP treatment in male rats did not alter performance on either spatial learning task, suggesting a uniquely beneficial effect of DINP treatment in female juvenile rats expressed by performance on the water maze task (Boberg et al., 2011). Overall, these findings demonstrate widely different behavioural outcomes on spatial learning tasks in phthalate-treated rodents. It appears these changes in spatial ability were highly dependent on the type of phthalate that was administered (DEHP versus DBP versus DINP), the dose of phthalate that was used, and the species and gender of the animal that was tested (rats versus mice).

In humans, phthalate exposure can be estimated by measuring the levels of phthalate metabolites in urine (Lyche et al., 2009). In one study, children exposed to higher concentrations of phthalates prenatally displayed poorer performance on working memory tasks at ages 4 and 9 (Engel et al., 2010). Similarly, higher urinary concentrations of phthalate metabolites in mothers during pregnancy was associated with an increase in behavioural and attention problems and lower Intelligence Quotient scores in school-age children (Cho et al., 2010; Kim et al., 2009). These research findings point to the importance of pursuing the experimental link between phthalate exposure and cognitive functioning.

Previous work has shown that extensive synaptic remodeling of mossy fiber projections to the CA3 hippocampal region occur between PND18 and PND24 in Long Evans rats, and correspond with the emergence of enhanced cognitive spatial function between PND19 and PND20 (Holahan et al., 2007; Keeley et al., 2010). Slight modifications in hippocampal development may produce long-lasting changes in hippocampal neurocircuitry, potentially leading to impairments in cognitive spatial function. In Chapters II and III, we reported widespread modifications in neurocircuitry (particularly in the CA3 hippocampal region) in male rats treated

with 10 mg/kg DEHP during postnatal (days 16-22) development. At the same dose, DEHP treatment did not alter hippocampal neurocircuitry in female rats (Chapter II and III), but did increase a number of hippocampal lipid species (Chapter IV). These results suggest a possible neuroprotective effect of elevated lipid species on the development of hippocampal neurocircuitry in DEHP-treatment female rats. The neurobehavioural impact of DEHP-induced alterations in hippocampal development in male and female rats has not been previously studied and was explored in this chapter.

Objectives and Hypotheses

The primary objective of the present study was to evaluate the effect of early developmental phthalate treatment (PND16-PND22) on neurobehavioural measures of cognitive performance in male and female, juvenile and young adult rats at four DEHP doses (0, 1, 10 and 20mg/kg). Rats were tested on a hidden platform spatial water maze task at two time points: as juveniles (PND30-PND34) and as young adults (PND74-PND78) to determine if DEHP differential altered spatial function over the lifespan. Given that synaptic remodeling in the CA3 region of the hippocampus from PND18 to PND21 coincides with an improvement in cognitive spatial function, and the negative impact of DEHP exposure on hippocampal development in male rats, it was expected that DEHP treatment in male rats would adversely affect spatial water maze performance and the underlying neurocircuitry in a dose-dependent manner (with the highest dose showing the largest deficit). It was also hypothesized that these deficits in spatial performance in male rats would be more noticeable during the juvenile period.

Since neurocircuitry in the hippocampus of female rats was unaffected by DEHP treatment, no differences were expected in spatial performance or hippocampal neurocircuitry in juvenile or

young adult rats. A cued water maze task was also conducted to ensure that differences observed in the hidden platform water maze task were not the result of DEHP exposure affecting motivation to find the hidden platform and/or swimming ability. No differences in cued learning were expected.

Materials and Method

Animals

Six untimed pregnant female Long Evans rats (approximately 13 days gestation) were purchased from Charles River Laboratories (St. Constant, Québec) for this experiment. The pregnant females were singly-housed in polycarbonate 48 x 26 x 20 cm³ cages within a temperature controlled environment. The day the pups were born was recorded as PND0. Pups (n = 26 males; n = 28 females) were weaned on PND22 and group-housed, with males and females in separate cages. All rats were on a 12 hour light-dark cycle (lights on at 8:00 a.m.) with *ad libitum* access to food (Purina rat chow) and tap water. All experiments were conducted at Carleton University and approved by the Institutional Animal Care Committee as per guidelines established by the Canadian Council on Animal Care.

DEHP Injections

Rats were injected i.p. with either 1, 10 or 20 mg/kg DEHP (Sigma-Aldrich; St. Louis, MO, USA) or vehicle (corn oil) daily from PND16 to PND22 inclusive: 1 mg/kg (n = 6 males; n = 7 females), 10 mg/kg (n = 7 males; n = 7 females) and 20 mg/kg (n = 6 males; n = 7 females). Each rat was injected in the late morning (between 10:30 and 11:30 AM) and was returned to their home cage following the injection. Rats were randomly assigned to treatment (DEHP) and control

(vehicle) groups counterbalanced across all litters. DEHP solutions were prepared using DEHP (1000 mg/kg) and corn oil immediately before each injection. Rats (n = 7 males; n = 7 females) receiving vehicle injections were injected with corn oil.

Water Maze Apparatus

All rats were trained in a standard water maze apparatus. The pool was 1.55 m in diameter and 0.6m in height. The platform was 14.5 cm in diameter and was submerged just below the water level. The pool was filled to a water depth of approximately 40 cm and the water temperature was kept constant at 21 °C throughout the trials. Visual cues (i.e. posters and cardboard shapes) were placed on the walls surrounding the pool which remained constant throughout the experiments. The experimenter stood in the same location for each trial to ensure consistency. The pool was located in a 2.5 x 2.5 test room which was illuminated by a single floor light. Movements were tracked using HVS Image 2100 Tracking System (HVS Image, Buckingham, UK).

Behavioural Procedures

All rats underwent three periods of water maze testing. Figure 5.1 shows a timeline of the experimental procedures. Each rat received water maze training using a hidden platform as juveniles (PND30-PND34) and again as young adults (PND74-PND78). Each rat was also trained on a water maze task with a cued platform on PND60. All rats were trained in the morning between 8:00AM and 12:00PM, and the testing order was randomized to eliminate order and time effects.

Hidden Water Maze

Acquisition Training (Days 1-4). All rats received 4 consecutive days of training (4 trials per day) to find the hidden platform submerged just below the water level. The platform was located in the southwest quadrant across all trials for juvenile training, and in the northeast quadrant for adult training. For each trial, rats started at a different location in the pool and had 60 seconds to reach the platform. If the platform was not located after 60 seconds, rats were guided to the platform location by the experimenter. Rats remained on the platform for 15 seconds. After which rats were towel dried and placed in a recovery cage for 30 seconds. Following the final trial, rats were towel dried and placed in their home cage on a heating pad for approximately 15 minutes.

Probe Test (Day 5). To determine memory retention for the platform location, each rat completed a 60 second free swim with no platform in the pool 24 hours after the final day of training. The placement of a circle 3 times (8 % of pool area) the diameter of the platform centered on the original platform location was used to infer memory retention of the target. The amount of time spent searching in this region was recorded, along with the amount of time spent searching in the opposite annulus, and the percent time spent swimming around the perimeter of the water maze pool (or thigmotaxis).

Cued Water Maze

All rats received one day of training on a cued water maze task (8 trials). The cued water maze task was completed to ensure that differences observed in the hidden platform task were not the result of DEHP exposure affecting motivation to find the hidden platform and/or swimming ability. Testing was completed in same room as the hidden platform water maze task with the same

spatial cues located on the walls around the pool. An additional cue (a flag) was taped to the submerged platform and served as a proximal cue to the platform's location. The location of the platform was randomized across the 8 trials and was located in four quadrants for two trials each. This was done so that the rats did not habituate to one particular quadrant of the pool, and learned to associate the proximal cue (the flag) with the platform's location rather than the spatial cues. There were also two start locations for each platform location, one distal and one proximal to the platform.

For each trial, rats had 60 seconds to reach the platform. If the platform was not located after 60 seconds, rats were guided to the platform location by the experimenter. Rats remained on the platform for 15 seconds. After which rats were towel dried and placed in a recovery cage for 30 seconds. Following the final trial, rats were towel dried and placed in their home cage on a heating pad for approximately 15 minutes.

Tissue Processing

Rats were euthanized on PND78 by live decapitation immediately after the water maze probe test. Brains were extracted and postfixed overnight in 4 % paraformaldehyde/ 0.01 M phosphate buffer. Brains were then cryoprotected in 30 % sucrose in 0.1 M PBS, and stored at 4 °C until sectioning.

Immunohistochemistry

The brain was sectioned into 60 µm coronal sections using a Leica CM1900 cryostat (Weztler, Germany). Sections were taken from the dorsal hippocampus and stored in a 0.1 % sodium azide solution in 0.01 M PBS at 4 °C until staining. Synaptophysin immunofluorescent

labeling, and DCX DAB staining were completed as described in Chapter II. DCX sections were visualized a 0.5 %, 3,3'-diaminobenzene solution and counterstained with fast green.

Synaptophysin Quantification

Fluorescent images of the CA3 region were captured at 10 x magnification using a Retiga-2000R camera and an Olympus BX61 microscope. Coronal sections were sampled from the dorsal hippocampus of each group. Approximate bregma levels were -3.14 to -3.30. Quantification was completed as described in Chapter II.

DCX Quantification

Images of the DG were captured at 20 x and 60 x magnification using an Olympus U-CMAD3 camera and an Olympus BX51 microscope with a motorized stage. Coronal sections were sampled from the hippocampus of each rat. Approximate bregma levels were -3.60 to -3.80. The optical fractionator method (West et al., 1991) was used to estimate the total number of DCX positive neurons in the DG. The quantification of DCX-positive neurons was completed as described in Chapter II.

Statistical Analyses

Acquisition data from the cued and hidden water maze training task were analyzed using mixed design, two-way Analysis of Variance (ANOVA) with dose (0, 1, 10, and 20 mg/kg DEHP) as the between-groups factor, and training day (1-4) as the within-groups factor. Probe test data was examined using one-way ANOVAs at the four DEHP doses. Synaptophysin and DCX staining were examined using one-way ANOVAs with dose (0, 1, 10, and 20 mg/kg DEHP) as the between-

groups factor. For all analyses, each gender was assessed separately. Post hoc analyses were conducted using Dunnett's t tests (with the vehicle group as the control group) where appropriate.

Results

A Biphasic Effect of DEHP Treatment on Acquisition in Juvenile Females

Male and female juvenile rats were trained for four consecutive days (PND30-PND33) on a spatial water maze task to evaluate whether acute postnatal DEHP exposure (0, 1, 10, 20 mg/kg DEHP) influenced the acquisition of the hidden platform location. Latency to reach the hidden platform (or escape latencies) and thigmotaxis were used as the operational measures of task performance.

Figure 5.2A-B shows the average latency to reach the hidden platform for each training day in male and female juvenile rats. There was a main effect of training day ($F(3,72) = 59.98, p < 0.001$) and DEHP dose ($F(3,24) = 3.38, p < 0.05$) in female rats, with increased escape latencies on training day 3 in female rats treated with 1 and 20 mg/kg DEHP compared to controls ($p < 0.05$; Figure 5.2B). No differences were found between female rats treated with 10 mg/kg DEHP and vehicle controls ($p > 0.05$), demonstrating a biphasic effect of DEHP dose on the acquisition of the spatial water maze task in female rats. In male rats, there was no effect of DEHP treatment on average escape latencies across the four training days ($p > 0.05$; Figure 5.2A). However, a significant downward linear relationship was found across training days ($F(3,66) = 67.67, p < 0.001$), indicating that the time required to reach the hidden platform decreased as training increased regardless of DEHP dose in male rats.

Figure 5.2C-D shows the percent of thigmotaxis for each training day in male and female juvenile rats. Similarly, there was a main effect of training day ($F(3,72) = 71.17, p < 0.001$) and

DEHP dose ($F(3,24) = 4.27, p < 0.05$) in female rats, with increased thigmotaxis observed in female rats treated with 1 mg/kg DEHP on training day 2 ($p < 0.01$) and 3 ($p < 0.05$), and in female rats treated with 20 mg/kg DEHP on training day 3 compared to female controls ($p < 0.01$; Figure 5.2D). In male rats, there was no effect of DEHP treatment on thigmotaxis across the four training days ($p > 0.05$; Figure 5.2C), but there was a main effect of training day ($F(3,66) = 86.93, p < 0.001$). Thigmotaxis for all rats followed a downward linear trend over time.

DEHP Treatment Impaired Retention of the Platform Location in Juvenile Females

A 60 second probe test was conducted 24-hours after the last training session. Memory retention of the platform's location was inferred from the amount of time each rat spent searching within an area three times the diameter of the platform centered on the location of the platform during training (i.e., the target annulus). Given the diameter of the pool and of the target annulus, it is expected that each rat would spend 8 % of the probe test searching within this region due to chance alone. The amount of time spent searching within the target annulus represents a good measure of memory retention. More searching in this region reflects better recall of the platform's location – in a positive, linear way. The amount of time spent searching in an annulus of the same size located in the opposite quadrant (i.e., the opposite annulus) and thigmotaxis were also examined.

Figure 5.3A shows occupancy plots for the entire 60 second probe test. Figure 5.3B-E shows the percent of time the rats spent swimming in the target and opposite annuli during the 60 second probe test. There were no selective differences in time spent in either annulus in male or female rats ($p > 0.05$). One sample t-tests revealed all male rats, regardless of DEHP dose, spent more time searching in the target annulus than what was expected from random chance ($p < 0.05$;

Figure 5.3B). In female rats, all DEHP groups (except the 20 mg/kg DEHP group) spent significantly more time in the target annulus than what was expected due to random chance ($p < 0.05$; Figure 5.3C). Figure 5.3E-F shows the percent of thigmotaxis during the 60 second probe test. Similarly, there was an effect of DEHP dose on thigmotaxis in female ($F(3,24) = 3.62, p < 0.05$; Figure 5.3G), but not male rats ($p > 0.05$; Figure 5.3F), with a significant increase in thigmotaxis in female rats treated with 20 mg/kg DEHP compared to controls ($p < 0.05$).

The first 15 seconds of the probe test was analysed to determine if initial searching for the former platform location differed following DEHP treatment. Figure 5.4A shows occupancy plots for the first 15 seconds of the probe test. Female rats treated with 20 mg/kg DEHP showed a significant reduction in the time spent searching in the target annulus during the first 15 seconds of the probe test compared to controls ($p < 0.05$; Figure 5.4C). There were no significant effects of DEHP dose in males ($p > 0.05$; Figure 5.4B).

DEHP Treatment Did Not Effect on Escape Latencies in Young Adult Rats

Male and female rats were re-trained for four consecutive days (PND74-PND77) on the water maze task (with the hidden platform in a new location) to evaluate the long-term effects of acute postnatal DEHP exposure on spatial function. Escape latencies and thigmotaxis were used as the operational measures of task performance.

Figure 5.5A-B shows the average latency to reach the hidden platform across the four training day in male and female adult rats. There was no effect of DEHP treatment on average escape latencies across the four training days in male ($p > 0.05$; Figure 5.5A) or female rats ($p > 0.05$; Figure 5.5B). However, escape latencies did follow a significant downward linear trend over time in males ($F(3, 66) = 37.70, p < .0001$) and females ($F(3, 72) = 21.41, p < .0001$), indicating

that performance on the water maze task was positively related to the amount of training received. Figure 5.5C-D shows the percent of thigmotaxis for each training day in male and female adult rats. There was a main effect of training day ($F(3,72) = 9.52, p < 0.001$) and DEHP dose ($F(3,24) = 5.20, p < 0.01$) in female rats, with increased thigmotaxis in female rats treated with 20 mg/kg DEHP on training day 3 ($p < 0.05$) and 4 ($p < 0.001$) compared to female controls (Figure 5.5D). In male rats, there was no effect of DEHP treatment on thigmotaxis across the four training days ($p > 0.05$; Figure 5.5C), but there was a main effect of training day ($F(3,66) = 42.60, p < 0.001$), with thigmotaxis for all male rats following a downward linear trend over time.

DEHP Treatment Impaired Retention of the Platform Location in Young Adult Females

A 60 second probe test was conducted 24-hours after the last training session. Time spent in the target and opposite annuli, and thigmotaxis served as operational measures of task performance. Figure 5.6A shows occupancy plots for the entire 60 second probe test. Figure 5.6B-E shows the percent of time the rats spent swimming in the target and opposite annuli during the 60 second probe test. All groups spent significantly more time searching in the target annulus than expected due to random chance ($p < 0.05$), and no significant differences were observed between DEHP dose in male ($p > 0.05$; Figure 5.6B) or female rats ($p > 0.05$; Figure 5.6C). Similarly, there was no effect of DEHP treatment on time spent in the opposite annulus in male ($p > 0.05$; Figure 5.6D) or female rats ($p > 0.05$; Figure 5.6E). Figure 5.6F-G shows the thigmotaxis during the 60 second probe test. A significant effect of DEHP dose on thigmotaxis was found in female rats ($F(3,24) = 4.31, p < 0.05$), with increased thigmotaxis in female rats treated with 1 and 20 mg/kg DEHP compared to female controls ($p < 0.05$; Figure 5.6G). No differences were significant in male rats ($p > 0.05$; Figure 5.6F).

Figure 5.7A shows occupancy plots for the first 15 seconds of the probe test. The first 15 seconds of the probe test was analysed to determine if initial searching for the former platform location differed following DEHP treatment (Figure 5.7B-C). In female rats, there was main effect of DEHP ($F(3,24) = 2.82, p < 0.06$; Figure 5.7C). Female rats treated with 1 and 20 mg/kg DEHP showed a significant reduction in the time spent searching in the target annulus compared to controls ($p < 0.06$; $p < 0.05$, respectively). No significant differences were found in male rats ($p > 0.05$; Figure 5.7B).

No Effect of DEHP Treatment on the Acquisition of a Cued Platform Location

Male and female rats received one day of training on a cued water maze task (8 trials). A flag was taped to the submerged platform and served as a proximal cue to the platform's location. Figure 5.8 shows the latency to reach the cued platform across the eight training trials in male and female rats. There was no effect of DEHP dose on escape latencies across the eight trials in male or female rats ($p > 0.05$; Figure 5.8), but there was a main effect of training trial in both genders (males: $F(3,22) = 2.91, p < 0.01$; females: $F(3,24) = 3.23, p < 0.01$) following a downward linear trend over time.

A Reduction in Immature Neurons Following DEHP Treatment in Males

Figure 5.9A shows representative images of DCX staining in the DG. There was no significant effect of treatment on the number of DCX positive neurons in the DG of female rats at any DEHP dose ($p > 0.05$; Figure 5.9C), but there was an effect of DEHP dose in male rats ($F(3,22) = 3.40, p < 0.05$; Figure 5.9B). There was a reduction in DCX-positive neurons in male rats treated with 1 mg/kg and 20 mg/kg DEHP had compared to controls ($p < 0.06$).

DEHP Treatment Increased Synaptophysin Staining in the CA3 SL Region in Females

Figure 5.10A shows representative images of synaptophysin staining in the CA3. Synaptophysin staining was delineated and measured in the CA3 SL and SO (microns squared). The ratio of staining in the SO to the SL (SO:SL), and area of staining (μm^2) in the SO and the SL were the bases of between group comparisons, where larger areas of SO staining signified increased axonal innervation to this region and larger ratios indicated more staining in the SO.

There was no effect of DEHP exposure on the SO:SL ratio (Figure 5.10B; $p > 0.05$) or the area of SL or SO staining (Figure 5.10C; $p > 0.05$) in male rats. In females, DEHP treatment significantly increased the area of SL staining (Figure 5.10E; $F(3,24) = 4.83, p < 0.01$), but did not alter the area of SO staining (Figure 5.10E; $p > 0.05$) or the SO:SL ratio (Figure 5.10D; $p > 0.05$). Post hoc analyses reveal a significant increase in synaptophysin staining in the SL of female rats treated with 10mg/kg and 20mg/kg compared to vehicle controls (Figure 5.10E; $p < 0.01$).

Discussion

Early developmental exposure to phthalates have been previously linked to abnormal neurodevelopment in rats (Chapters II, II, and IV; Andrade et al., 2006; Bell et al., 1979; Ishido, et al., 2004; Li et al., 2010; Li et al., 2013a,b; Xu et al., 2007). The impact of phthalate-induced neurodevelopment changes on cognitive functioning has not been widely studied and was the basis for the present study. Previous work that investigated the impact of phthalates (DINP and DBP) on spatial learning and memory tasks has demonstrated both beneficial and detrimental effects in both genders (Boberg et al., 2011; Li et al., 2009; 2010; 2013a). The present study was the first to examine the effects of postnatal (days 16 to 22) DEHP exposure on a spatial water maze task in male and female rats. Performance on a cued water maze task was also evaluated in both genders

to ensure that differences observed in the spatial water maze task were not the result of DEHP exposure affecting motivation to find the hidden platform and/or swimming ability. No differences in cued learning were observed in male or female rats, indicating that any performance deficits/enhancements on the spatial water maze task were not related to motivation or physical ability.

Male and female rats were assessed on the spatial water maze task as juveniles and as adults to evaluate the effect of DEHP treatment on spatial function over the lifespan. DEHP treatment in male rats had no effect on spatial learning or memory when assessed as juveniles or as young adults. This was unexpected given the detrimental effect of postnatal exposure to 10 mg/kg DEHP on hippocampal development in male juvenile rats. It is possible that some degree of functional re-organization or adaptation had occurred in the hippocampi of DEHP-treated male rats, such that spatial functioning in these rats was preserved. Consistent with this, there were no detectable differences in axonal innervation to the CA3 SL or SO regions in male rats compared to controls when assessed as young adults, suggesting that while exposure to DEHP during postnatal development was initially detrimental to hippocampal development it did not lead to any lasting changes in hippocampal connectivity in young adulthood. Perhaps axonal innervation to the CA3 SO region was only delayed by postnatal DEHP treatment and over time the normal pattern of CA3 connectivity emerged.

Male rats treated with 1 and 20 mg/kg DEHP had reduced levels of DCX-positive granule cells in the DG compared to controls when evaluated during young adulthood. DCX is a protein expressed by neuronal precursor cells and immature neurons, and can be used as a marker of neurogenesis in the adult brain (Couillard-Despres et al., 2005; Rao and Shetty, 2004). Low levels of neurogenesis are correlated with memory impairments on the spatial water maze task (Drapeau

et al., 2003; Jessberger et al., 2009), suggesting some compensatory mechanism had occurred in male rats exposed to 1 and 20 mg/kg DEHP that was responsible for their recovery in spatial function. It would be of interest to evaluate the effects of postnatal exposure to 1 and 20 mg/kg DEHP on neurogenesis during the juvenile developmental period to determine if low levels of neurogenesis were present during early development or if these reductions in neurogenesis had gradually appeared over time. Contrary to the findings reported in Chapter II, exposure to 10 mg/kg DEHP did not affect the density of DCX-positive granule cells in the DG in male rats assessed as young adults. This finding was surprising, but indicates that while the 10 mg/kg DEHP dose initially reduced levels of neurogenesis in the DG, over time its impact on neurogenesis had diminished.

Unlike male rats, DEHP treatment in female rats led to deficits in spatial performance during the juvenile and adult water maze sessions. Female rats treated with 1 and 20 mg/kg DEHP showed increased escape latencies on training day 3 of juvenile acquisition, suggesting exposure to the lowest (1 mg/kg) and the highest (20 mg/kg) dose of DEHP delayed the acquisition of the platform location. By training day 4, these deficits in spatial information processing were no longer present, indicating the 1 and 20 mg/kg female DEHP groups quickly “caught-up” to the female controls, and were able to reach the platform after similar latencies. The thigmotaxis data during juvenile acquisition further supports the idea of delayed acquisition of the water maze task in the 1 and 20 mg/kg female DEHP groups as both groups spent more time swimming around the perimeter of the pool instead of searching for the hidden platform on training day 3.

Memory retention for the hidden platform location was examined in a probe test conducted 24 hours after the last juvenile acquisition training session. All rats were expected to spend approximately 8% of the probe test searching in the target annulus due to random chance. Increased

time spent in this location indicates better retention of the former platform location. In female rats, all groups (except the 20 mg/kg DEHP group) spent more time in the target annulus than due to random chance— demonstrating a strong memory for the platform location. Female rats treated with 20 mg/kg DEHP displayed no preference for the target annulus, and showed increased thigmotaxis during the probe test, indicating poor memory retention for the platform location. Together, these findings suggest a significant impairment in spatial memory function in this group.

When female rats were examined for a second time on the spatial water maze task during young adulthood, no differences in escape latencies were observed across the four acquisition training sessions. However, probe test data revealed a significant reduction in the time spent searching in the target annulus during the initial 15 seconds of the probe test in female rats treated with 1 and 20 mg/kg DEHP when compared to controls. The 1 and 20 mg/kg DEHP groups also showed increased thigmotaxis throughout the 60 second probe test; indicating poor memory retention for the platform location.

Overall, postnatal DEHP treatment in female rats had a biphasic effect on performance in the spatial water maze task. Female rats treated with the highest DEHP dose (20 mg/kg) displayed the most significant spatial memory impairments during the juvenile and adult water maze testing sessions. Exposure to the lowest DEHP dose (1 mg/kg) also led to disruptions in spatial function in female rats. These deficits were more prominent during the adult water maze sessions; there was no accompanying deficit in memory retention during the juvenile probe test (only a delay in acquisition of the task during training). Spatial memory impairments in the 1 mg/kg group become more severe over time, demonstrating that while this group appeared to function normally during development, abnormalities in these same functions appeared later in life. Postnatal DEHP

treatment with 10 mg/kg (the intermediate DEHP dose) did not affect performance in the spatial water maze task – creating a u-shaped dose-response curve in female rats.

Alterations in neurobiology may contribute to this biphasic effect of DEHP treatment on spatial functioning. In the present study, DCX and synaptophysin staining in the DG and CA3, respectively, were evaluated. No differences in the density of DCX-positive granule cells or in axonal innervation to the CA3 SO region in female rats. Only a slight increase in CA3 SL synaptophysin staining was detected in female rats treated with 10 and 20 mg/kg DEHP. These findings were unexpected in the 1 and 20 mg/kg DEHP groups as deficits in spatial memory performance are associated with a reduction in both neuronal markers, and suggest that there may be other neuromodulators underlying these spatial memory deficits.

In Chapter IV, we postulated that elevated levels of lipids in female juvenile rats treated with 10mg/kg had a neuroprotective effect on the hippocampus preventing DEHP-induced abnormalities in hippocampal neurocircuitry. It is possible that up-regulation of hippocampal lipid in DEHP-treated female rats may also have a protective effect on spatial functioning. Interestingly, no deficits in spatial function were detected in female rats treated with 10 mg/kg DEHP when assessed as juveniles or young adults, suggesting the up-regulation of hippocampal lipid may protect female rats from deficits in spatial function. Further investigation is required in order to confirm this hypothesis.

Conclusion

Data from the current study revealed negative effects of postnatal (days 16 to 22) DEHP exposure on performance in a spatial water maze task in female rats – with impairments in spatial function observed in the 1 and 20 mg/kg DEHP groups. No performance deficits in the spatial

water maze task were found in male rats, despite a reduction in the density of immature neurons in the DG following treatment with 1 and 20 mg/kg DEHP. These data highlight the importance of investigating the impact of a variety of DEHP doses on neurodevelopment and behavioural measures as postnatal DEHP treatment appears to have a biphasic effect on spatial functioning and its associated neurocircuitry (with differential affects across gender). In addition, comprehensive investigations which assess DEHP-induced alterations in performance on other behavioural measures of cognitive function are needed and will provide an opportunity to thoroughly evaluate the toxic potential of DEHP.

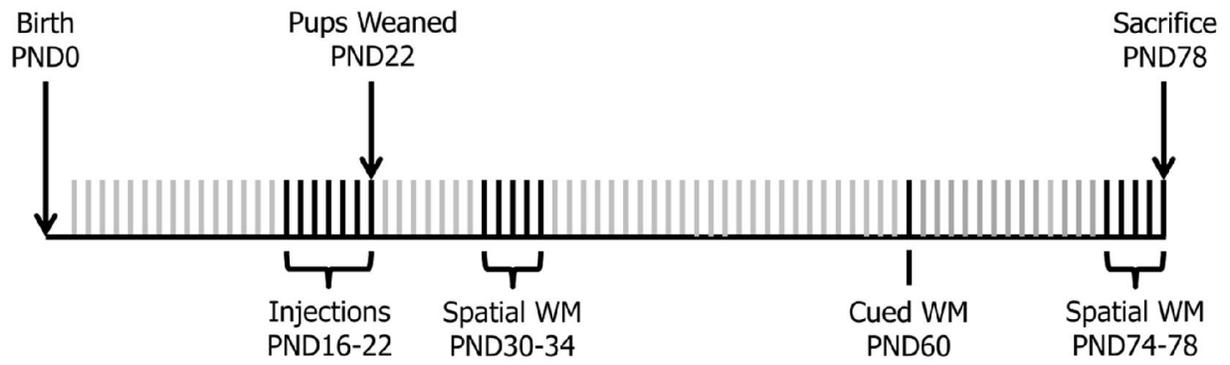


Figure 5.1: Timeline of experimental procedures. A timeline of di(2-ethylhexyl) phthalate treatment and water maze sessions. PND: Postnatal day; Injections: Injections of 1, 10, 20 mg/kg di(2-ethylhexyl) phthalate or vehicle; WM: water maze.

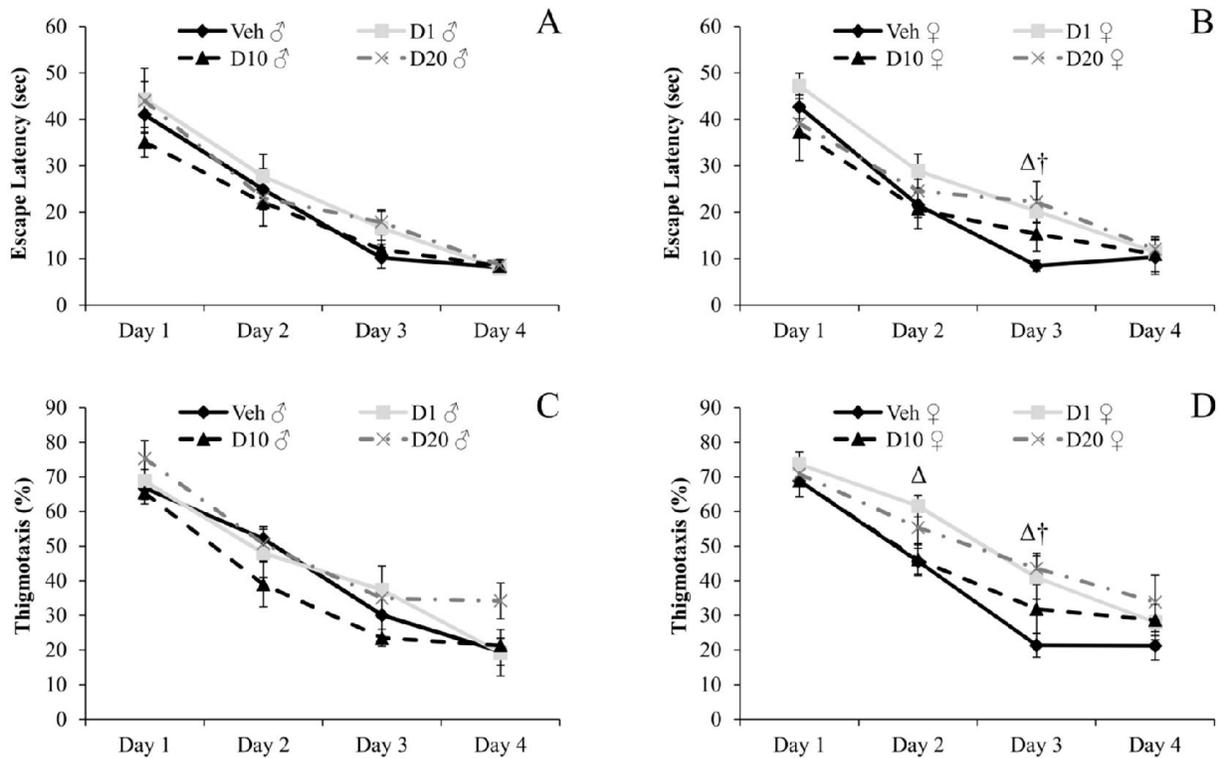


Figure 5.2: Daily latencies and thigmotaxis during juvenile spatial water maze acquisition. Latency to reach the hidden platform averaged across four trials of daily training from PND30 to PND33 for male (A) and female (B) rats exposed to 1, 10, 20 mg/kg di(2-ethylhexyl) phthalate (DEHP) compared to controls (Veh). Percent time spent swimming around the perimeter of the pool (thigmotaxis) averaged across four trials of daily training for male (C) and female (D) rats. The Δ denotes a significant difference between the 1mg/kg DEHP dose and vehicle controls and the \dagger denotes a significant difference between the 20mg/kg DEHP dose and vehicle controls ($p < .05$). Female rats treated with 1 and 20 mg/kg DEHP showed an increase in latency to locate the hidden platform and increased thigmotaxis on training day 3 compared to controls. There was no effect of DEHP treatment on performance during the acquisition training session in male rats. D: DEHP. Error bars represent standard error of the mean.

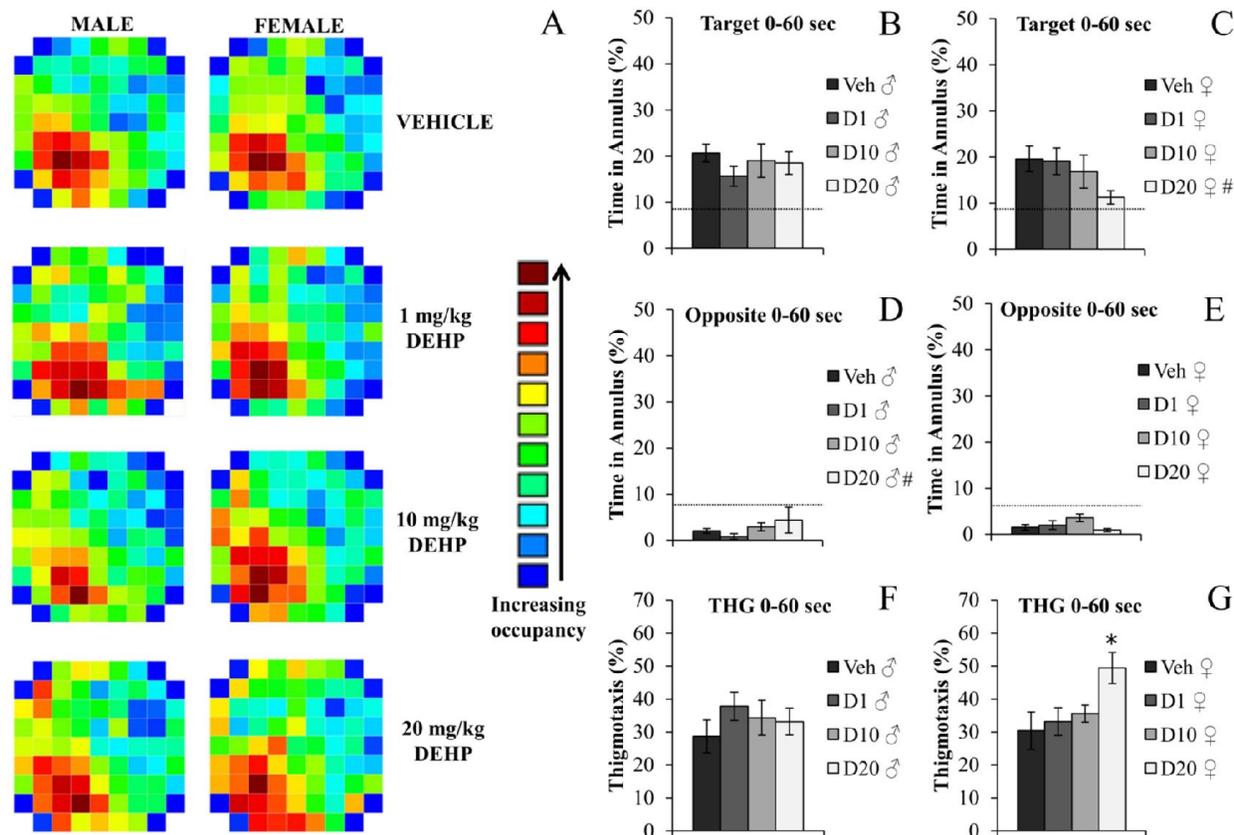


Figure 5.3: Retention of platform location during juvenile probe test 0-60 seconds. Occupancy plots of the 60 second probe test (A). Target annulus is located in the southwest quadrant. Percent time spent swimming in the target annulus during the 60 second water maze probe test (PND34) for male (B) and female (C) rats exposed to 1, 10, 20 mg/kg di(2-ethylhexyl) phthalate (DEHP) compared to controls (Veh). Percent time spent swimming in the opposite annulus during the 60 second probe test for male (D) and female (E) rats. Percent time spent swimming around the perimeter of the pool (thigmotaxis) during the 60 second probe test for male (F) and female (G) rats. The dashed line represents chance performance (8%). The # denotes treatment group was not significantly different from chance performance. All groups (except the 20 mg/kg DEHP female group) spent significantly more time in the target annulus than what was expected due to random chance. Female rats treated with 20 mg/kg DEHP also showed increased thigmotaxis during the 60 second probe test compared controls. No differences in the amount of time spent in the target annulus were detected between DEHP-treated females and controls. There was no effect of DEHP treatment on performance during the probe test in male rats. D: DEHP; * = $p < 0.05$. Error bars represent standard error of the mean. Occupancy plots were generated using Wintrack freeware (<http://www.dpwolfer.ch/wintrack/>).

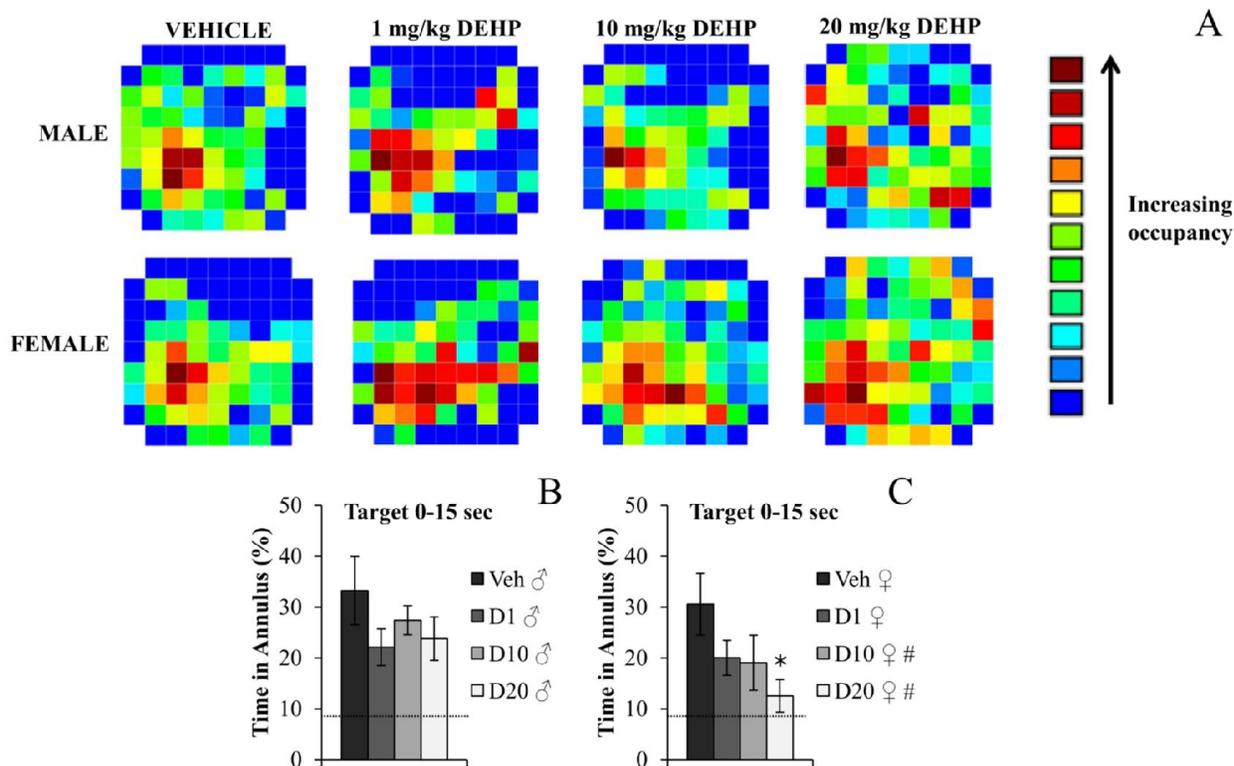


Figure 5.4: Retention of platform location during juvenile probe test 0-15 seconds. Occupancy plots of the first 15 seconds of the probe test (A). Target annulus is located in the southwest quadrant. Percent time spent swimming in the target annulus during the first 15 seconds of the water maze probe test (PND34) for male (B) and female (C) rats exposed to 1, 10, 20 mg/kg di(2-ethylhexyl) phthalate (DEHP) compared to controls (Veh). The dashed line represents chance performance (8%). The # denotes treatment group was not significantly different from chance performance. All groups (except the 10 and 20 mg/kg DEHP female groups) spent significantly more time in the target annulus than what was expected due to random chance. Female rats treated with 20 mg/kg DEHP spent significantly less time searching in the target annulus during the first 15 seconds of the probe test compared to controls. No differences were detected in male rats. D: DEHP; * = $p < 0.05$. Error bars represent standard error of the mean. Occupancy plots were generated using Wintrack freeware (<http://www.dpwolfer.ch/wintrack/>).

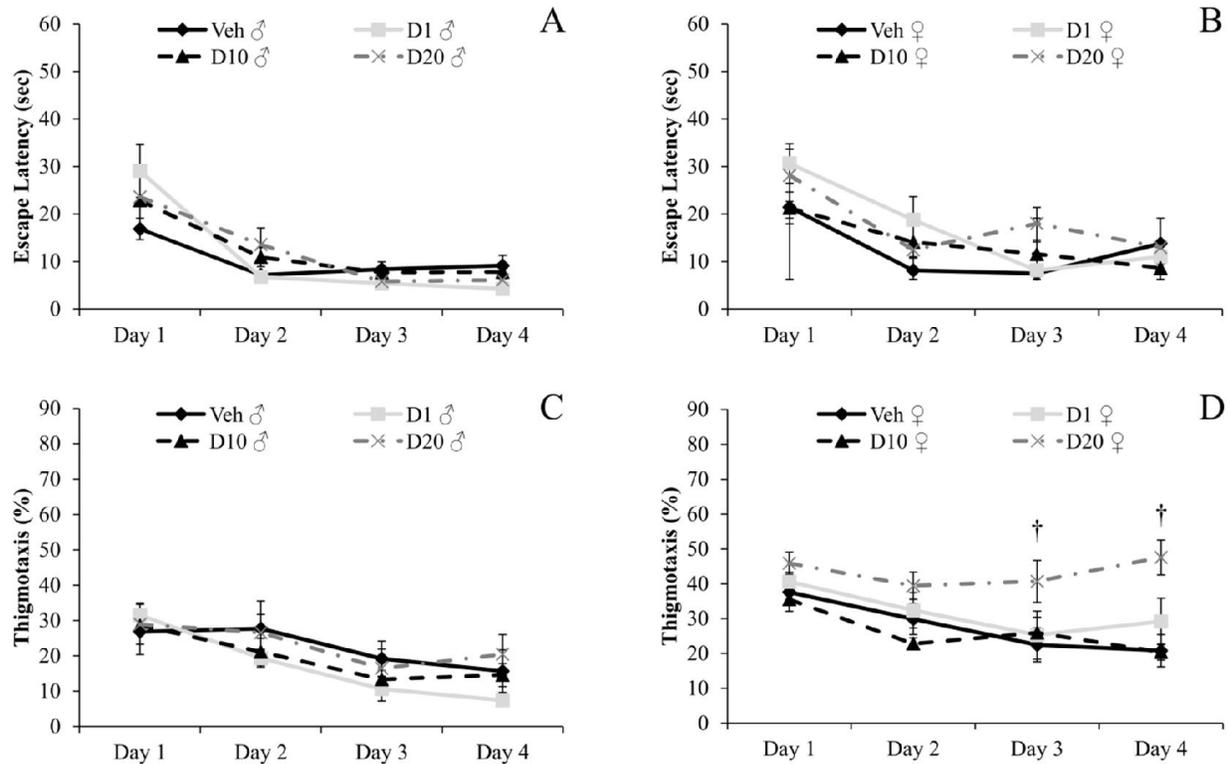


Figure 5.5: Daily latencies and thigmotaxis during adult spatial water maze acquisition. Latency to reach the hidden platform averaged across four trials of daily training from PND74 to PND77 for male (A) and female (B) rats exposed to 1, 10, 20 mg/kg di(2-ethylhexyl) phthalate (DEHP) compared to controls (Veh). Percent time spent swimming around the perimeter of the pool (thigmotaxis) averaged across four trials of daily training for male (C) and female (D) rats. The † denotes a significant difference between the 20mg/kg DEHP dose and vehicle controls ($p < .05$). Female rats treated with 20 mg/kg DEHP showed increased thigmotaxis on training days 3 and 4 compared to controls. There were no differences in latency to locate the hidden platform between DEHP-treated females and controls. There was no effect of DEHP treatment on performance during the acquisition training session in male rats. D: DEHP. Error bars represent standard error of the mean.

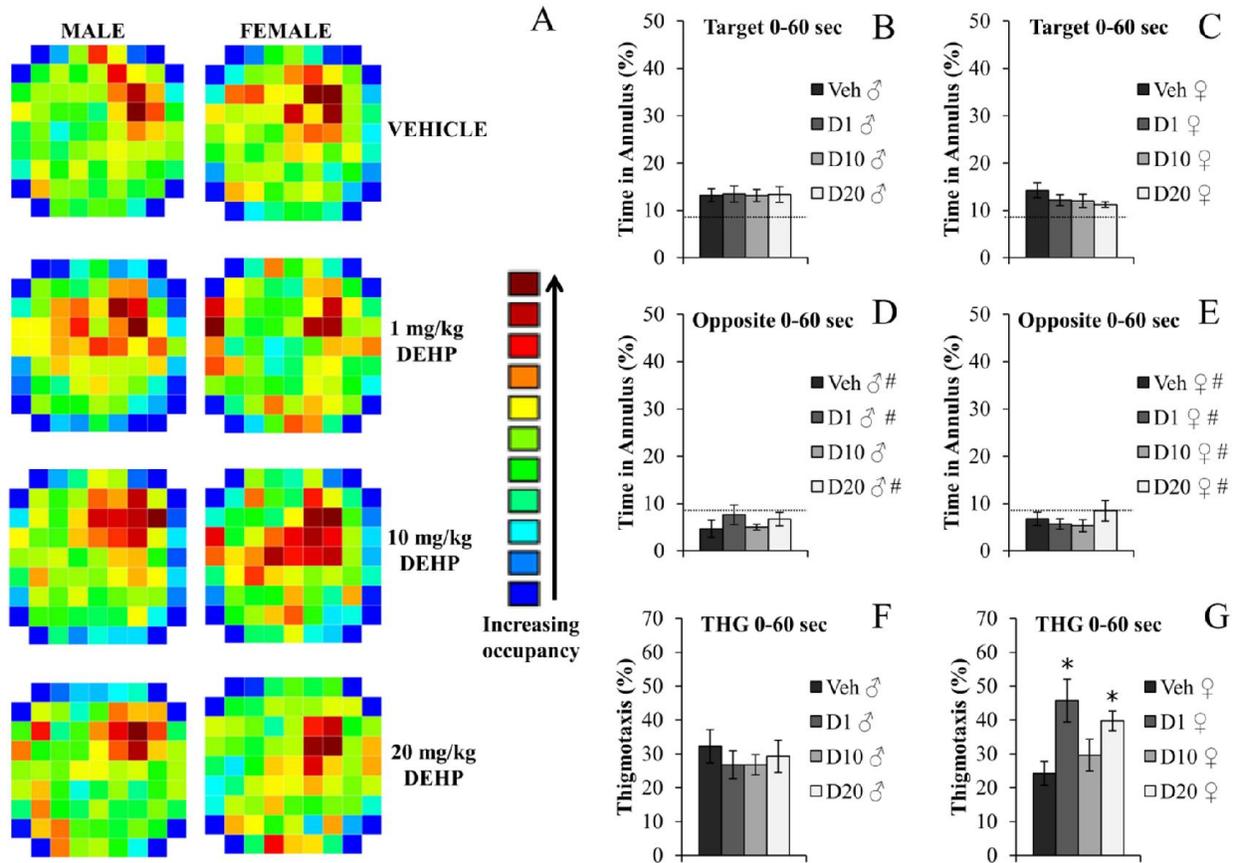


Figure 5.6: Retention of platform location during adult probe test 0-60 seconds. Occupancy plots for the entire 60 second probe test (A). Target annulus is located in the northeast quadrant. Percent time spent swimming in the target annulus during the 60 second water maze probe test (PND78) for male (B) and female (C) rats exposed to 1, 10, 20 mg/kg di(2-ethylhexyl) phthalate (DEHP) compared to controls (Veh). Percent time spent swimming in the opposite annulus during the 60 second probe test for male (D) and female (E) rats. Percent time spent swimming around the perimeter of the pool (thigmotaxis) during the 60 second probe test for male (F) and female (G) rats. The dashed line represents chance performance (8%). The dashed line represents chance performance (8%). The # denotes treatment group was not significantly different from chance performance. All groups spent significantly more time in the target annulus than what was expected due to random chance. Female rats treated with 1 and 20 mg/kg DEHP showed increased thigmotaxis during the 60 second probe test compared to controls. There were no differences in the amount of time spent in the target annulus between DEHP-treated females and controls. There was no effect of DEHP treatment on performance during the probe test in male rats. D: DEHP; * = $p < 0.05$; + = $p < 0.06$. Error bars represent standard error of the mean. Occupancy plots were generated using Wintrack freeware (<http://www.dpwolfer.ch/wintrack/>).

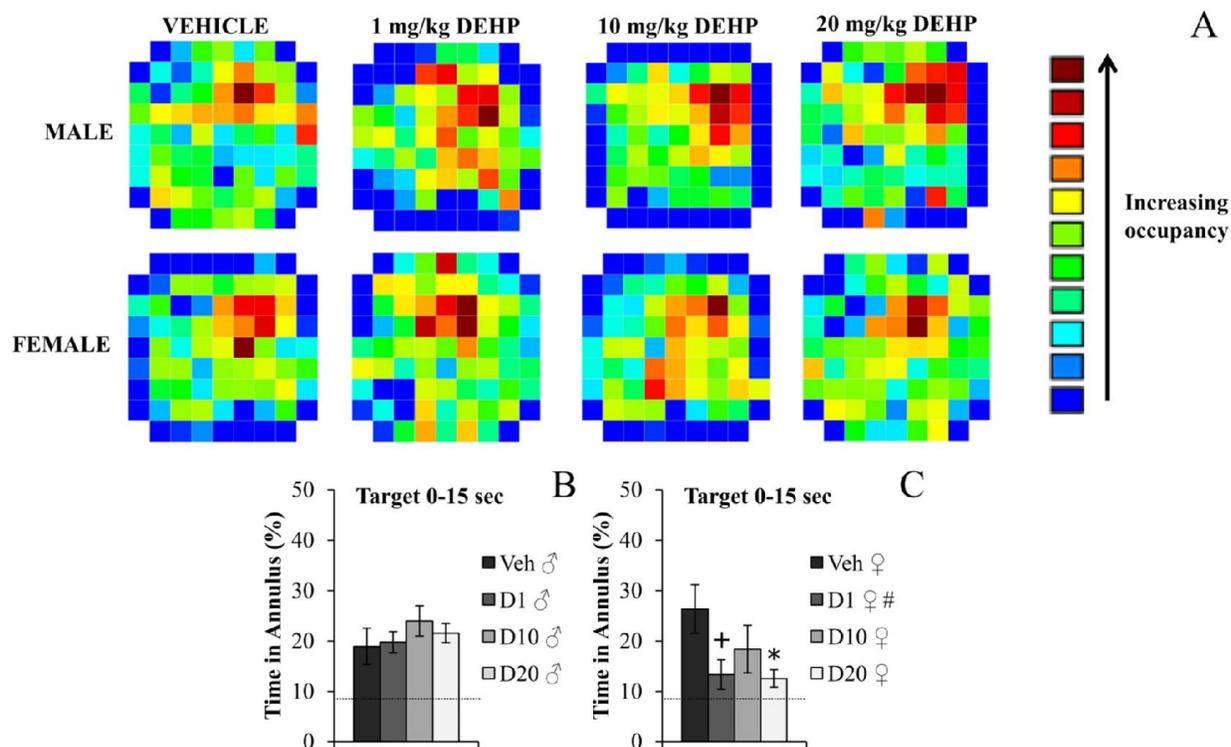


Figure 5.7: Retention of platform location during adult probe test 0-15 seconds. Occupancy plots of the first 15 seconds of the probe test (A). Target annulus is located in the northeast quadrant. Percent time spent swimming in the target annulus during the first 15 seconds of the water maze probe test (PND78) for male (B) and female (C) rats exposed to 1, 10, 20 mg/kg di(2-ethylhexyl) phthalate (DEHP) compared to controls (Veh). The dashed line represents chance performance (8%). The dashed line represents chance performance (8%). The # denotes treatment group was not significantly different from chance performance. All groups (except the 1 mg/kg DEHP female group) spent significantly more time in the target annulus than what was expected due to random chance. Female rats treated with 1 and 20 mg/kg DEHP spent significantly less time searching in the target annulus during the first 15 seconds of the probe test compared to controls. No differences were detected in male rats. D: DEHP; * = $p < 0.05$; + = $p < 0.06$. Error bars represent standard error of the mean. Occupancy plots were generated using Wintrack freeware (<http://www.dpwolfer.ch/wintrack/>).

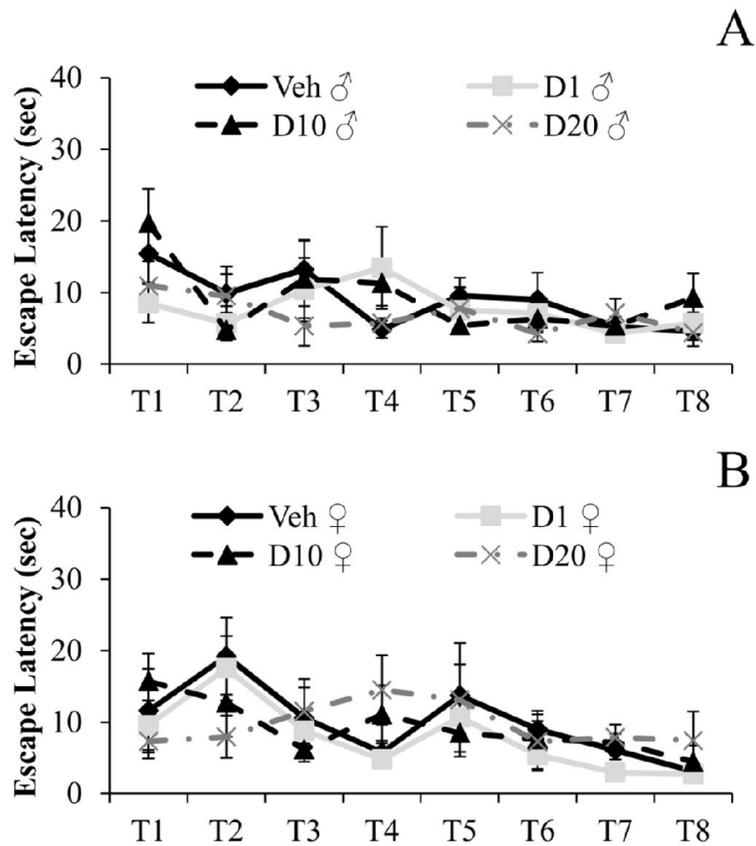


Figure 5.8: Latencies during cued water maze acquisition. Latency to reach the hidden platform across eight trials for male (A) and female (B) rats exposed to 1, 10, 20 mg/kg di(2-ethylhexyl) phthalate (DEHP) compared to controls (Veh). There were no differences in latency to locate the visible platform across the 8 training trials in male or female rats. Error bars represent standard error of the mean. D: DEHP; T: trial. Error bars represent standard error of the mean.

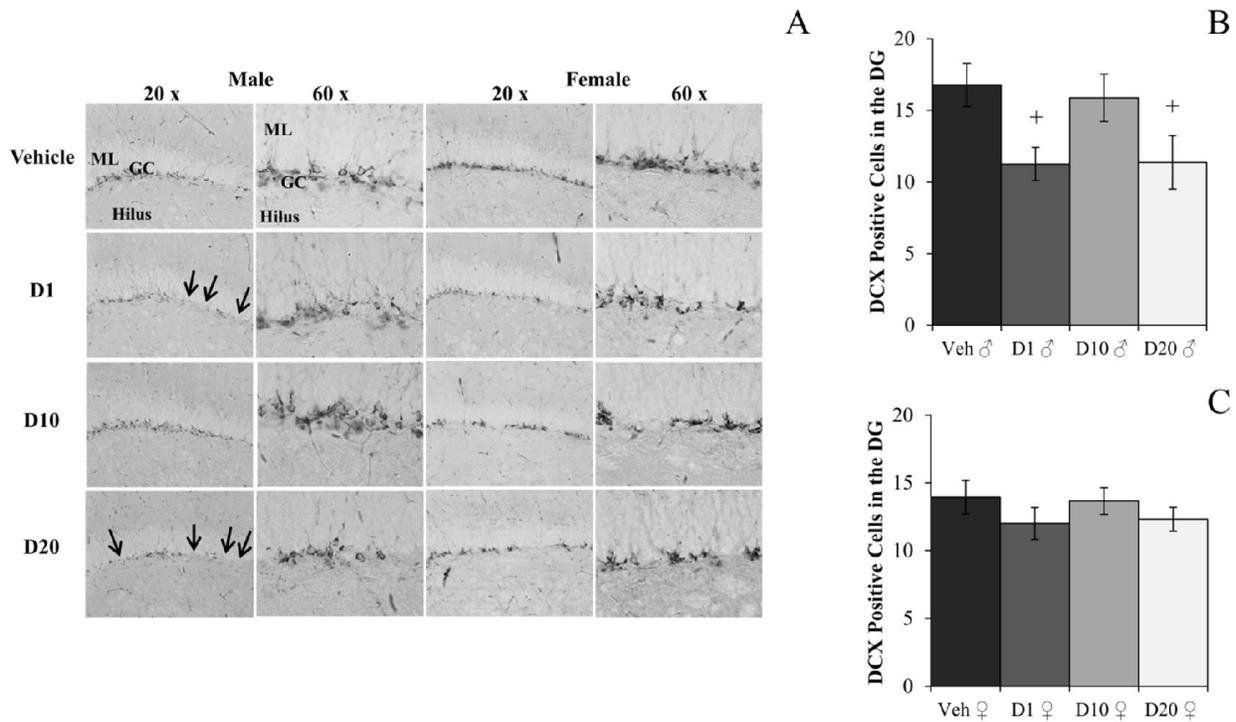


Figure 5.9: Doublecortin staining in the dentate gyrus. (A) Representative photomicrographs (20 x and 60 x) of doublecortin (DCX) staining in the dentate gyrus (DG). Quantification of the number of DCX-positive cells in the DG granule cell layer (GC) in male (B) and female (C) rats exposed to 1, 10, 20 mg/kg di(2-ethylhexyl) phthalate (DEHP) compared to controls (Veh). Male rats exposed to DEHP showed a significant reduction in DCX positive cells in the GC compared to controls indicated by downward arrows in image. There were no differences detected in the total number of DCX-positive cells in the DG of female rats treated with DEHP compared to controls. D: DEHP; + = $p < 0.06$. Error bars represent standard error of the mean.

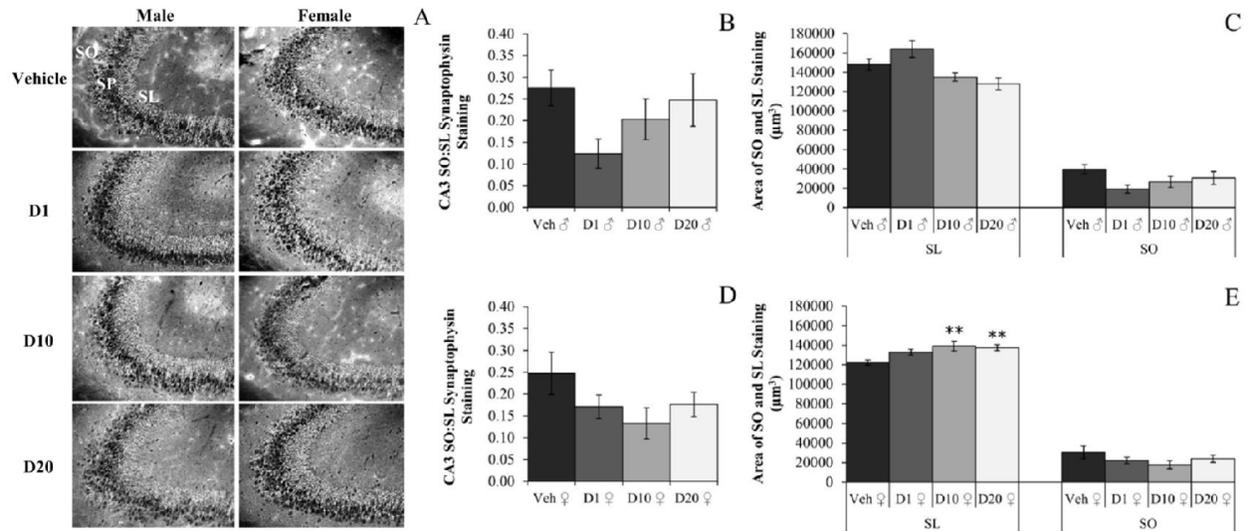


Figure 5.10: Synaptophysin staining in the CA3 region. (A) Representative photomicrographs of synaptophysin staining in the CA3 region (10 x). The ratio of synaptophysin staining in the SO to the SL quantified for male (B) and female (C) rats exposed to 1, 10, 20 mg/kg di(2-ethylhexyl) phthalate (DEHP) compared to controls (Veh). The area of synaptophysin staining (μm^2) in the stratum oriens (SO) and the stratum lucidum (SL) of the CA3 region quantified for male (D) female (E) rat. Female rats exposed to 10 and 20 mg/kg DEHP showed a significant increase in the CA3 SL synaptophysin staining compared to controls. No differences in synaptophysin staining were detected between DEHP-treated males and controls. D: DEHP; ** = $p < 0.01$. Error bars represent standard error of the mean.

Chapter VI

Perseverative Behaviour and Hyperactivity in Rats

Following Acute Postnatal Exposure to Phthalates

In Chapters II-V, we examined the impact of postnatal DEHP exposure on cognitive spatial functioning and its underlying hippocampal neurocircuitry. An interesting pattern of DEHP-induced morphological and behavioural changes emerged. DEHP treatment in male rats did not modify behavioural performance on a spatial water maze task, despite altered hippocampal connectivity observed in juvenile and adult male rat brains. In contrast, exposure to DEHP had a biphasic response on spatial function in female rats, with the lowest (1 mg/kg) and highest (20 mg/kg) doses impairing spatial memory performance, while a mid-range dose (10 mg/kg) had no behavioural effects. Treatment with 10 mg/kg DEHP elevated levels of hippocampal lipids in juvenile female rats, which may have a downstream protective effect on hippocampal neurobiology and spatial function. In this chapter, we explored the impact of postnatal DEHP exposure on other behavioural measures of cognitive functioning, and its underlying neurocircuitry.

The dopaminergic pathways play a critical role in reward-based learning and movement. Dopaminergic axons from VTA neurons project to the prefrontal cortex and the limbic system, including the nucleus accumbens, and form the basis of the reward pathway in the brain (Ikemoto, 2007; Woodward et al., 2006). Dopamine efflux has been reported to increase during food-based operant extinction procedures (Ahn & Phillips, 2007). Dopamine agonists increase non-rewarded operants while dopamine receptor antagonists suppress the extinction of operants (Duarte et al.,

2003; Joel & Doljansky, 2003; Rick et al., 2006). Pre-treatment with D₁- or D₂-like dopamine receptor antagonists has been shown to reduce lever pressing associated with food-based conditioning procedures (Beninger et al., 1987). Dopamine transporter knockout mice, that show elevated levels of dopamine in the synaptic cleft, also show resistance to extinction for food-based operants (Hironaka et al., 2004). Systemic injections of the NMDA receptor antagonist, MK-801 (which elevates dopamine efflux in the nucleus accumbens) also increases lever pressing during non-rewarded, extinction session. This effect can be suppressed experimentally through the administration of dopamine antagonists (Davis-MacNevin et al., 2013; Holahan et al., 2010; 2011; 2012; Yan et al., 1997).

Known environmental toxicants, such as DCHP, have been shown to disrupt dopaminergic function in male rats following a single treatment (87 nmol/10 μ L; Ishido et al., 2004). A reduction in tyrosine hydroxylase staining and an increase in TUNEL-positive staining were observed in the substantia nigra 8 weeks after DCHP treatment, indicating probable degeneration of dopaminergic neurons in this brain region. Elevated levels of spontaneous motor activity were also observed during the nocturnal periods following DCHP exposure (Ishido et al., 2004). Activity levels increased in a dose-dependent manner with the highest dose (29 μ g/kg) displaying the largest increase in hyperactivity.

Objectives and Hypotheses

The primary goal of this work was to establish whether acute postnatal exposure to DEHP would alter performance on a reward-based operant conditioning task in male and female rats. It was expected that DEHP exposure would interfere with reward-based learning in a dose dependent manner (with the highest dose leading to the greatest disruption in learning), and these behavioural

changes would be more pronounced in males than in females. Locomotor activity was also evaluated throughout the operant conditioning task and it was expected that DEHP treatment would produce elevated levels of activity in both male and female rats. Given that behavioural performance on the operant conditioning task is sensitive to dopamine function, abnormalities in dopaminergic neurocircuitry were also expected in male and female rats (with the highest dose showing the largest deficit).

Materials and Method

Animals

Six untimed pregnant female Long Evans rats (approximately 13 days gestation) were purchased from Charles River Laboratories (St. Constant, Québec) for this experiment. The pregnant females were singly-housed in polycarbonate 48 x 26 x 20 cm³ cages within a temperature controlled environment. The day the pups were born was recorded as PND0. Pups (n = 30 males; n = 30 females) were weaned on PND22 and group-housed, with males and females in separate cages. All rats were on a 12 hour light-dark cycle (lights on at 8:00 a.m.) with *ad libitum* access to food (Purina rat chow) and tap water. All experiments were conducted at Carleton University and approved by the Institutional Animal Care Committee, as per guidelines established by the Canadian Council on Animal Care.

DEHP Injections

Rats were injected i.p. with 1, 10 or 20 mg/kg DEHP (Sigma-Aldrich; St. Louis, MO, USA) or vehicle (corn oil) daily from PND16 to PND22 inclusive. Each rat was injected in the late morning (between 10:30 and 11:30 AM) and was returned to their home cage with their mother

following the injection. The 1 mg/kg (n = 7 males; n = 7 females), 10 mg/kg (n = 8 males; n = 8 females) and 20 mg/kg (n = 8 males; n = 8 females) DEHP solutions were prepared fresh using DEHP (1000 mg/kg) and corn oil immediately before each injection. Control rats (n = 7 males; n = 7 females) were injected with corn oil.

Operant Chambers

Six operant conditioning chambers (Coulbourn Instruments, Pennsylvania, USA; 30.5 cm wide, 30.5 cm high and 25.5 cm deep) were used for each testing session. Each chamber was located inside a second insulated chamber to minimize outside noise during testing. Each operant conditioning chamber was equipped with two levers (one to the right and one to the left of the food hopper). When the left lever was pressed twice (FR2 schedule) the house light turned off, the lights directly above the left lever changed from red to green, and a 45 mg chocolate pellet (BioServe, New Jersey) was released into the food hopper. The right lever was inactive and did not lead to a food reward no matter how many times it was pressed. The conditioning chambers were equipped with infrared detectors for collection of locomotor activity data throughout each session. Cumulative correct lever presses and locomotor activity were recorded for each rat every 5 minutes during the testing sessions, and served as the operational measures of task performance.

Behavioural Procedures

Figure 6.1 shows a timeline of the experimental procedures. The rats were singly-housed and food restricted to 90 % of their baseline weight 10 days prior to the start of operant testing. Rats were weighed daily and fed approximately 8-15 grams of standard rat chow depending on

their daily weight. The rats also received 5 chocolate pellets per day and had *ad libitum* access to tap water.

Acquisition. Rats were trained for 5 days (PND55-59) to lever press for chocolate pellets in an operant conditioning chamber on a FR2 schedule (30 minutes each day). One chocolate pellet was placed on the correct (left) lever prior to the start of testing on each day to prime the rat to press the correct lever.

Extinction. Seventy-two hours following the final acquisition session, rats underwent two extinction sessions (PND62-63) for 30 minutes each day. When the rat pressed the correct lever the house light turned off and the lights above the lever changed from red to green, but no food reward was given.

Re-acquisition. Following a 7 day recovery period, rats were re-trained to lever press for chocolate pellets for 5 days (PND71-75) as described in the acquisition section above.

Progressive Ratio. Immediately following the final re-acquisition session, rats were given free access to food for 48 hours. Seventy-two hours (PND78) later, all rats underwent one 45 minute progressive ratio test where two additional correct lever presses from the previous session were required to obtain a chocolate pellet (PR2 schedule).

Tissue Processing

Rats were euthanized on PND78 by live decapitation immediately after the progressive ratio test. Brains were extracted and postfixed overnight in 4 % paraformaldehyde in 0.01 M phosphate buffer solution. Brains were then cryoprotected in 30 % sucrose in 0.01 M PBS and then stored at 4 °C. The brain was sectioned into 60 µm coronal sections using a Leica CM1900

cryostat (Weztler, Germany). Sections were stored in a 0.1 % sodium azide solution in 0.01 M PBS at 4°C until staining.

Tyrosine Hydroxylase Immunofluorescent Labeling

Sections from the striatum were chosen for each group and labeled for tyrosine hydroxylase (TH), a dopamine precursor protein expressed in dopaminergic neurons. Sections were washed in 0.1 %/T-PBS, pH 7.4, for 15 minutes then transferred to AFB in T-PBS; Vector Laboratories, Burlingame, CA, USA) for 60 minutes to prevent non-specific binding. Tissue was incubated overnight in a rabbit polyclonal anti-TH primary antibody (1:1000; Millipore; Billerica, MA, USA) in AFB/T-PBS at room temperature. Sections were washed the following day for 15 minutes in T-PBS and incubated for 2 hours at room temperature with a fluorescently-labeled (Alexa Fluor 488) goat anti-mouse secondary antibody (1:500; Invitrogen; Burlington, ON, Canada) in AFB/T-PBS. Sections were rinsed for 15 minutes in PBS, mounted onto glass slides and coverslipped using Fluormount (Sigma-Aldrich) mounting medium. Slides were stored at 4°C in slide boxes to protect from light exposure.

Tyrosine Hydroxylase Immunohistochemical Staining

Sections from the substantia nigra pars compacta (SNc) and the ventral tegmental area (VTA) were stained for TH. Sections were washed 3 times in T-PBS for 5 minutes per wash then incubated in 0.3 % hydrogen peroxide/ T-PBS for 30 min to remove endogenous peroxidase activity. Sections were washed 3 more times in T-PBS for 5 minutes per wash, transferred into 1x AFB in T-PBS for 60 minutes then incubated overnight at room temperature in rabbit polyclonal anti-TH primary antibody (1:1000) in AFB/ T-PBS.

The following day, tissue was washed 3 times in T-PBS for 5 minutes per wash then incubated for 2 hours with a biotinylated goat anti-mouse secondary antibody (1:500; Vector Laboratories) in AFB/ P-TBS. Sections were washed 3 times for 5 minutes each wash in T-PBS then incubated in ABC in T-PBS for 2 hours. Sections were rinsed three times in PBS for 5 minutes each and staining was visualized with a 0.5 %, 3,3'-diaminobenzene solution. Sections were washed in PBS for 5 minutes then mounted onto glass slides. TH sections were counterstained with cresyl violet the following day.

Tyrosine Hydroxylase Immunofluorescent Quantification

Fluorescent images of the striatum were captured at 10 x magnification using a Retiga-2000R camera and an Olympus BX61 microscope, using the same exposure time to reduce photobleaching and to equalize intensities across each section. Coronal sections were sampled from the striatum of each group. Approximate bregma levels were +0.84 to +1.08. Five circles (radius = 5 mm) were generated across the striatum and measures of staining intensity were collected at each circle. A sixth circle was generated over the anterior commissure to normalize measures of staining intensity. An average normalized TH intensity profile was then generated for each section. An experimenter who was blind to group assignment carried out all analyses.

Tyrosine Hydroxylase Immunohistochemical Quantification

Images of the SNc and VTA were captured at 4 x magnification using an Olympus U-CMAD3 camera and an Olympus BX51 microscope. Coronal sections were sampled from the SNc and VTA of each rat. Approximate bregma levels were -5.20 to -5.30. The optical fractionator method (West et al., 1991) was used to estimate the total number of TH-positive and cresyl violet

positive neurons in these brain regions. The entire SNc and VTA were outlined using the tracing function in the Stereo Investigator software at 4 x magnification. Counting parameters were set to a counting frame of 60 x 60 μm , a grid size of 100 x 100, and a dissector depth of 15 μm centered between top and bottom guard zones. TH-positive and cresyl violet positive neurons were counted at 60x magnification (oil immersion) when the top of the cell body of stained neurons was in focus within the counting frame and the dissector depth. An experimenter who was blind to group assignment carried out all analyses. Analyses were conducted using the estimate of total neurons by number weighted section thickness as it was a more accurate reflection of the total number of neurons. Final quantification values were represented as the total number of neurons per 10,000 μm^2 to adjust for slight variances between each rat.

Statistical Analyses

Operant data from the acquisition, extinction, and re-acquisition training sessions were analyzed using a two-way ANOVA, with dose (0, 1, 10, and 20 mg/kg DEHP) as the between-groups factor and training day (1-4) as the within-groups factor. The progressive ratio data was analyzed in 5 minute intervals across the single testing session using a two-way ANOVA, with dose (0, 1, 10, and 20 mg/kg DEHP) as the between-groups factor and time interval (5, 10, 15, 20, 25, 30, 35, 40, and 45 minutes) as the within-groups factor. TH staining was examined using one-way ANOVAs, with dose (0, 1, 10, and 20 mg/kg DEHP) as the between-groups factor. For all analyses, each gender was assessed separately. Post hoc analyses will be conducted using Dunnett's t tests (using the vehicle group as the control group) where appropriate.

Results

Elevated Lever Pressing in DEHP-Treated Female Rats

Acquisition. Rats were trained over 5 days (PND55-59) to lever press for a chocolate pellet reward. Figures 6.2A and 6.2B show correct lever presses during each acquisition training session in male and female rats. No significant group differences in DEHP dose were present on the first training day in male ($p > 0.05$; Figure 6.2A) or female rats ($p > 0.05$; Figure 6.2B). On days 2 to 5 of acquisition training, there was a main effect of day ($F(3,78) = 20.09, p < 0.001$) and a main effect of dose ($F(3,26) = 4.20, p < 0.05$) in male rats (Figure 6.2A). Post hoc analyses revealed no significant differences between each of the DEHP doses (1, 10, 20mg/kg) and male controls. The significant main effect of dose was attributed to the increase in lever pressing in rats treated with 10mg/kg dose DEHP compared to rats treated with 1mg/kg DEHP ($p < 0.05$). These findings indicate that male rats treated with DEHP (regardless of the dose) acquired the task similarly during the 5 day acquisition period compared to the control group. Analysis of the correct lever presses in female rats (Figure 6.2B) on days 2 to 5 of acquisition revealed a main effect of day and ($F(1,26) = 19.84, p < 0.001$) dose ($F(3,26) = 4.714, p < 0.01$), with an increase in correct lever presses on days 3 and 5 following treatment with 10mg/kg DEHP compared to controls ($p < 0.05$).

Extinction. Rats underwent two extinction sessions (in which lever pressing did not result in a chocolate pellet reward) seventy-two hours after the last acquisition day. Figures 6.2A and 6.2B show correct lever presses during each extinction session in male and female rats. In male rats, DEHP treatment did not affect non-reward operants during either extinction session ($p > 0.05$; Figure 6.2A). However, there was a main effect of day ($F(1,26) = 34.11, p < 0.001$), with all male rats (regardless of DEHP treatment group) showing a significant decrease in lever pressing between day 1 and 2 of the extinction session (Figure 6.2A). Female rats treated with 10 mg/kg

DEHP failed to extinguish high levels of lever pressing in the absence of a reward during either extinction session (main effect day: $F(1,26) = 19.84, p < 0.001$; main effect dose: $F(3,26) = 4.71, p < 0.01$; Figure 6.2B)

Re-acquisition. One week after the last extinction session, all rats were re-training over 5 training sessions to lever press for a chocolate pellet reward. Figures 6.3A and 6.3B show correct lever presses during each re-acquisition training session in male and female rats. Performance on the first day of re-acquisition was compared to performance during the previous extinction session. No carry-over effects were observed in males ($p > 0.05$; Figure 6.3A). In female rats (Figure 6.3B) lever pressing remained elevated on the first day of re-acquisition in 10mg/kg group compared to female controls. ($p < 0.05$). Analyses of correct lever presses on days 2 to 5 of re-acquisition revealed no effect of DEHP treatment in male or female rats ($p > 0.05$).

Progressive ratio. All rats underwent a single 45-minute progressive ratio training session seventy-two hours after the last re-acquisition session. This type of training was used to determine the length of time to extinction of lever pressing for a food reward, when the number of lever presses before the reward (and time between the instances of reward) was increased. This assesses the motivational component of reward-based learning. Cumulative correct lever presses were recorded every 5 minutes across the 45 minute testing session for a total of 9 time intervals of 5 minutes each.

Figures 6.4A and 6.4B show cumulative correct lever presses for each 5 minute time interval during progressive ratio testing in male and female rats. In female rats (Figure 6.4B) there was a significant interaction between time interval and dose ($F(24, 208) = 2.40, p < 0.001$). Female rats treated with the highest dose of DEHP (20 mg/kg) showed an increase in cumulative correct lever presses across all 5 minute time intervals when compared to vehicle-treated female rats ($p <$

0.05). The 20 mg/kg female group continued to press at a high rate throughout the 45 min test which suggests that this group of rats was very highly motivated to receive a reward. Female rats treated with 1 and 10 mg/kg DEHP and female controls showed breakpoints (indicating the behavioral response was beginning to be extinguished) approximately 35 min into the test. No differences in progressive ratio responding were observed in males ($p > 0.05$; Figure 6.4A).

DEHP-Induced Hyperactivity in Male and Female Rats

Acquisition. Figures 6.2C and 6.2D show locomotor activity during each acquisition training session in male and female rats. Treatment with DEHP elevated locomotor activity during the acquisition training sessions in male and female rats (Figure 6.2). In male rats, there was a main effect dose ($F(3,26) = 4.18, p < 0.05$) and day ($F(4,104) = 7.49, p < 0.001$). Male rats exposed to 20 mg/kg DEHP were more active on days 1, 2, 3 and 5 of acquisition training compared to controls ($p < 0.05$; Figure 6.2C). There was also a main effect dose ($F(3,26) = 3.65, p < 0.05$) and day ($F(4,104) = 7.19, p < 0.001$) in female rats. Female rats treated with 10mg/kg DEHP displayed elevated locomotor activity on days 2 and 5 compared to controls ($p < 0.05$; Figure 6.2D). Similarly, female rats treated with 20mg/kg DEHP were more active than controls on days 2 and 5, and days 1, 3, 4 and 5 ($p < 0.05$; Figure 6.2D).

Extinction. Figures 6.2C and 6.2D show locomotor activity during each extinction session in male and female rats. In male rats there was a significant main effect of day ($F(1,26) = 24.10, p < 0.001$), with all rats showing a decrease in locomotor activity between days 1 and 2 of extinction (Figure 6.2C). Analysis of locomotor activity in female rats revealed a significant main effect of dose ($F(3,26) = 3.80, p < 0.05$) and day ($F(1,26) = 6.64, p < 0.05$). Female rats treated with 10 and

20 mg/kg DEHP displayed elevated levels of locomotor activity with respect to vehicle controls on days 1 and 2 of extinction ($p < 0.05$; Figure 6.2D).

Re-acquisition. Figures 6.3C and 6.3D show locomotor activity during each re-acquisition training session in male and female rats. In male rats there was a main effect of day ($F(4,104) = 5.25, p < 0.001$) and dose ($F(3,26) = 3.08, p < 0.05$). Treatment with 10 mg/kg DEHP led to hyperactivity in male rats during days 4 and 5 compared to controls ($p < 0.05$; Figure 6.3C). Similarly, male rats treated with 20 mg/kg DEHP were more active on days 2, 4, and 5, respectively compared to controls ($p < 0.05$; Figure 6.3C). Analysis of locomotor activity in female rats revealed a significant interaction between training day and DEHP dose ($F(12,104) = 4.216, p < 0.001$) with DEHP-induced hyperactivity in the 20 mg/kg DEHP dose on days 4 and 5 of re-acquisition with respect to the female control group ($p < 0.05$; Figure 6.3D).

Progressive Ratio. Figures 6.4C and 6.4D show the cumulative correct lever presses for each 5 minute time interval during the progressive ratio training session in male and female rats. Analyses revealed a significant interaction between time interval and dose in female rats ($F(24,208) = 4.82, p < 0.0001$) with the 20mg/kg DEHP dose displaying significantly elevated levels of activity at each time interval compared to female controls ($p < 0.05$; Figure 6.4D). No differences in locomotor activity were observed between DEHP-treated male rats and male controls during progressive ratio training ($p > 0.05$; Figure 6.4C).

DEHP Treatment Reduced TH-Positive Neurons in the SNc and VTA of Male Rats

Figure 6.5A shows representative images of TH and cresyl violet staining in the SNc. The ratio of the estimated number of TH-positive neurons to the estimated number cresyl violet positive neurons (TH:CV) was calculated to adjust for slight variances between each rat. There was a

significant effect of DEHP treatment on the TH:CV ratio in male rats ($F(3,23) = 5.26$, $p < 0.01$; Figure 6.5D). Male rats exposed to 10 mg/kg and 20mg/kg DEHP had smaller TH:CV ratios compared to control males ($p < 0.01$; $p < 0.05$; respectively). The estimated number of cresyl violet positive neurons was also evaluated to examine whether DEHP-induced reductions of SNc neurons were specific to dopaminergic neurons or if other cell types in this brain region were affected. The number of cresyl violet positive neurons in the SNc of male rats was not affected ($p > 0.05$; Figure 6.5B), suggesting that dopaminergic neurons were uniquely sensitive to DEHP treatment. No differences were observed in the TH:CV ratio, or cresyl violet positive neurons in the SNc of female rats ($p > 0.05$; Figure 6.5C; 6.5E).

Figure 6.6A shows representative images of TH and cresyl violet staining in the VTA. There was a significant effect of DEHP treatment in male rats on the TH:CV ratio ($F(3,23) = 7.30$, $p < 0.001$; Figure 6.6D). Male rats treated with 10 mg/kg and 20mg/kg DEHP displayed smaller TH:CV ratios compared to male controls ($p < 0.001$; $p < 0.05$; respectively), indicating a reduction in TH-positive neurons in the VTA. No differences were observed in the TH:CV ratio in the VTA of female rats ($p > 0.05$; Figure 6.6E). The number of cresyl violet positive neurons in the VTA of male and female rats was not affected ($p > 0.05$; Figure 6.6B; 6.6C).

Figure 6.7A shows representative images of TH-positive axonal terminal fields in the striatum. There was no effect of DEHP treatment on TH-positive labeling in the striatum of male and female rats ($p > 0.05$; Figure 6.7B and 6.7C), suggesting that exposure to DEHP did not interfere with TH-positive axonal projections from the SNc and VTA to the striatum in either gender.

Discussion

The present set of experiments evaluated the effect of acute postnatal administration of DEHP on a reward-based learning task, and the underlying neurobiology in male and female rats. DEHP treatment had no effect on lever pressing during the rewarded and non-rewarded operant sessions in male rats; but led to a heightened level of responding in female rats, particularly at the 10 mg/kg dose. Treatment with 10mg/kg DEHP increased rewarded operants during day 3 and 5 of the acquisition phase in female rats, suggesting this group more readily acquired the action-reward association. The elevated levels of responding persisted in female rats treated with 10 mg/kg DEHP during non-rewarded extinction sessions. A normal control rat will learn during extinction training that the operant is no longer predictive of the reward and will gradually reduce its behavioural response. In this case, exposure to 10 mg/kg DEHP in female rats appeared to induce a behavioral pattern characteristic of perseveration, such that these rats failed to extinguish this type of responding even after removal of the reward. Locomotor activity recorded during acquisition and extinction supports the idea of behavioral perseveration in female rats treated with 10 mg/kg DEHP. This increase in lever pressing in the 10 mg/kg group was not simply a by-product of increased locomotor activity, as the 20 mg/kg group showed an increase in activity, but no subsequent increase in lever pressing.

Female rats that were exposed to 20 mg/kg DEHP only exhibited elevated levels of lever pressing during the progressive ratio training session in which two additional lever presses from the previous session were required to obtain a chocolate pellet. Progressive ratio training is used to determine the length of time to extinction when the number of lever presses before the reward (and time between the instances of reward) is increased. Breakpoints were observed for female rats treated with 1 and 10 mg/kg DEHP and female controls at approximately 35 min into the

progressive ratio session, indicating the behavioral response was beginning to be extinguished. Female rats treated with 20 mg/kg DEHP continued to lever press at a high rate throughout the 45 min session, demonstrating a response characteristic of perseveration.

Overall, these behavioural findings suggest an inverted u-shaped dose-response curve in female rats in which acute postnatal exposure to a low dose of DEHP (1 mg/kg) appeared to have no behavioural effects, a mid-range dose of DEHP (10 mg/kg) significantly altered operant performance and a high dose of DEHP (20 mg/kg) produced very minimal alterations in reward-based learning. Interestingly, in Chapter V we reported a contrasting DEHP dose-response curve when evaluating spatial function using identical doses in female rats. Treatment with 10 mg/kg DEHP had no effect on spatial functioning in female rats, whereas significant spatial memory impairments were observed following treatment with 1 and 20 mg/kg DEHP. It was surprising that the same DEHP doses produced radically different behavioural outcomes on spatial and reward-based learning tasks in female rats. These data emphasize the importance of investigating the impact of DEHP exposure on a variety of behavioural measures of cognitive performance when evaluating its toxic potential.

Pretreatment with the NMDA receptor antagonist MK-801, which directly elevates dopamine levels in the nucleus accumbens, has been shown to induce perseverative behaviour during non-rewarded, extinction sessions (Davis-MacNevin et al., 2013; Holahan et al., 2010; 2011; 2012; Yan et al., 1997). This phenomenon can be suppressed experimentally through the administration of dopamine antagonists (Davis-MacNevin et al., 2013; Holahan et al., 2010; 2011; 2012). Ishido et al., (2004) reported that a single dose of DCHP (87 nmol/10 μ L) disrupted dopaminergic functioning in the brain and speculated that DCHP treatment may lead to increased extracellular dopamine levels in the striatum (an area which includes the nucleus accumbens).

Therefore, it is probable that dopaminergic dysfunction (and in particular, elevated dopamine levels in the striatum) may underlie this perseverative operant response during non-rewarded extinction sessions in DEHP-treated female rats.

Rat dopaminergic pathways develop during the embryonic stage beginning with the formation of the first dopaminergic neurons on embryonic day 13 in the ventral prosencephalon (Voorn et al., 1987). Over the next few days, dopaminergic projections undergo rapid expansion and growth, and by PND20 attain a mature pattern of connectivity (Voorn et al., 1987). In the present study, male and female rats were exposed to DEHP between PND16 and PND22 before the dopaminergic system was fully developed. It is therefore possible that DEHP treatment may have disrupted dopaminergic development.

DEHP treatment had no effect on the density of dopaminergic neurons in the SNc and VTA, or dopaminergic projections to the striatum in female rats, despite observing significantly elevated levels of lever pressing during rewarded and non-reward sessions. This indicates the probable involvement of other neural mechanisms in mediating DEHP-induced changes in reward-based learning in female rats, such as alterations in dopamine synthesis/release, or the up/down-regulation of dopamine receptors and transporters. Dopaminergic projections to the striatum were spared in male rats; however, treatment with 10 and 20 mg/kg DEHP led to drastic reductions in the density of dopaminergic neurons in the SNc and VTA. This was finding was unexpected as such an extreme reduction in VTA neurons produced no discernable effects on operant learning in male rats. Perhaps some type of functional adaptation had occurred in the brains of DEHP-treated males, diminishing the consequences of VTA neuronal degeneration on operant responding.

Postnatal DEHP exposure also induced a hyperactive behavioural state in male and female rats. Treatment with 10 and 20 mg/kg DEHP robustly elevated locomotor activity throughout the

operant conditioning task in both genders. This is consistent with findings reported by Ishido and colleagues (2004) who found DCHP-induced hyperactivity in male rats that was also accompanied by dopamine cells loss in the SNc, the down-regulation of dopamine D₄ receptors in the midbrain and striatum, and the down-regulation of dopamine transporters in the midbrain.

Dysregulation of dopaminergic pathways have been linked to hyperactivity and other movement disorders (Costall et al., 1984; Ishido et al., 2004; van der Kooji & Glennon, 2007; Zhang et al., 2001). Neonatal chemical lesions of dopaminergic neurons resulted in hyperactivity and increased dopamine D₄ receptors in the striatum of juvenile rats (Zhang et al., 2001). Locomotor hyperactivity can also be induced in juvenile rats by an infusion of dopamine into the nucleus accumbens (Costall et al., 1984). Partial chemical lesions of SNc dopaminergic neurons in adult rats increased the synthesis and the release of dopamine from the remaining SNc neurons to the striatum (Hefti et al., 1980). Together these findings suggest that the DEHP-induced hyperactive behavioural state in male and female rat may be regulated in part by the loss of SNc dopaminergic neurons.

Conclusion

Overall, these data establish a strong link between early developmental exposure to DEHP and disruptions in reward-based learning in female rats. Exposure to 10 mg/kg DEHP induced a behavioral pattern characteristic of perseveration in female rats during non-rewarded operant conditioning procedures. DEHP treatment also led to robust increases in locomotor activity in male and female rats at the highest doses (10 and 20 mg/kg). These behavioural outcomes suggest the involvement of the dopaminergic pathways. Reductions in the density of SNc and VTA dopaminergic neurons were only found in male rats, indicating DEHP-induced perseverative

behaviours and hyperactivity may involve other aspects of dopaminergic function. Possible neuromodulators may include reductions in synaptic efficacy in hippocampal and dopaminergic pathways, modifications in neurotransmitter synthesis/release, and/or the up/down-regulation of receptors. This awaits confirmation by further research.

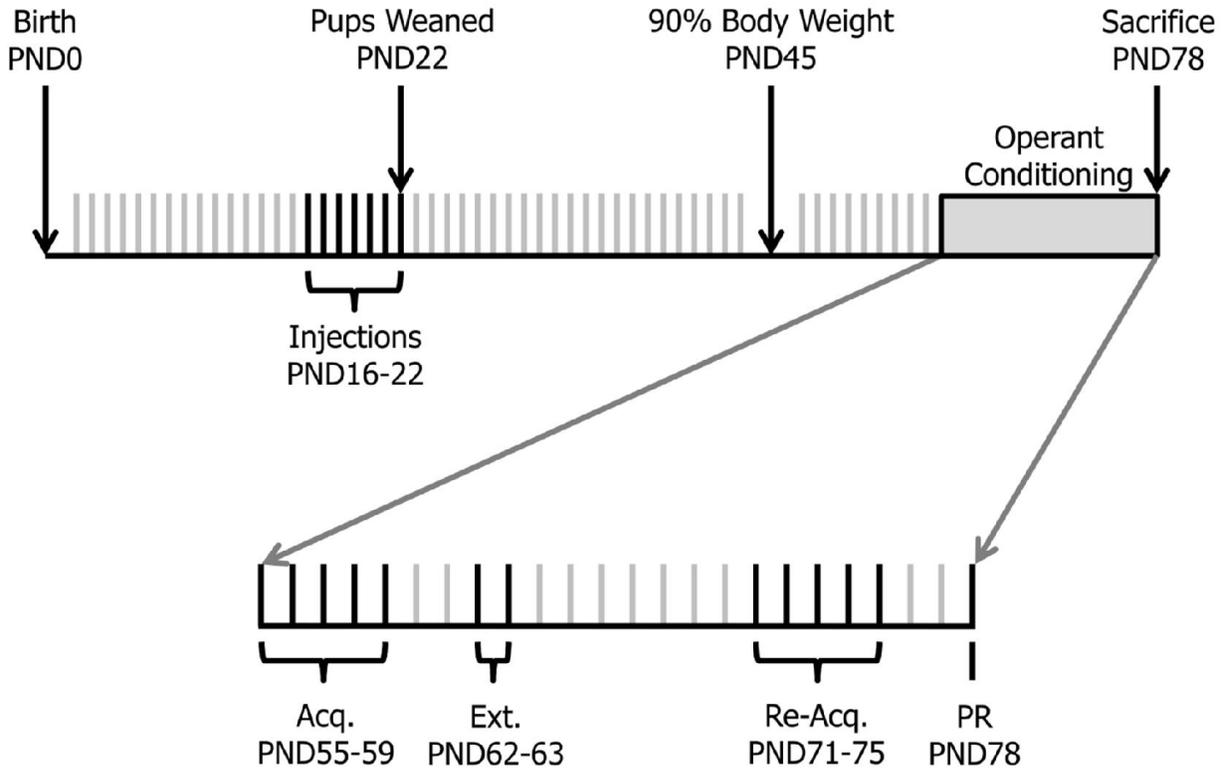


Figure 6.1: Timeline of experimental procedures. A timeline of di(2-ethylhexyl) phthalate treatment and operant conditioning sessions. PND: Postnatal day; Injections: Injections of 1, 10, 20 mg/kg di(2-ethylhexyl) phthalate or vehicle; Acq: Acquisition; Ext: Extinction; Re-Acq: Re-Acquisition; PR: Progressive Ratio.

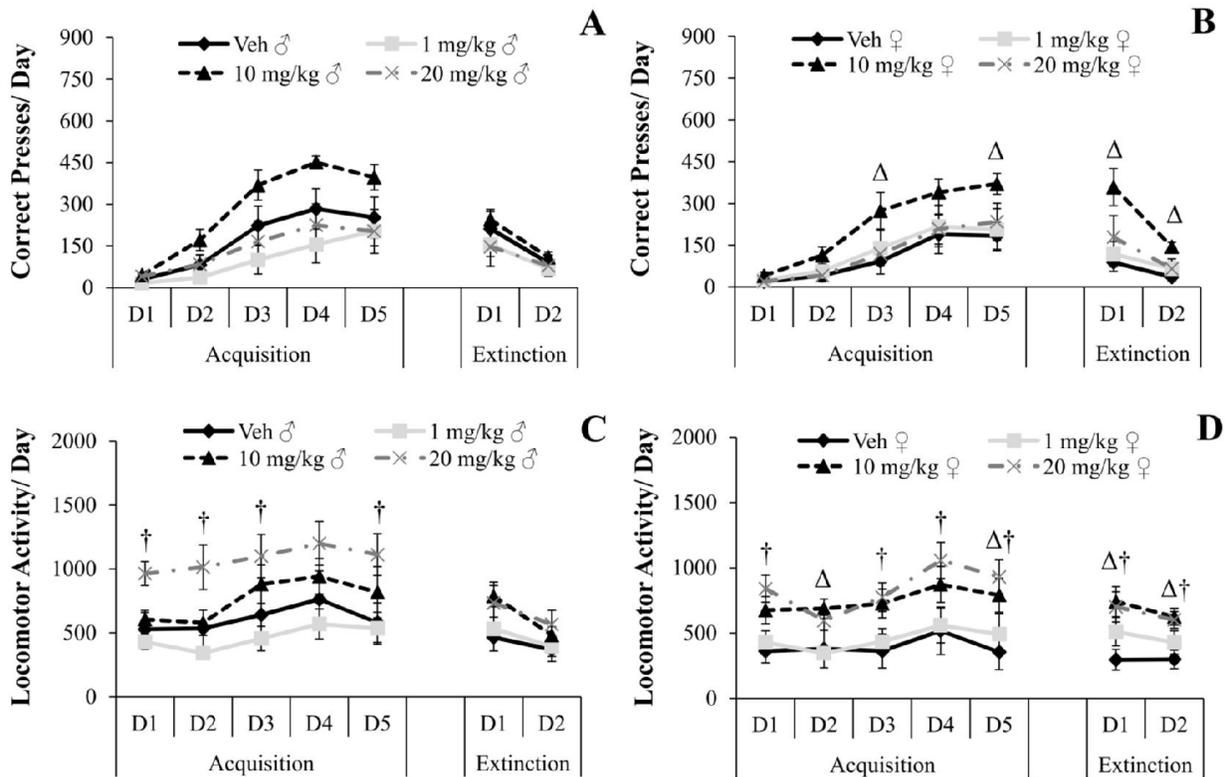


Figure 6.2: Correct lever presses and locomotor activity during acquisition and extinction. The total number of correct lever presses during acquisition and extinction operant sessions per training day in male (A) and female (B) rats exposed to 1, 10, 20 mg/kg di(2-ethylhexyl) phthalate (DEHP) compared to controls (Veh). Locomotor activity during acquisition and extinction operant sessions per training day in male (C) and female (D) rats. Treatment with 10mg/kg DEHP increased rewarded operants during day 3 and 5 of the acquisition phase in female rats compared to controls. The elevated levels of responding persisted in female rats treated with 10 mg/kg DEHP during non-rewarded extinction sessions. No differences in operant responding were detected during the acquisition or extinction sessions between DEHP-treated males and controls. Female rats exposed to 10 and 20 mg/kg DEHP were more hyperactive through the acquisition and extinction sessions compared to controls. Treatment with 20 mg/kg also led to a hyperactive behavioural state in male rats compared to controls. The Δ denotes a significant difference between the 10mg/kg DEHP dose and vehicle controls ($p < .05$) and the \dagger denotes a significant difference between the 20mg/kg DEHP dose and vehicle controls ($p < .05$). D: day.

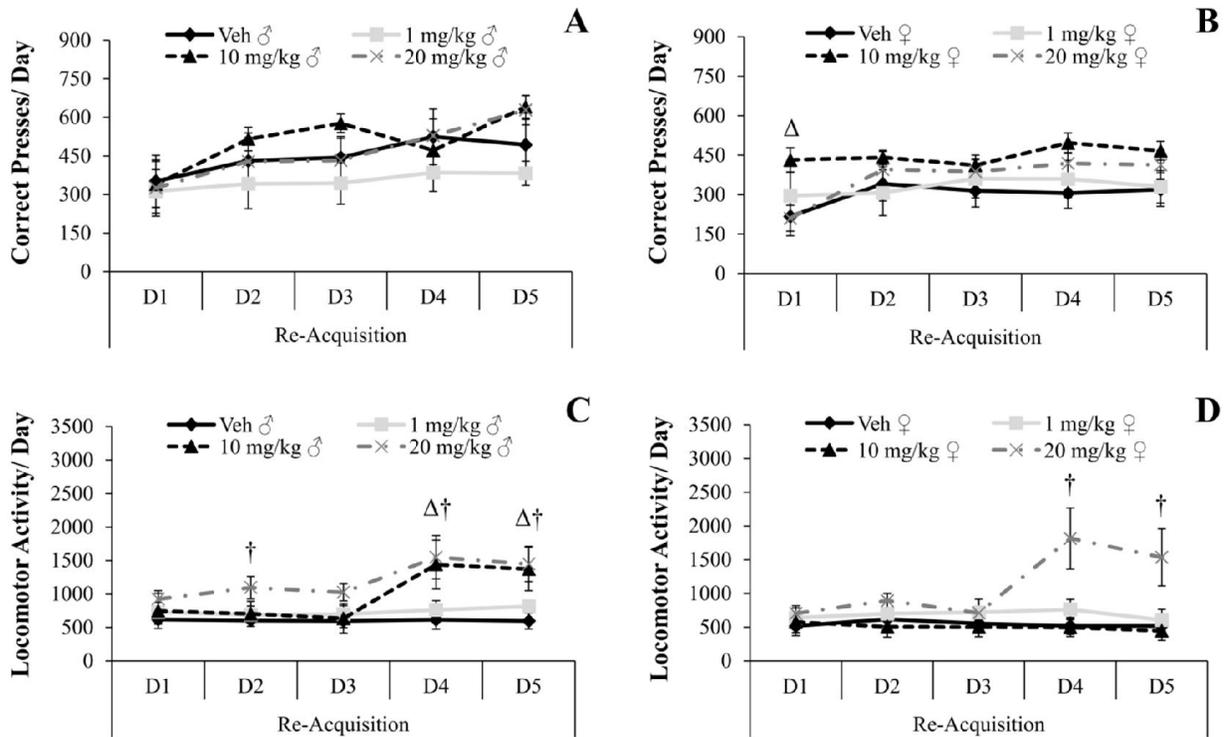


Figure 6.3: Correct lever presses and locomotor activity during re-acquisition. The total number of correct lever presses during the re-acquisition operant session per training day in male (A) and female (B) rats exposed to 1, 10, 20 mg/kg di(2-ethylhexyl) phthalate (DEHP) compared to controls (Veh). Locomotor activity during the re-acquisition operant session per training day in male (C) and female (D) rats. Treatment with 10mg/kg DEHP increased rewarded operants on day 1 of re-acquisition in female rats compared to controls. No differences in operant responding were detected during the re-acquisition sessions between DEHP-treated males and controls. Female rats exposed to 20 mg/kg DEHP were more hyperactive on day 4 and 5 of re-acquisition compared to controls. Treatment with 10 and 20 mg/kg also led to a hyperactive behavioural state in male rats compared to controls. The Δ denotes a significant difference between the 10mg/kg DEHP dose and vehicle controls ($p < .05$) and the \dagger denotes a significant difference between the 20mg/kg DEHP dose and vehicle controls ($p < .05$). D: day.

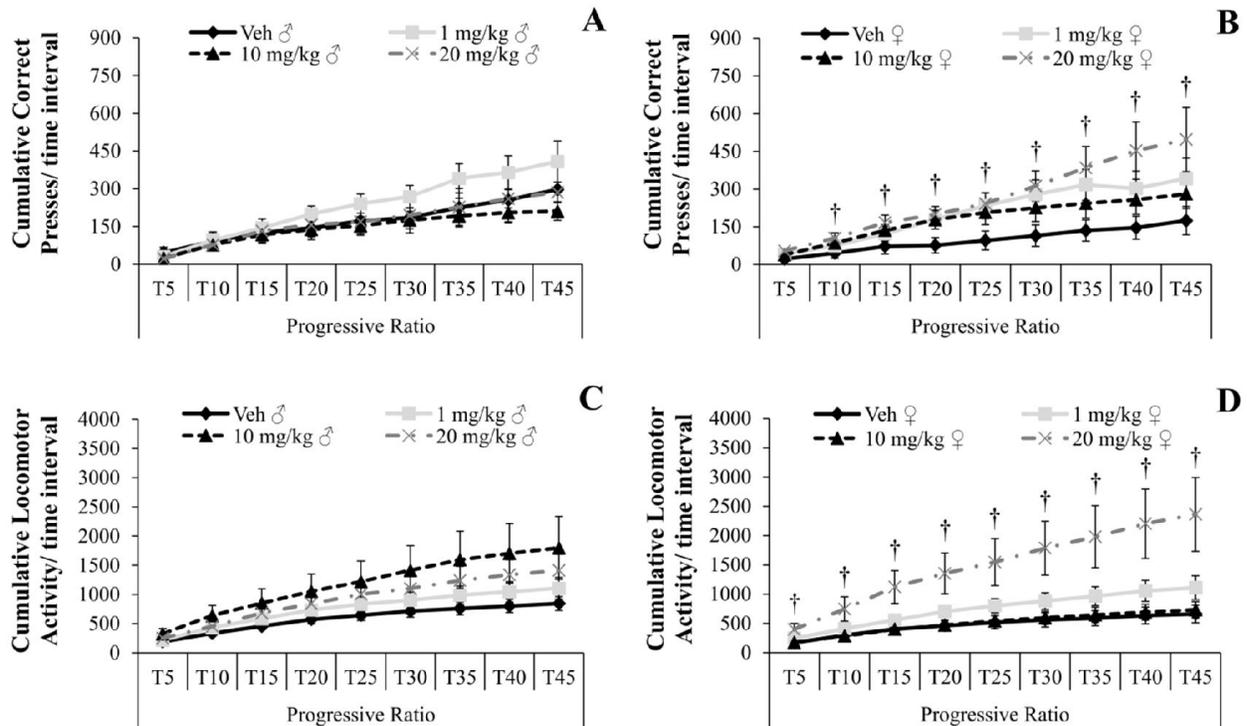


Figure 6.4: Correct lever presses and locomotor activity during progressive ratio. The cumulative correct lever presses during the progressive ratio operant session per 5-minute time interval in male (A) and female (B) rats exposed to 1, 10, 20 mg/kg di(2-ethylhexyl) phthalate (DEHP) compared to controls (Veh). Locomotor activity during the progressive ratio operant session per 5-minute time interval in male (C) and female (D) rats. Treatment with 20mg/kg DEHP increased rewarded operants during the progressive ratio sessions in female rats compared to controls. Female rats exposed to 20 mg/kg DEHP were also more hyperactive through the acquisition and extinction sessions compared to controls. No differences in operant responding or locomotor activity were detected during the acquisition or extinction sessions between DEHP-treated males and controls. The † denotes a significant difference between the 20mg/kg DEHP dose and vehicle controls ($p < .05$). T: time interval.

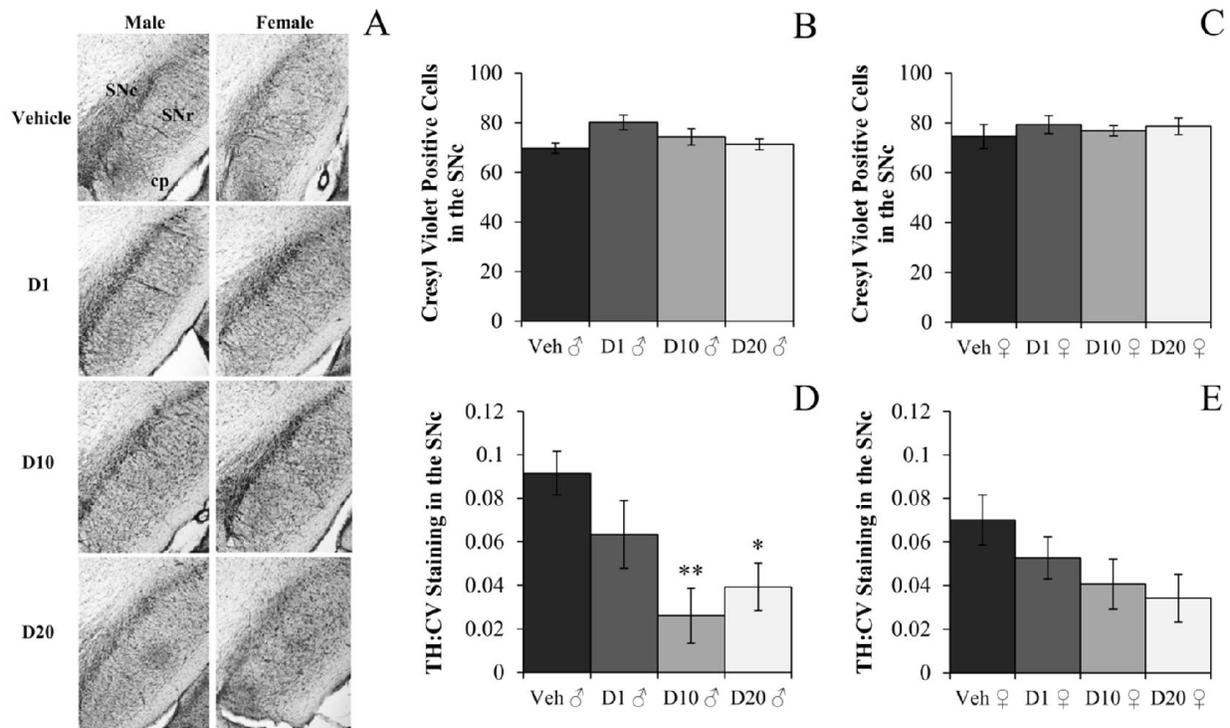


Figure 6.5: Tyrosine hydroxylase staining in the substantia nigra pars compacta. (A) Representative photomicrographs (4 x) of tyrosine hydroxylase (TH) staining in the substantia nigra pars compacta (SNc). Quantification of the number TH-positive cells the SNc in male (B) and female (C) rats exposed to 1, 10, 20 mg/kg di(2-ethylhexyl) phthalate (DEHP) compared to controls (Veh). Other cell types in the SNc were quantified for male (D) and female (E) using cresyl violet staining. The ratio of TH-positive to cresyl violet positive neurons (TH:CV) in the SNc quantified for male (F) and female (G) rats. Male rats exposed to DEHP showed a significant reduction in TH-positive neurons in the SNc compared to controls. There were no differences detected in the total number of TH-positive neurons in the SNc of female rats treated with DEHP compared to controls. D: DEHP; cp; cerebral peduncle; SNr: substantia nigra pars reticulata; * = $p < 0.05$; ** = $p < 0.01$; *** = $p < 0.001$. Error bars represent standard error of the mean.

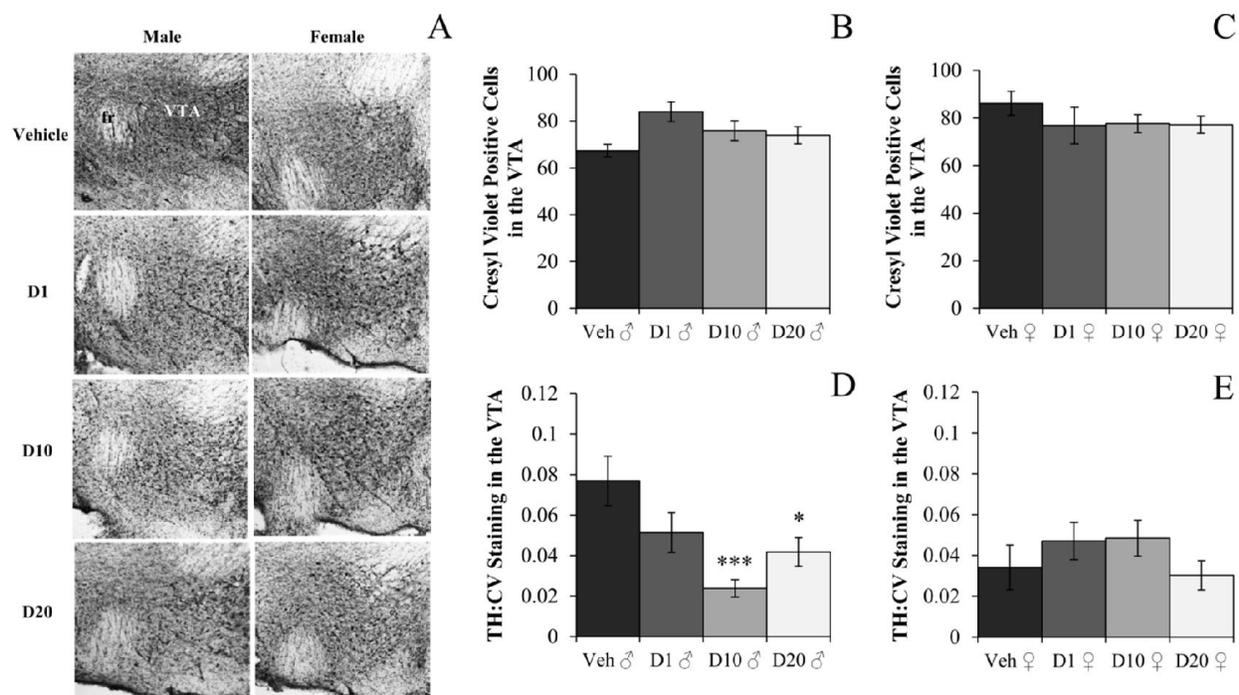


Figure 6.6: Tyrosine hydroxylase staining in the ventral tegmental area. (A) Representative photomicrographs (4 x) of tyrosine hydroxylase (TH) staining in the ventral tegmental area (VTA). The ratio of TH-positive to cresyl violet positive neurons (TH:CV) in the VTA quantified for male (D) and female (E) rats exposed to 1, 10, 20 mg/kg di(2-ethylhexyl) phthalate (DEHP) compared to controls (Veh). Other cell types in the VTA were quantified for male (B) and female (C) using cresyl violet staining. Male rats exposed to DEHP showed a significant reduction in TH-positive neurons in the VTA compared to controls. There were no differences detected in the total number of TH-positive neurons in the VTA of female rats treated with DEHP compared to controls. D: DEHP; fr: fasciculus retroflexus; * = $p < 0.05$; *** = $p < 0.001$. Error bars represent standard error of the mean.

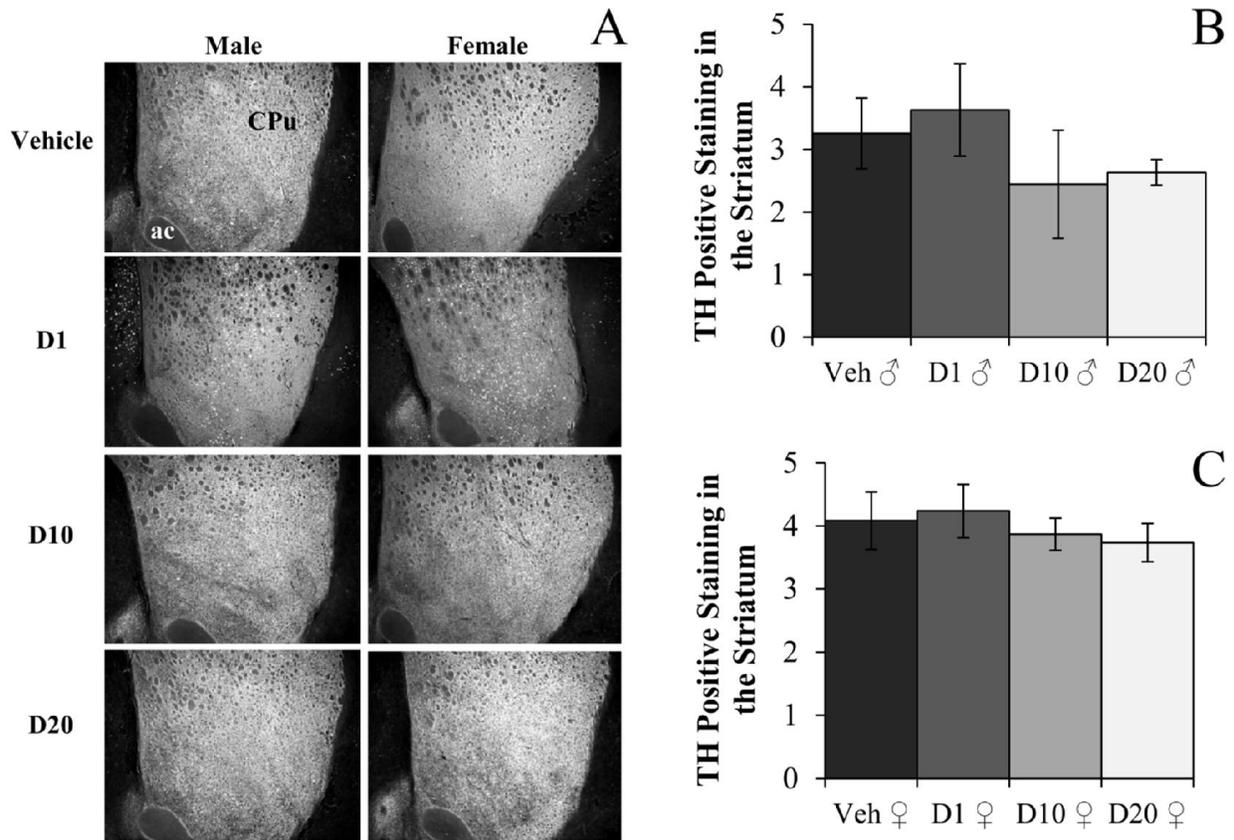


Figure 6.7: Tyrosine hydroxylase staining in the striatum. (A) Representative photomicrographs (10 x) of tyrosine hydroxylase (TH) staining in the ventral tegmental area (VTA). Quantification of the intensity of TH staining in the striatum of male (B) and female (C) rats exposed to 1, 10, 20 mg/kg di(2-ethylhexyl) phthalate (DEHP) compared to controls (Veh). Intensity of staining in the anterior commissure (ac) was used to normalize TH intensity. There were no differences detected in the intensity of TH staining in the striatum (CPu) of male or female rats treated with DEHP compared to controls. D: DEHP. Error bars represent standard error of the mean.

Chapter 7

General Discussion

This dissertation explored the effects of acute postnatal administration of di(2-ethylhexyl) phthalate (DEHP) on brain structure and function in the rat. Experimental data presented in Chapter II demonstrated for the first time that acute postnatal administration of DEHP (10 mg/kg; PND16-22) interfered with the development of the hippocampus in male juvenile rats (PND26). Developmental exposure to DEHP disrupted CA3 hippocampal neurocircuitry (as shown by reductions in CA3 SO synaptophysin staining) and reduced the density of immature neurons in the DG and mature neurons in the CA3 of male, but not female rats. Male rats exposed to DEHP during young adulthood (PND68 to PND74) also showed disruptions in CA3 hippocampal connectivity, but these effects were limited to reductions in axonal innervation to the CA3 SO region. Given that more widespread deficits in the hippocampus were observed in male rats following postnatal DEHP treatment, these findings suggest that male rats may be more susceptible to the neurotoxic effects of DEHP. These results were consistent with a study by Li and colleagues (2013a), who reported a reduction in hippocampal synaptophysin protein and a decrease in the density of hippocampal neurons in male and female rats on PND5 and PND21 (but not at PND60) following perinatal treatment with a different phthalate, DBP (500 mg/kg).

Experiments outlined in Chapter III and IV built on the original findings presented in Chapter II to further characterize the impact of postnatal DEHP exposure (10 mg/kg) on hippocampal development in juvenile rats (PND26). DEHP treatment reduced dendritic spine density on CA3 apical and basal dendrites in male, but not female rats, which were congruent with the findings in Chapter II. The formation and maintenance of synaptic connections in developing

organisms is critical for proper dendritic arborisation, and the failure to form these connections in development can disrupt dendritic outgrowth (Frotscher et al., 2000; McAllister, 2000; Tailby et al., 2005). It is possible that the reduction in axonal innervation to the CA3 SO region in DEHP-treated male rats may have contributed to the reduction in spine development on CA3 pyramidal neurons.

The up-regulation of a pro-apoptotic LPC lipid was also observed in developing male rats treated with DEHP. This is congruent with decreased CA3 cell density in DEHP-treated male rats as reported in Chapter II. Interestingly, the administration of DEHP did not affect mRNA caspase-3 expression when assessed on PND26. It is possible that caspase-3 activity may have been up-regulated during the DEHP treatment days (PND16-22) leading to reduced CA3 hippocampal cell density. Once DEHP treatment ended on PND22, caspase-3 activity would have returned to baseline levels and thus, no differences in caspase-3 mRNA expression would have been detected when assessed on PND26. This hypothesis is consistent with a previous study that showed increased caspase-3 expression only on days during which phthalate treatment occurred (Li et al., 2013a). When caspase-3 expression was measured 5 weeks after the last day of phthalate treatment, no differences in caspase-3 expression were present (Li et al., 2013a).

Hippocampal BDNF mRNA expression was shown to be down-regulated in DEHP-treated male rats but BDNF expression was unchanged in female rats treated with DEHP. BDNF plays a critical role in neurite outgrowth and synaptic enhancement (Danzer et al., 2001; Rabacchi et al., 1999; Scharfman et al., 2003). Decreased hippocampal BDNF *in vitro* and *in vivo* has been associated with reduced dendritic spine outgrowth on hippocampal neurons (Kellner et al., 2014; Zagrebelsky & Korte, 2014). BDNF is also important for regulating cell apoptosis via caspase-3 suppression (Han et al., 2000). Therefore, DEHP-induced decreases in hippocampal BDNF

expression may represent a possible molecular mechanism underlying these reductions in CA3 dendritic spine density, CA3 axonal innervation, and CA3 cell density in DEHP-treated male rats. Additional experimental studies are necessary to confirm the role of BDNF in DEHP-mediated hippocampal dysfunction. For instance, would the hippocampus of DEHP-treated male rats develop normally if BDNF levels were restored experimentally via systemic injections of recombinant BDNF? Similarly, would comparable disruptions in hippocampal neurobiology be observed in DEHP-treated female rats if BDNF was experimentally blocked using the neurotrophin antagonist Y1036?

One interesting finding in Chapters II and III was the gender-selective detrimental effect of postnatal DEHP administration on hippocampal development in male rats. No differences in hippocampal connectivity or morphology were observed in female rats treated with DEHP during the same postnatal time period. The differential effects of DEHP across genders may be mediated in part by DEHP-induced alterations in circulating levels of gonadal hormones. Decreased serum testosterone levels and aromatase enzyme activity in the brain have been reported in DEHP-treated male rats (Akingbemi et al., 2001; Andrade et al., 2006; Borch et al., 2004; 2006; Ge et al., 2007; Jones et al., 1993; Noreiga et al., 2009; Parks et al., 2000), while decreased levels of estradiol and progesterone were found in female rats treated with DEHP and MEHP (Davis et al., 1994a; 1994b; Lovekamp-Swan & Davis, 2001; Svechnikova et al., 2007). Gonadal hormones are critically involved in the organization and maintenance of synaptic connections in the brain (Cooke & Woolley, 2004; Kovacs et al., 2003; Leranthe et al., 2003; 2004) and normal physiological levels of these hormones have been shown to have a neuroprotective effect against apoptotic neurodegeneration (Liu et al., 2001; Nguyen et al., 2005; Pike, 1999; 2001).

The findings from Chapters II and III suggest that altered testosterone and/or aromatase enzyme activity may be involved in the DEHP-induced hippocampal dysfunction given that abnormalities in the hippocampus were only detected in male rats exposed to DEHP. Further investigation is required to determine whether these changes in hippocampal neurobiology in DEHP-treated male rats can be attributed to low levels of testosterone and/or the suppression of aromatase activity. It would also be interesting to assess circulating levels of gonadal hormones in female rats to uncover possible reasons why DEHP treatment had no effect on female hippocampal neuroconnectivity.

The up-regulation of SM and PC hippocampal lipids in DEHP-treated female rats may protect the female rat hippocampus from damage by early developmental DEHP exposure. Perhaps the combined effect of elevated SM and PC lipids in DEHP-treated female rats had a downstream protective effect on hippocampal neurons, promoting their survival. Elevated levels of SM lipids with C18-C24 fatty acid side chains were found in the hippocampus of female rats treated with DEHP. Previous studies indicated that the down-regulation of sphingolipids (which include SM lipids) with C18-C24 fatty acid side chains were linked to the degeneration of cerebellar Purkinje and granule cells, and the accumulation of lipofuscin and ubiquitin in the CA3 hippocampal region of mice (Imgrund et al., 2009; Zhao et al., 2011). This suggests that a reduction in sphingolipids, particularly those with C18-C24 fatty acid side chains may be detrimental to neuronal health. Therefore, the up-regulation of SM lipids with C18-C24 fatty acid side chains in the hippocampus of DEHP-treated female rats may protect against DEHP-induced reductions in hippocampal connectivity and cell density.

Unsaturated fatty acid-containing PC lipids were also up-regulated in the hippocampus of female rats following postnatal DEHP treatment. The carbon-carbon double bonds in unsaturated

PC lipids amplify the oxidization potential thereby increasing the vulnerability to apoptotic cell death (Esposti, 2002; Cantrel et al., 2009). Conversely, the presence of specific polyunsaturated fatty acids in lipids (such as DHA and AA) is neuroprotective, and promotes neuronal survival by attenuating apoptosis (Bazan, 2005; Hoguey et al., 2003; Strokin et al., 2006; Wang et al., 2003; 2006). A previous study had shown that *in utero* exposure to DEHP decreased the concentration of DHA and AA in membrane lipids in fetal rat brains (Xu et al., 2007). These findings indicate a possible role for polyunsaturated fatty acid containing membrane lipids in modulating the effects of DEHP. Unfortunately, identifying concentrations of specific fatty acid containing lipids was not possible with the method used in Chapter IV. In future studies, it would be of interest to evaluate the effects of DEHP on fatty acid composition in the hippocampus as potential mediators of hippocampal toxicity in DEHP-treated male rats, and hippocampal protection in DEHP-treated female rats.

The long-term effects of postnatal administration of DEHP on neural and behavioural measures of cognitive performance were evaluated in Chapters V and VI. When the hippocampus of male rats was assessed during young adulthood, only relatively minor changes in hippocampal neurobiology were observed. This was unexpected given the detrimental effect of postnatal exposure to DEHP on hippocampal development in male juvenile rats. One possibility is that postnatal exposure to DEHP initially disrupted the development of hippocampal connectivity in male juvenile rats, but as these rats matured (and DEHP treatment ended) compensatory processes were activated and typical hippocampal connectivity patterns emerged.

Interestingly, DEHP treatment did not impair performance on a spatial water maze task when male rats were tested as juveniles or as young adults. These findings suggest that compensatory mechanisms in the brain may have counteracted the initial disruptions to

hippocampal development, preserving spatial function in DEHP-treated male rats. It is also possible that the water maze task was too simple and by increasing the demands of the task, deficits in spatial learning/ memory would become evident. Alternatively, the cognitive effects of DEHP may only emerge under certain conditions, such as chronic stress or in aging.

Postnatal DEHP treatment (10 and 20 mg/kg DEHP) led to drastic reductions in the density of dopaminergic neurons in the VTA of male rats. Remarkably, this extreme reduction in dopaminergic neurons produced no discernable effects on reward-based learning. These results suggest that compensatory mechanisms in the brain of DEHP-treated male rats may underlie the unimpaired reward-based learning. It would be of interest to determine at what time point this reduction in VTA dopaminergic neurons had begun. Did postnatal DEHP treatment immediately reduce the density of dopaminergic neurons, or did this reduction gradually occur over time as these rats matured? It would also be important to establish whether reductions in VTA dopaminergic neurons would be magnified with chronic stress or during aging and whether this would coincide with the emergence of impairments in reward-based learning.

Unlike male rats, postnatal exposure to DEHP led to impairments on spatial learning/ memory, and reward-based learning tasks in female rats. The most unexpected findings were the unique and contradictory dose-response curves that emerged for each behavioural task. In both cases the dose-response curves were non-monotonic – meaning the slope of the dose-response curve gradually reversed as the dose of DEHP increased. The behavioural findings for the spatial learning/ memory task revealed a u-shaped dose-response curve with spatial memory impairments observed at the lowest (1 mg/kg) and the highest (20 mg/kg) doses of DEHP. The highest dose of DEHP led to minor impairments in spatial memory retention during the water maze probe test when female rats were examined as juveniles and as young adults, while the lowest dose of DEHP

only impaired memory retention as young adults. Treatment with the intermediate dose of DEHP (10 mg/kg) had no effect on spatial performance in female rats.

Conversely, an inverted u-shaped dose-response curve emerged for DEHP-treated female rats on the reward-based operant conditioning task with the intermediate dose of DEHP (10 mg/kg) showing the greatest response. The highest dose of DEHP (20mg/kg) produced very minimal alterations in reward-based learning and the lowest dose of DEHP (1 mg/kg) appeared to have no behavioural effects. It was surprising that the same DEHP doses produced radically different behavioural outcomes on spatial and reward-based learning tasks in female rats. These data emphasize the importance of investigating the impact of DEHP exposure on a variety of behavioural measures of cognitive performance when evaluating its toxic potential.

Interestingly, other than a slight increase in CA3 SL synaptophysin staining in female rats treated with 10 and 20 mg/kg DEHP there was no effect of DEHP treatment on hippocampal or dopaminergic neurobiology in female rats. These findings suggest the probable involvement of other neural mechanisms in mediating DEHP-induced changes in spatial learning/ memory, and reward-based learning in female rats. Possible neuromodulators may include reductions in synaptic efficacy in hippocampal and dopaminergic pathways, modifications in neurotransmitter synthesis/release, and/or the up/down-regulation of receptors.

Postnatal DEHP exposure also induced a hyperactive behavioural state in male and female rats. Treatment with 10 and 20 mg/kg DEHP robustly elevated locomotor activity throughout the operant conditioning task in both genders. This is consistent with findings reported by Ishido and colleagues (2004) who found DCHP-induced hyperactivity in male rats that was also accompanied by dopamine cells loss in the SNc, the down-regulation of dopamine D₄ receptors in the midbrain and striatum, and the down-regulation of dopamine transporters in the midbrain.

Dysregulation of dopaminergic pathways have been linked to hyperactivity and other movement disorders (Costall et al., 1984; Ishido et al., 2004; van der Kooji & Glennon, 2007; Zhang et al., 2001). Neonatal chemical lesions of dopaminergic neurons resulted in hyperactivity and increased dopamine D₄ receptors in the striatum of juvenile rats (Zhang et al., 2001). Locomotor hyperactivity can also be induced in juvenile rats by an infusion of dopamine into the nucleus accumbens (Costall et al., 1984). Partial chemical lesions of SNc dopaminergic neurons in adult rats increased the synthesis and the release of dopamine from the remaining SNc neurons to the striatum (Hefti et al., 1980). Together these findings suggest that the DEHP-induced hyperactive behavioural state reported in male and female rat in Chapter VI may be regulated in part by the loss of SNc dopaminergic neurons. It is not known whether this reduction in SNc dopaminergic neurons increased the release of dopamine in the striatum. This awaits confirmation by further research.

Genetic abnormalities in dopamine D₄ receptors and/or dopamine transporters are prevalent in individuals with attention deficit-hyperactivity disorder (ADHD) – a developmental disorder in which hyperactivity, impulsivity, and attentional problems are common (Tripp & Wickens, 2008; van der Kooji & Glennon, 2007; Zwi et al., 2000). A correlation has been established between the concentration of phthalate metabolites in the urine of school-age children and reported symptoms of ADHD (Kim et al., 2009). These findings suggest a potential connection between phthalate treatment, hyperactivity, and abnormalities in dopaminergic function. While further experimental research is necessary to reach a conclusion regarding the link between phthalate exposure and ADHD. The resemblance of rat phthalate behavioural effects and ADHD pathology suggests phthalate exposure may be a risk factor for ADHD.

Conclusion

Data reported in this thesis represent a novel assessment of the toxic potential of DEHP on developing rats. Male and female rats exhibited distinct neurodevelopmental and behavioural outcomes following postnatal exposure to DEHP. Widespread disruptions in hippocampal and dopaminergic neurocircuitry were reported in DEHP-treated male rats while only minimal changes in neurobiology were observed in DEHP-treated female rats. The cognitive effects of postnatal DEHP exposure were marginal and were only evident in female rats. It is possible that exposure to chronic stress or during aging noticeable deficits in cognitive functioning would emerge due to DEHP treatment. The biological contributors underlying DEHP-induced changes in neurodevelopment and behaviour are not understood, but it is likely that the effects of DEHP are mediated by different mechanisms in male and female rats. Decreased BDNF expression may be a potential candidate for the near-selective detrimental effect of DEHP exposure on neurodevelopment in male rats. The up-regulation of hippocampal lipids may serve a neuroprotective role in DEHP-treated female rats and may underlie the resistance of the female rat hippocampus to modification by DEHP. Comprehensive investigations which simultaneously assess the neurodevelopmental and behavioural correlates of DEHP exposure are needed and will provide an opportunity to thoroughly evaluate the toxic potential of DEHP.

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