Changes in Hippocampal Plasticity and HPA Axis Function Following Postnatal Exposure to Di(2-ethylhexyl) Phthalate in Rats

By

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ABSTRACT

Phthalates are known to affect the development of regions within the hippocampus, however the mechanisms of these toxic effects are not well known. Stress is also known to have detrimental effects on the hippocampus. This study aimed to determine whether phthalates affect hippocampal development, and whether there is an associated activation of the HPA axis. DEHP was injected in juvenile rats daily from PND 16 – PND 22. Brain tissue samples were analyzed by immunohistochemistry to quantify developing neurons. The Golgi – Cox Impregnation method was used for detailed morphological analyses. Blood samples were analyzed for corticosterone levels. The CA3 of males showed a decrease in the size of the area of basal dendrites at the middle and highest dose of DEHP treatment, while the DG of females showed a decrease in number of spines at the highest dose, and a decrease in the dendrite length at only the lowest dose.

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ABBREVIATIONS

adrenocortiocotrophic hormone (ACTH); attention deficit hyperactive disorder (ADHD); animal free blocker (AFB); α-amino-3-hydroxy-5-methyl-4 isoxazole propionic acid (AMPA); AMPA receptor (AMPAR); brain derived neurotrophic factor (BDNF); 5-Bromo-5'-deoxyuridine (BrdU); corticotrophic releasing hormone (CRH); doublecortin (DCX); di-(2-ethylhexyl) phthalate (DEHP); dentate gyrus (DG); embryonic (E); glucocorticoid receptors (GR); hypothalamic-pituitary-adrenal (HPA); locus ceruleus-norepinephrine (LC-NE); long term depression (LTD); long term potentiation (LTP); mineralocorticoid receptors (MR); Morris water maze (MWM); NMDA receptor channel complexes (NMDAR); phosphate buffer solution (PBS); postnatal day (PND); polyvinyl chloride (PVC); subgranular zone (SGZ); subventricular zone (SVZ)

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INTRODUCTION

Overview

The hippocampus serves several neural functions including an overall facilitation of information coding leading to short and long term memory formation particularly in the realm of spatial and declarative forms of memory (Khazal-Nazzal, 2013). In order for the proper neural substrates to be available for this enduring function, the hippocampus undergoes extensive remodeling postnatally and well into adulthood. While this continued remodeling is essential for memory formation, it makes the hippocampus particularly sensitive to toxins that may affect not only its development but its function throughout the lifespan (Bayer, 1980). Phthalates, toxins found extensively in the environment, are known to affect the development of regions within the hippocampus (Smith, Holahan, 2011). However, the mechanisms through which phthalates exert their toxic effects on hippocampal plasticity are not well known and are the focus of the current thesis.

Hippocampus

Anatomy

The hippocampal formation includes the hippocampus itself, as well as the subicular zone (subdivided into the subiculum, presubiculum, and parasubiculum) and the entorhinal cortex (subdivided into the medial and lateral subregions, each containing 6 cellular layers) (Jarrard, 1993, Khazal-Nazzal and 2013). The hippocampus is comprised of two main regions, the dentate gyrus (DG) and the hippocampus proper, separated by the hippocampal sulcus (Amaral and Witter, 1989). The DG is the first main input into the hippocampus. It is divided into three

layers, the polymorphic cell layer, the granule cell layer, and the molecular layer. The polymorphic layer is the most superficial layer and is comprised of a variety of cell types including mossy cells and interneurons. The granule cell layer is the middle layer containing the cell bodies of the granule cells and is the location of neurogenesis in the hippocampus. The mostly cell-free molecular layer is the deepest layer containing the dendrites of the granule cells where many connections between regions (e.g., from the entorhinal cortex) are made. (Amaral and Witter, 1989, Khalaf-Nazzal, 2013).

The hippocampus proper is divided into three fields: CA1, CA2 and CA3. These regions are further divided into specific lamina, each receiving different inputs. The deepest layer, the alveus, contains axons from pyramidal cells and is the main output from the hippocampus. The stratum oriens contains the basal dendrites of pyramidal cells and is the location of inputs from the other fields of the hippocampus proper as well as the DG. The stratum pyramidale contains the cell bodies of the pyramidal cells, which are the primary excitatory cells in the hippocampus proper. The stratum lucidum is found only in the CA3 region and is where the majority of mossy fibers (axons that arise from the granule cells) project to. The stratum radiatum in the CA1 subfield contains the Schaffer collateral fibres, which are connections from the CA3 to the CA1. The stratum lacunosum-moleculare is the most superficial layer and is composed of the apical dendrites from pyramidal cells. This CA1 layer contains Schaffer collateral fibres form CA3 as well as perforant path inputs from the entorhinal cortex. (Amaral and Witter, 1989, Anderson et al., 2007, Khalaf-Nazzal, 2013).

The hippocampus receives a majority of its input from association brain regions. Somatosensory and auditory associational cortices as well as olfactory inputs project sensory information to the perirhinal cortex whereas visual and somatosensory association cortices

project to the postrhinal cortex (Burwell, at al., 1995). These regions then relay information to the entorhinal cortex. The entorhinal cortex layers 2 and 3 feed a direct projection to the DG, the first main excitatory input to the hippocampus. The entorhinal cortex also has secondary projections to the CA1 and CA3 subregions (Jarrard, 1993, Witter, et al., 2000). The entorhinal cortex projects to the DG molecular layer via the perforant pathway. The perforant path also projects to the distal dendrites of the CA1 stratum radiatum and CA3 region**s.** Hippocampal output from the CA1 and subiculum is then relayed back to entorhinal cortex and from there, back to the perirhinal and postrhinal cortices (Amaral and Witter, 1989).

Within the hippocampus, the lamellar hypothesis describes how connections are mostly unidirectional and activated in succession (Amaral and Witter, 1989). The DG granule cells project to the CA3 stratum lucidum and oriens via mossy fibers. These axons collateralize in the polymorphic cell layer to be able to produce associational, or recurrent, connections within the DG. The CA3 pyramidal cells contain recurrent collaterals forming looped connections within the CA3 region itself. The CA3 pyramidal cells then project via Schaffer collaterals to the proximal dendrites of the CA1 stratum radiatum (Amaral and Witter , 1989, Teyler et al., 1983, Jarrard, 1993, Witter, 2007). There are also less studied connections from the CA3 back to the DG (Witter, 2007). The CA1 neurons send inputs to both the subiculum and back to the entorhinal cortex layer 5 (Witter et al., 2000). These connections allow for information to be transmitted into the hippocampus and then back out to the cortical regions where the projections originated essentially completing a circuit (Khazal-Nazzal, 2013).

There is also recent evidence of a more minor pathway where neurons project from the entorhinal cortex and synapse onto CA2 neurons. The CA2 neurons then project onto the CA1 neurons, driving them to undergo strong depolarization and ultimately, long term potentiation

(LTP) (Chevaleyre and Siegelbaum, 2010, Khazal-Nazzal, 2013). These connections may have important involvement in spatial learning (Chevaleyre and Siegalbaum, 2010).

Development

Hippocampal development starts during embryonic stages and continues postnatally (Pokorny and Yamamoto, 1981).The hippocampus originates in the dorso-medial region of the telencephalon (Khazzal-Nazzal, 2013). The specification of subregions begins early in hippocampal development, around embryonic day 10 (E10) and at different time points according to region. The cells of the CA1 and CA2 start to develop around E10, peak at E15 (Bayer, 1980) and finish expansion by postnatal day 7 (PND7) (Pokorny and Yamamato, 1981). The developmental emergence of CA3 neurons begins at E11, peaks at E14 and achieves maturity at PND 24 (Khazzal-Nazzal, 2013, Crain et al., 1973). Granule cells, located in the granular layer of the DG, are the primary neuron type in the DG (Khalaf-Nazzal, 2013). Neuron development in the DG starts at E10, peaks at E16 and continues into the first week postnatally (Bayer, 1980).

Dendritic spines are protrusions that are found on excitatory synapses in the nervous system (Bosch and Hayashi, 2012). They are the main target of excitatory input with generally one glutamergic synapse present per spine. In the cortex, approximately 90% of excitatory synapses occur on the spines (Zhang et al. 2010). During development, there is a large increase and then pruning of these spines with changes occurring well into adulthood. Spine structure is extremely variable, as well as being plastic and mobile. Spine structure can change after long term potentiation (LTP) induction, leading to more efficient transmission of electrical current. There is also an increase in number of synapses present following LTP, which would also lead to

an increase in transmission efficiency (Bosch and Hayashi, 2012). Dendritic spines in the DG of the hippocampus develop on the basal dendrites at PND 5 and are fully developed by PND 10 with spine density increasing from PND 10 to PND 25 (Seress, Pokorn, 1980).

Neurogenesis in the DG

Seib and Martin-Villalba (2014) have described neurogenesis as "the process of generation, migration, maturation, and functional integration of new neurons into the pre-existing neuronal network". It is an exceptional form of plasticity in the brain, where whole neurons are generated and selected for survival (Castren and Hen, 2013). The DG is one of only two areas in the brain where neurogenesis occurs well into adulthood; the other region being the subventricular zone (SVZ) lining the lateral ventricles, where neural stem cells and progenitors generate new neurons that migrate to the olfactory bulb via the rostral migratory stream (Erikkson et al., 1998). Neurogenesis in the DG arises through the development of progenitor cells in the subgranular zone (SGZ) of the DG, located between the granular cell layer and the hilus. Pluripotent cells in this region have been shown to continuously divide then migrate and incorporate into the DG as mature granule cells. These newly-matured cells then send mossy fibers into the CA3 to become an active part of neural transmission in the hippocampus. (Ehninger and Kempermann, 2007).

Hippocampal progenitor cells are multipotent cells that can develop into neurons, astrocytes and oligodendrocytes *in vitro* (Palmer et al., 1999, Ehninger and Kempermann, 2007). The existence of neural stems cells in the DG, which are pluripotent, is highly debated, but recent evidence has shown the presence of these cells (Julian et al., 2013). There also exist pools of stem and progenitor cells that reside in non-neurogenic regions of the brain that once placed

within the neurogenic regions of either the DG or the SVZ, demonstrate the ability to develop into mature neurons. This suggests the presence of some extracellular factors in the DG and SVZ that may regulate the maturation of these cells (Shihabuddin et al., 2000).

Neurogenesis has been found in all mammals, including humans (Erikkson et al., 1998). This process has been shown to be important for certain types of memory formation including fear conditioning and spatial memory (Seib and Matin-Villalba, 2014). It also has been shown to be involved in the effects of some types of antidepressants (Castren and Hen, 2013). The regulation of neurogenesis is multi-factorial and can be affected by developmental enrichment, activity (e.g., exercise), and learning (Deng et al., 2010). Neurogenesis has also been shown to be increased following apotosis of granule cells in the DG (McEwen and Margarinos, 2001). Acute and chronic stress has been shown to decrease proliferation and survival of cells; two common measures of neurogenesis (Castren and Hen, 2013). This reduction in neurogenesis is thought to be caused by increased levels of circulating glucocorticoids (Mirescu, Gold, 2006).

Neurogenesis in the adult hippocampus contributes strongly to learning and memory especially in the context of pattern separation and is part of the reason why there is turnover of granule cells. This neurogenesis allows the hippocampus to adjust to needs for learning (Sherry et al., 1992, Bruel-Jungerman, 2007, Deng at all., 2010). Physical activity and enriched environments, which are known to increase neurogenesis in the DG, are associated with enhanced performance on spatial memory tasks (Nilson et al., 1999, Van Praag et al., 1999).

Regulation of hippocampal neurogenesis is especially pronounced in spatial navigation learning in the Morris water maze (MWM), a test that is specifically designed to assess spatial

memory function (Deng et al., 2010). In this test, rats must learn the location of a platform in the water to be able to escape the water then remember the location of the platform during a probe test when the platform is removed (Morris, 1984). A study showed that learning in the MWM promoted the survival of newly formed granule cells born 7 days before the start of training, while promoting the death of granule cells formed 3 days before the training. This is thought to occur as after a week of establishment in the DG, the granule cells start to make synapses and are able to be positively influenced by learning, while the newer cells have not yet reached this stage. If this apoptosis of the newer neurons is blocked, spatial learning is inhibited (Dupret et al., 2007, Deng et al., 2010).

Neurogenesis in the adult hippocampus also contributes to the loss of old memories, known as forgetting. As new neurons are incorporated into the DG, they form connections with previously existing neurons, while at the same time replacing some connections that had previously been established, but were not currently in use. This replacement of old synapses with new synapses removes the old memory, making room for the new memory to be integrated (Deng at el., 2010).

Memory

Plasticity

The brain has the ability to store what seems to be an infinite amount of information and experiences in the form of memory, which can last decades (Malenka and Nicoll, 1999). Memory is processed and stored in the brain in the form of changes in synaptic activity efficiency (Squire, 1992). The ability to store memories by changes in synaptic connectivity and activity is known as synaptic plasticity and is exemplified as the ability of the brain to modify

circuits in response to experiences. The transmission can either be enhanced or depressed and is stored in spatio-temporal patterns that allow for specificity to individual inputs (Citri and Malenka, 2008). This was first shown in the hippocampus, where repetitive activation of excitatory synapses caused an increase in the synaptic strength which could last for days (Malenka and Nicoll, 1999).

Two different forms of plasticity have been proposed: functional and structural plasticity. Functional plasticity refers to changes in synaptic transmission such as strengthening or downgrading synaptic strength by LTP or long term depression (LTD). Structural plasticity refers to physical changes in network connectivity such as axonal patterning, dendritic spine densities, and incorporation of neurons by neurogenesis. Functional plasticity and structural plasticity are associated as functional plasticity is thought to lead to structural plasticity (Granger and Nicoll, 2014, Bosch and Hayashi, 2012).

LTP is thought to play a key role in long term memory formation. It is triggered rapidly and can last for hours to days. LTP is measured as a lasting increase in the size of the synaptic component of a response and is input specific (Squires, 1992). This means that repetitive stimulation at one set of synapses results in an enhanced response at those specific synapses, and this enhanced response will not be observed at other synapses on the same cell. This is important to ensure that the responses are specific, making it possible for greater storage capacity on individual neurons (Malenka and Nicoll, 1999).

LTP is mediated primarily by NMDA receptor channel complexes (NMDAR) (Squires, 1992). For the NMDAR to open, two steps must occur. The membrane must locally depolarize to expel Mg^{2+}, which blocks the channel, and at the same time, L-glutamate must be bound to the

receptor to promote the opening of the channel (Squires, 1992). Depolarization of the membrane often arises through glutamate binding on the α-amino-3-hydroxy-5-methyl-4 isoxazole propionic acid (AMPA) receptors (AMPARs). Activation of these receptors provides most of the inward sodium current that provides the depolarizing stimulus for removal of the Mg^{+2} block of the NMDAR. The depolarization strength depends on several variables, including frequency, pattern and strength of the stimulus, the amount of inhibition, the excitability of the membrane, and many other variables (Granger and Nicoll, 2014). Once these events occur, Ca^{2+} enters the cell via the NMDAR to initiate the LTP process in that region of the neuron. The increase in intracellular Ca^{2+} is localized to individual dendritic spines, which allows the input to be very specific (Citri and Malenka, 2008). The increased Ca^{2+} then leads to second messenger cascades, which are essential for the induction of LTP (Malenka and Nicholl, 1999). Although there are many proposed mechanisms of how LTP is induced through second messengers, a few have received particular attention. Additional AMPARs are known to be added to the membrane following LTP to allow easier depolarization with less input (Malenka and Nicholl, 1999, Citri and Malenka, 2008). Other proposed mechanisms for LTP induction is through changes in AMPAR subunit expression, changes in dendritic spine density and conformation, as well as increased extracellular glutamate released from the presynaptic neuron to initiate the depolarization by the AMPARs (Malenka and Nicholl, 1999, Citri and Malenka, 2008, Bosch and Hayashi, 2012).

Types of memory

Primary knowledge regarding the division of memory systems comes from studies in patients receiving damage to certain regions of the brain, while all other regions remain intact. One of the most influential studies on memory came from patient H.M., who received bilateral

hippocampal surgery to relieve seizures. This patient was able to perform normally on short-term memory tasks, but was unable to transfer these memories to long-term storage. Other patients who received damage to the temporo-parietal cortex showed the opposite, where they were able to use long-term stored memories, but were unable to perform short-term memory tasks (Baddeley, 2010).

Memory can be divided into short-term and long-term memory (Izquierdo et el., 1999, Baddeley, 2010). Short-term memory, also known as working memory, is the temporary storage of small amounts of information and is dependent on electrical activity in the prefrontal cortex (Izquierdo et al., 1999). Although many estimates of the exact number of information pieces that can be used in working memory at one time have been proposed, newer evidence suggests that this number is difficult to estimate as it is dependent on many factors, such as relevancy to the other pieces and context of the situation (Cowan, 2005). Long-term memory is the more permanent storage of memory over longer periods of time. This type of memory can further be divided into declarative and nondeclarative memory and from there further divided into multiple subsections (Izquierdo et al., 1999).

Role of hippocampus

The hippocampus is especially important in the formation of spatial and declarative memories (Jarrard, 1993, Citri and Malenska, 2008). Most extensive studies of memory and LTP have arisen from experiments carried out in the CA1 region of the hippocampus. Animal studies have been performed in which lesions of the hippocampus are made and memory abilities are tested. Animals with hippocampal lesions were unable to perform spatial memory tasks, such as the MWM (Morris et al., 1982), were unable to form fear conditioning memories (Chen et al.,

1996), and were impaired on tests such as the trace eyeblink conditioning paradigm (Kim et al., 1995). The hippocampus can also be temporarily inactivated through use of certain antagonists (Corcoran et al., 2005). Studies using hippocampus inactivation have shown disruptions in context-specific retrieval of fear extinction. This is where a conditioned response to a conditioned stimulus which is paired to an aversive unconditioned aversive stimulus, decreases when the unconditioned stimulus is removed, however this decrease in response to specific to certain contextual factors (e.g., location) (Corcoran and Maren, 2001, 2004). Studies on LTP have also been conducted which either decrease or enhance NDMARs in the hippocampus. Blocking NMDAR activity by the use of antagonists, as well as studies in mice lacking NMDARs in the hippocampus, have the effect of blocking LTP as well as spatial learning, while studies in mice overexpressing NMDARs showed increased LTP and enhanced spatial learning (Morris and Frey, 1997, Citri and Malenska, 2008).

Phthalates

Properties

Phthalates are diesters of 1-2 benzenedicarboxylic acid, also known as phthalic acid, which are produced in a two-step dehydrolosis reaction (Kim, et al., 2011, Graham, 1973). They are colourless and odorless chemicals (Graham, 1973). Phthalates, first produced in the 1920's, are used as plasticizers to increase the flexibility and extensibility of different polymers. They are ideally suited to this due to their stability, fluidity and low volatility (Cousins et al., 2003).

Phthalates are widely used in building and construction materials, home furnishings, cars, and to a limited extent, food packaging (Stales et al., 1997). The industrial plasticizer di-(2-ethylhexyl) phthalate (DEHP), is the most common phthalate and is widely used in the

production of polyvinyl chloride (PVC) plastics, which are used extensively in household construction and medical devices (Wu et al., 2014, Tickner et al., 2001). Over three billion kilograms of DEHP are produced annually and, due to their global presence in the environment, exposure to some degree is unavoidable (Lyche et al., 2009). DEHP provides the flexibility and toughness to the PVC and is found in many medical devices, including intravenous fluid bags and tubing, blood bags, feeding tubes, catheters and gloves (Tickner et al., 2001). Some soft PVC can be composed of up to 40% DEHP (Koch et al., 2005). Phthalates are known to escape into the environment from the products in which they are present (Latini, 2005). A study by Sharman et al. in 1994 measured the average DEHP levels from several dairy based products, which are known to contain high levels of DEHP due to their high fat content. The following results were obtained: skimmed milk (0.02 mg/kg), cream (0.24-4.2 mg/kg), cheese (0.2-16.8 mg/kg), butter (3-7.4 mg/kg). The study concluded that the DEHP levels found in these products were too high to have arisen from the milk alone and must have been introduced to the products by alternative means, including the packaging (Sharman et al., 1994).

DEHP is a branched-chain phthalate with the molecular formula $C6H4(C8H17COO)_2$ and a molecular weight of 390.57g/mol (Howard, 1996). It is excreted in the urine in the form of several different metabolites and has a half-life of 6-12 hours (Tickner at el., 2001). In 2004, Kock et al. measured the clearance of DEHP in a single male volunteer at three different doses. After 24 hours, around 67% of DEHP was excreted in urine in the form of five of the major metabolites, comprised of around 23% 5-OH-MEHP, 18.5% 5cx-MEPP, 15% 5oxo-MEHP, and 4.2% 2cx-MEPH. This metabolism did not appear to be dose dependent, as there was no difference noted between the three dosage levels.

Although DEHP is known to be a toxicant to reproduction and development, the Federal Hazardous Substance Act (FHSA) classifies it as a chronic toxicant with no acute toxicity (CPSA, 2010). The European Union has banned DEHP, along with other phthalates, in toys used by children (European Union, 2005). Canada and the United States have banned children's toys with a concentration of DEHP greater than 0.1 percent (Health Canada, 2011, CPSIA, 2008)

Estimates of Exposure

Exposure to phthalates can occur from the environment (through food, water or air) and through use of medical devices (Ticker et al., 2001). The length of the alkyl chain is associated with the primary route of exposure. Exposure to phthalates with long alkyl chains primarily occurs via ingested materials while exposure to phthalates with shorter alkyl chains often occurs via percutaneous absorption or inhalation if volatility is high (Koch et al., 2003). DEHP, with its long and branched alkyl chain, is fat soluble and therefore is found in high levels in fatty foods. However, it should be noted that most food, fatty or otherwise, contains low, albeit measurable, amounts (Stales et al., 1997). Estimations of exposure can be done by either measuring concentrations of DEHP in the environment or by measuring metabolite levels in urine (Lyche et al., 2009). The disadvantage of measuring DEHP in the environment is the need to calculate the amount in all exposure types as well as how much exposure happens, which can be difficult to measure accurately. The disadvantage of measuring DEHP metabolites in urine is that DEHP has a short half-life so it can only be used as a point estimate as opposed to long-term exposure rates (Lyche et al., 2009).

Ingested DEHP is converted to the monoester form, 5-OH-MEHP, by gut lipases then absorbed into the blood stream (Tickner et al., 2001). This conversion from DEHP to MEHP, as

well as the absorption is known to occur more rapidly and to a greater degree in young rodents than in adults (Gray and Gangolli, 1986).Once absorbed, it is distributed throughout the body, mostly in fat, the lungs, the gastrointestinal tract, the liver and the kidneys, but is also found in the heart, the spleen, the reproductive tract, muscles and the brain (Gray and Gangolli, 1986).

The average exposure to DEHP in an individual is difficult to estimate and there are large variations of results between studies and within a research group. In the US, it is estimated that the average exposure to DEHP is 0.27 mg per day for any given individual, however these estimates are conservative as individuals exposed to medical settings have a much greater exposure than the general population (Stales et al., 1997). A study estimating the daily exposure to DEHP in the German population was conducted by measuring the urine concentrations of DEHP and its secondary metabolites. This study concluded that the average person is exposed to 13.8 µg/ kg body weight/ day (Koch et al., 2004). Another study by the same group concluded a median exposure in an adult to be 5.6 µg/ kg body weight/ day with the highest levels found to be 21 µg/ kg body weight/ day. The same study reported a median exposure in children to be 7.7 µg/ kg body weight/ day with the highest levels found to be 25 µg/ kg body weight/ day respectively (Koch et al., 2005).

DEHP exposure is significantly higher in children than in adults. Infants ingest more calories per body weight than adults with a higher amount of the foods being high in fat including breast milk (Lyche et al., 2009). DEHP is also found in the toys which infants chew on leading to a higher exposure potential (Lyche et al., 2009). Neonatal infants may be exposed to 2.5 mg/kg/day, which is largely attributed to their potential need for medical devices such as i.v. administration, which are known to contain high concentrations of DEHP (Schettler, 2005).

Toxic potential

Although it has been estimated that the toxic effects of DEHP are around ten times lower than other phthalates, studies have shown there to be a toxic effect of DEHP on many organs (Koch et al., 2003). The effects of DEHP on the male reproductive organs have been studied extensively, however there is a paucity of information to date about the effects on the nervous system.

Human studies show various effects of DEHP exposure in males and females. High DEHP levels in males are correlated with lower plasma testosterone levels, decreased sperm volume, and increased sperm DNA damage. Females with high DEHP levels show increased rates of endometriosis as well as earlier term pregnancies (Lyche et al., 2009).

Fetal exposure to DEHP is of particular concern, as phthalate metabolites are known to cross the placenta and have been found in amniotic fluid, placental tissue, cord blood and neonatal meconium (Polanska et al., 2014). DEHP effects the development of the fetus in utero, and exposure has been linked to malformed reproductive organs, decreased anogenital distance, and retained nipples, as well as decreased mating, pregnancy and fertility of the offspring (Koch et al., 2005, Polanska et al., 2014).

Phthalate toxicity of testicular function has been found following prenatal, neonatal and postnatal exposure in rats (Chauvigne et al., 2009). Juvenile male rats exposed to DEHP showed decreased testis weight, seminal vesicle weight and prostate weight, along with malformed epididymis, vas deferens, seminal vesicles, prostate, external genitalia and cryptorchidism (Latini et al., 2006, Lyche et al. 2009, Gray and Gangolli, 1986). Male rats also showed increased germ

cell apoptosis following exposure to MEHP, the active metabolite of DEHP (Giammona et al., 2002).

Higher levels of phthalate metabolites in utero, measured by the levels of urine metabolites in pregnant woman are associated with delayed motor development in the first few years of life (Polanska et al., 2014). Children exposed to higher level of phthalates also have been found to score lower on psychomotor developmental indices, such as cognitive, language and motor abilities, than children exposed to lower levels of phthalates in utero (Kim et al., 2011). These studies suggest that phthalate exposure in young organisms may have detrimental effects on the development of the nervous system.

A study by Park et al. (2014) showed an association between increased levels of DEHP and attention deficit hyperactive disorder (ADHD) and cortical thickness. Boys between the ages of 6 and 15 years with ADHD showed higher levels of DEHP metabolites in urine compared to boys without ADHD. This study also showed that concentrations of DEHP metabolites were negatively correlated with cortical thickness in the right middle and superior temporal gyri, consistent with previous studies showing structural abnormalities, such as decreased volume of the frontal and temporal cortices in individuals with ADHD (Park et al., 2014). This study could not conclude whether the DEHP was a cause of the reduced cortical thickness, but provided a suggestive link between DEHP levels and ADHD in children.

Xu et al. (2015) reported that DEHP exposure was associated with depressive and anxiety-like behaviours in mice. Mice exposed to DEHP from E 7 to PND 21 were subjected to behavioral tests at 6 and 12 weeks of age. Both male and female mice at 6 weeks of age showed increased anxiogenic-like behaviours, while at 12 weeks, only females showed this persistent

anxiety. For both sexes, and at both time points, depressive-like behaviours were seen in association with DEHP exposure compared to the control group. These results suggest that early life DEHP exposure may predispose mice to develop depressive and anxiety-like behaviours during adolescence and adulthood.

Wu et al. (2014) demonstrated DEHP toxicity in cultured neurons. This study showed that when primary neuron cultures were exposed to a dose of DEHP as low as 1nmol/L, there was a significant increase in the concentration of reactive oxygen species, which are used as a marker of oxidative stress. They also showed that the number of neurons and neurite outgrowth in the cultured neurons were reduced with increased DEHP concentrations.

Smith et al. (2011) demonstrated detrimental acute effects of DEHP exposure on the development of regions within the hippocampus. There was a reduction in axonal markers in the CA3 in DEHP exposed males, as well as a reduction in cell density in immature and mature neurons in the CA3 and DG of DEHP exposed males. No difference was noted in the same regions in DEHP exposed females compared to control females or within the CA1 of either males or females. The study concluded that DEHP exposure can impair region specific developmental processes in a gender-dependent way.

A second, related, study found that male rats exposed to DEHP had reduced hippocampal spine density and brain derived neurotrophic factor (BDNF) expression (Smith and Holahan, 2014). BDNF plays an important role in neuron and synapse development and is important for neuron survival (Horch, 2004). While no effect of DEHP was noted on cell body size or branching in the DG, CA1 or CA3 of either male or female rats, spine density of CA3 neurons in males exposed to DEHP was reduced along with a reduction in BDNF mRNA expression

compared to control males (Smith and Holahan, 2014). This is again suggestive of gender and region specific alterations in the hippocampus due to developmental DEHP exposure.

Stress Axis

Overview

Survival of an organism depends on the ability to remain within a state of dynamic equilibrium, known as homeostasis (Frodl and O'Keane, 2013). This homeostasis is seen at the molecular, cellular, physiological and behavioural levels. Stress is known to act as a threat to the equilibrium of biological systems (Chrousos and Gold, 1992). The ability to react appropriately to stressors and to adapt and control the stress reaction confers increased survival adaptability and is known as allostasis (O'Connor et al., 2000, Frodl and O'Keane, 2013). If stress becomes chronic, or if the organism is unable to adapt appropriately, stress system activation may have detrimental effects on the organism (Chrousos, 1998). In this case, the body may be prevented from returning to a healthy state of homeostasis and the stress response becomes nonspecific (Frodl and O'Keane 2013). This detriment, known as the allosteric load, can affect the whole organism, including the immune, cardiovascular, neuroendocrine and central nervous systems (Chrousos, 1998). Long term stress has been shown to be a contributing factor in many acute and chronic diseases (Nicolaides et al., 2015).

Anatomy

The stress system receives information from neurosensory and blood-borne signals (Chrousos, 1998). Activation of the stress system consists of physiological and behavioural adaptive responses that are initiated to be able to properly respond to the threat (Chrousos, 1998).

The stress system is comprised of two separate, yet dependent systems, called the hypothalamic-pituitary-adrenal (HPA) axis and the locus ceruleus-norepinephrine (LC-NE)/autonomic (sympathetic) nervous system (O'Conner et al., 2000). The LC-NE system is part of the autonomic nervous system. This system directly innervates many tissues, including the cardiovascular, respiratory, gastrointestinal, renal and endocrine systems, for a rapid response to stressors (Chrousos, 1998). In response to activation, NE is released to target tissues with a range of responses depending on which tissue is activated (O'Connor et al., 2000). NE also inhibits CRH secretion from the hypothalamus in a negative feedback loop (O'Conner et al., 2000).

The principle regulator of the HPA axis is corticotrophic releasing hormone (CRH) (Frohl and O'Keane, 2013). During non-stress situations, CRH is released from the hypothalamus into the portal system at basal levels following a circadian rhythm (Chrousos, 1998). During acute stress, CRH secretion increases, which in turn stimulates the release of adrenocortiocotrophic hormone (ACTH) from the anterior pituitary gland (Frohl and O'Keane, 2013). ACTH is released into the peripheral circulation and acts on the cortex of the adrenal glands to stimulate release of glucocorticoids (Frohl and O'Keane, 2013, Chrousos, 1998). Activation of the HPA axis results in release of glucocorticoids throughout the body. Cortisol is the main glucocorticoid released by the human HPA axis, while corticosterone is the principle glucocorticoid of the rodent HPA system (Tamashiro, 2005). Circulating glucocorticoids function in a negative feedback loop on the HPA axis through receptors in the hypothalamus and hippocampus to regulate CRH secretion (Frohl and O'Keane 2013).

Glucocorticoids have various functions such as binding to receptors on tissue targets that are necessary for meeting the demands of a stressed system in order to mobilize or store energy (Frohl and O'Keane, 2013). These functions include glucose and fat mobilization, bone

metabolism, cardiovascular activity and immune function (Chrousos, 1998, Frohl and O'Keane, 2013). They also modify brain function via two types of receptors: the high-affinity glucocorticoid receptors (GR), which are located in the hippocampus, and the low-affinity mineralocorticoid receptors (MR), located throughout the brain (Chrousos, 1998).

Stress and the Hippocampus

Stress and the resulting increased levels of glucocorticoids are known to have effects on hippocampal structure and memory formation and retrieval. The hippocampus has a high concentration of glucocorticoid receptors, making it particularly vulnerable to circulating glucocorticoid levels (Brown, et al., 2014, Kim and Diamond, 2002). The high concentration of receptors in the hippocampus is necessary due its role in terminating the stress response through negative feedback loops (McEwen and Sapolsky, 1995).

Studies have shown altered morphology in different sub regions of the hippocampus following exposure to elevated levels of glucocorticoids. Adult male rats subjected to 21 days of subcutaneous injections of corticosterone were shown to have decreased branching and length of apical dendrites on CA3 pyramidal cells with no differences observed in CA1 pyramidal cells or DG granule cells (Woolley, et al., 1990, reviewed by McEwen and Margarinos, 2001). Another study subjected adult male rats to restraint stress to determine whether behavioral stress would affect hippocampal neuron morphology. Similar to the previous study, CA3 pyramidal neurons in the hippocampus showed a significant reduction in apical dendrite length and branching, with no significant differences in CA1 neurons or DG neurons of stressed vs non stressed animals (Watanabe, 1992). This stress-induced atrophy can be blocked by treatment with cyanoketone, which is an adrenal steroid synthesis blocker (McEwen and Margarinos, 2001).

Stress has also been shown to suppress neurogenesis in the DG of the hippocampus. Acute stress in animals, such as exposure to the smell or sight of their predators, has been shown to reduce neurogenesis (McEwen and Magarinos, 2001). Animals exposed to psychosocial stress in the form of dominant/subordinate relationships showed a rapid decrease in neurogenesis in the subordinate animal immediately following the establishment of the relationship. Even following this first encounter, the subordinate animal continued to show an increased stress response in the presence of the dominant animal, resulting in a continued suppression of neurogenesis (Gould et al., 1999). Another study demonstrated that rats, after removal of the adrenal glands, which are the source of circulating glucocorticoids, while supplementing low doses of glucocorticoids to maintain diurnal rhythms, eliminated stress induced decreases in neurogenesis (Mirescu et al., 2004).

Circulating glucocorticoid levels are thought to be the primary cause of inhibition of neurogenesis following stress (Mirescu and Gould, 2006). Blocking glucocorticoid release by removal of the adrenal glands, which removes the source of glucocorticoids, or by blockers of HPA activity, lead to an increase in neurogenesis in young, adult, and aged rats (Gould et al., 1992, Mirescu and Gold. 2006). Increasing glucocorticoid levels, such as by administering exogenous corticosterone, is known to block neurogenesis in early postnatal and adult rats (Gould et al., 1991).

Hypothesis

It is hypothesized that acute exposure to DEHP in juvenile rats will have detrimental effects in remodeling and impairments of plasticity in the hippocampus. This detriment will be in a dose dependent manner, in which higher doses will have a greater impairment effect than lower

doses and this will be associated with elevated corticosterone levels. Granule cells of the DG and pyramidal cells of the CA3 are hypothesized to show a decrease in both newly dividing neurons and immature neurons, shown by a decrease in staining of BrdU and DCX respectively. The granule cells are also hypothesized to show a decrease in plasticity following DEHP exposure, with a decrease in branching and spine density, as assessed by Golgi staining. These changes in the hippocampus are hypothesized to be due to increased HPA axis activation and therefore an increase in circulating corticosterone levels, as DEHP may act as a stressor to the rat.

MATERIALS AND METHODS

Animals

Four untimed pregnant females (approximate gestational day 13) were ordered from Charles River Laboratories (St Constant, Quebec). The day the pups were born was recorded as PND0. Pregnant rats were single housed in 48 x 26 x 20 cm^3 polycarbonite cages in a temperature controlled environment and pups were kept with their mother. Rats were kept on 12 hour light-dark cycle, with lights on at 8:00 am. Rats were fed *ad libitum*. The study was carried out at Carleton University in compliance with and approved by the Institutional Animal Care Committee along the guidelines of the Canadian Council on Animal Care.

Injections

Bis(2-ethylhexyl) phthalate (DEHP) (Sigma-Aldrich; St. Louis, MO, USA) or vehicle (sunflower oil), and 5-Bromo-5'-deoxyuridine (BrdU) (Sigma-Aldrich; St. Louis, MO, USA) was injected i.p. once daily, on alternating sides, into awake rat pups from PND16 to PND 22. This time point was chosen as previous studies have shown this to be a crucial period of hippocampal development (Smith et al.,2011, Smith and Holahan, 2014). DEHP solution, diluted in sunflower oil and BrdU, dissolved in 0.9% NaCl, was prepared just prior to injections. Rats were injected between 14h00 and 15h00 and were returned to their home cage following injections. Rats were randomly assigned to treatment groups (0.1 mg DEHP/kg, 1mg DEHP/kg, or 10mg DEHP/kg), or control. All rats received an injection of BrdU. Each group consisted of 5 males and 5 females.

Tissue Collection

Rats were euthanized by rapid decapitation on PND22, 2 hours after the final injections. Brains were extracted and sectioned in half sagitally, with one half of each brain being randomly assigned between immunohistochemical staining and Golgi-Cox Impregnation method. Whole blood was collected in microtubes with EDTA. Whole blood was centrifuged at 3000 rpm for 8 minutes and plasma was collected and stored at -80^{0}C until further analysis.

Immunohistochemical Staining

Tissue Processing

One half of each brain, sectioned sagitally, was used for immunohistochemical staining. Immediately following extraction, brains were put into 4 % paraformaldehyde/0.01 M phosphate buffer solution (PBS) and stored at 4^{o}C for 24 hours. Brains were then cryoprotected in 30% sucrose at 4^{o}C for a minimum of 3 days before sectioning. Hippocampus sections of 60 µM were obtained using a ThermoScientific cryostat. Sections were stored in 0.1% sodium azide/0.01 M phosphate buffer solution at 4^{o}C until further analysis.

Immunohistochemistry

Hippocampus sections were stained for doublecortin (DCX) and adjacent sections for BrdU. DCX is a marker expressed by immature neurons (Seib and Martin-Villalba, 2014). BrdU is a marker of neurogenesis and is a thymidine analog that is incorporated into the DNA of dividing cells which can be detected using immunohistochemistry (Erikkson et al., 1998).

Sections stained with DCX were washed three times in 0.01M phosphate buffered solution in Triton-X (PBS-TX) for 5 minutes, then transferred to 0.3% H_2O_2 in PBS-TX for 15 minutes, followed by three more washes in PBS-TX for 5 minutes. Sections were then

transferred to 1x animal free blocker (AFB) (Vector Laboratories, Burlingame, CA, USA) in PBS-TX for 30 minutes to block non-specific binding. Sections were then incubated overnight in the primary antibody, goat anti-doublecortin (1:200, Santa Cruz Biotechnologies, Santa Cruz, CA, USA), in AFB/PBS-TX. The primary antibody binds to specific antigens on the target of interest. The following day, sections were washed three times in PBS-TX for 10 minutes then incubated in the secondary antibody, biotinylated donkey anti-goat (1:1000; Vector Laboratories, Burlingame, CA, USA) in AFB/PBS-TX for 1 hour. The secondary antibody binds to an antigen on the primary. Sections were washed three times in PBS for 10 minutes, and then incubated in an avidin-biotin complex (ABC; Vector Laboratories) in PBS for 1 hour, followed by three more washes in PBS for 5 minutes. Staining was visualized with a 0.5 %, 3,3'-diaminobenzene (Sigma-Aldrich) solution, enhanced with 1 % cobalt chloride and 1 % nickel ammonium sulfate. Sections were washed three times in PBS for five minutes then mounted onto glass slides. After the sections were dry, the slides were rinsed in distilled water for 1 minute, then dehydrated in 50%, 75% an d 100% ethanol for 1, 5 and 10 minutes respectively. Slides were then transferred to clearene for 20 minutes, then coverslipped with Permount (Sigma-Aldrich) hardset mounting medium.

Sections stained with BrdU were washed in PBS-TX for 15 minutes, and then transferred to 1x AFB in 0.01M (PBS-TX) for 60 minutes to block non-specific binding. Sections were then incubated overnight in the primary antibody, goat anti-BrdU (1:200, Santa Cruz Biotechnologies, Santa Cruz, CA, USA). The following day, sections were washed in PBS-TX for 15 minutes then incubated in the secondary antibody, DyLight anti-goat 488 (1:500, Vector Laboratories, Burlingame, CA, USA), for 2 hours. In immunofluorescent staining, the secondary antibody is tagged with a fluorescent marker. Sections were then washed in PBS for 15 minutes. Sections

were mounted on slides then coverslipped with Vectashield Hard Set Mounting Medium with DAPI (Vector Laboratories, Burlingame, CA, USA) and stored at 4^{o}C until further analysis.

Analysis

Images of the dentate gyrus sections were analyzed on a DCX Olympus BX61 microscope, and analyzed using Image J. A picture was taken at 10x magnification of an overview the DG region, and pictures were taken at 4x of the DG and the CA3 regions of the hippocampus for analysis in Image J. For the CA3, area and light intensity was compared between the basal region and the apical region. For the DG, number of particles was counted to determine number of DCX stained cell bodies, using the parameters of a size between 50-500, and a roundness of 50-100%. Light intensity was also compared between the dendrite staining in the DG, to a region just outside of the DG.

BrdU was not analyzed due to not being able to visualize or localize the staining appropriately.

Golgi-Cox Impregnation Method

Tissue Processing

One half of each brain, sectioned sagitally, was used for Golgi-Cox impregnation method. Brains were prepared using the Golgi-Cox technique. Immediately following extraction, brain were put in potassium dichromate, mercuric chloride and potassium chromate solution (Golgi fix solution) for 4 days. This was followed by 3 washes in distilled water (dH_2O), for 4 hours, 3 then hours, then overnight. Brains were then cryoprotected in graduated sucrose solutions of 10% for 8 hours, 20% overnight and 30% for a minimum of 4 days. Hippocampus

sections of 200μM were obtained using a Vibratome and mounted on double gelatinized slides. These slides were placed in a dark, humidified box for 24 hours. Slides were then rinsed in dH_2O for 1 minute, submerged in 28% Ammonium Hydrozide for 40 minutes, washed in dH_2O for 1 minute, submerged in Kodax film fix A (diluted 1:1 with dH_2O) for 40 minutes, and washed twice in dH_2O. Following the washes, slides were submerged in 50%, 70% then 95 % ethanol for one minute each. Slides were then washed 3 times in desiccated 100% ethanol for 5 minutes each, then immersed in desiccated 33% ethanol, 33% chloroform and 33% clearene solution for 10 minutes, followed by two final submersions in desiccated 100% clearene for 15 minutes each. Desiccated solutions are made 24 hours in advance using 3A molecular sieve 1/16" pellets to remove any trace amounts of water in the solutions. Sections were coverslipped with Permount mounting solution and placed in a dark, desiccated box for a minimum of four days.

Morphological Analysis

Individual neurons within the dentate gyrus were reconstructed and analyzed at 100x on a Olympus BX51 microscope (MBF Bioscience Inc.,Williston, VT). A total of 3 neurons were traced within each hippocampal region, and the results were averaged across the region. For each neuron, the cell body and dendrites were reconstructed. Measurements were recorded for cell body size, number of branching points, total dendrite length and spine density.

Plasma Corticosterone Analysis

`Plasma corticosterone levels were measured using a commercial radioimmunoassay kit. Samples were run in duplicate on a single run and averaged to avoid inter-assay variability (Liu et al., 2014).

Statistical Analysis

One way Analysis of variance with Tukey post-hoc tests were conducted to compare treatment groups within a gender, and Univariate Analysis of Variance tests were performed to compare between genders and to test for interactions between gender and treatment. DCX stained regions of the CA3 cells were analyzed for differences in light intensity and area of the basal dendrites over the apical dendrites. DCX stained regions of the DG were analyzed for number of stained cell bodies, and light intensity of the dendrites over a region just outside the DG. Golgi stained cells were analyzed for differences in cell body size, dendritic branching, total dendritic length, and spine density. Plasma corticosterone levels were analyzed.

RESULTS

DCX staining in the DG

There was no significant effect of treatment on the intensity of DCX staining in the dendrites of either females (Figure1A; One-Way ANOVA $F(3, 12) = .252$, $p = .858$) or males (Figure1B; One-Way ANOVA $F(3, 11) = 3.016$, $p = .076$). There was also no differences found in the number of DCX stained neurons in the DG of either females (Figure2A; One-Way ANOVA $F(3, 12) = 1.438$, $p = .280$) or males (Figure 2B; One-Way ANOVA $F(3, 11) = .547$, $p = .660$). There was no significant difference between gender, or interaction between gender and treatment for the intensity of DCX staining (Figure 1C; Univariate Analysis of Variance $F(1, 23) = .297$, $p = . 591$ [gender]; $F(2,23) = 1.324$, $p = .291$ [interaction]), or in number of DCX stained neurons (Figure 2C; Univariate Analysis of Variance $F(1, 23) = 2.066$, $p = .164$ [gender]; $F(2,23) = 1.340$, $p = .286$ [interaction]).

DCX staining in the CA3

There was no significant effect of treatment on the intensity of the DCX stained area of the basal dendrites over the intensity of the DCX stained area of the apical dendrites in either females (Figure 3A; One-Way ANOVA $F(3, 12) = .666$, $p = .589$) or males (Figure 3B; One-Way ANOVA $F(3, 11) = 1.011$, $p = .360$). There was no significant effect of treatment in ratio of the size of the DCX stained area of the basal dendrites over the size of the DCX stained area of the apical dendrites of the CA3 in females (Figure 4A; One-Way ANOVA $F(3, 12) = 1$, $p = .426$). Males exposed to 1 mg/kg and 10 mg/kg of DEHP showed a significant reduction in the ratio of the size of the DCX stained area of the basal dendrites over the size of the DCX stained area of the apical dendrites compared to controls (Figure 4B; One-Way ANOVA $F(3, 11) =$

7.537, p = 0.005; Tukey post –hoc p= .045 and p = .004 respectively). There was no significant difference between gender, or interaction between gender and treatment for the intensity of DCX stained area (Figure 3C; Univariate Analysis of Variance F(1, 23) = .659, p = .425 [gender]; F(2,23) = 1.324, p = .291 [interaction]), or in the ratio of the size of the DCX stained area (Figure 4C; Univariate Analysis of Variance F(1, 23) = .080, p = .779 [gender]; F(2,23) = 2.159, p = .120 [interaction]).

Golgi Staining in the DG

There was no significant effect of treatment in the DG on cell body size of females (Figure 5A; One-Way ANOVA F(3, 8) = .058, p = .980) or males (Figure 5B; One-Way ANOVA F(3, 8) = 2.019, p = .190), dendritic branching of females (Figure 6A; One-Way ANOVA F(3, 8) = 2.978, p = .096) or males (Figure 6B; One-Way ANOVA F(3, 8) = .623, p = .620), or spine density of females (Figure 7A; One-Way ANOVA F(3, 8) = 1.928, p = .204) or males (Figure 7B; One-Way ANOVA F(3, 8) = 3.921, p = .054). Females exposed to 10 mg/kg of DEHP showed a decrease in total number of spines compared to controls (Figure 8A; One-Way ANOVA F(3, 8) = 5.004, p = .031; Tukey post –hoc p= .042). Females exposed to 0.1mg/kg of DEHP showed a decrease in dendrite length compared to controls (Figure 9A; One-Way ANOVA F(3, 8) = 4.455, p = .040; Tukey post –hoc p= .048). No differences were seen in males in total number of spines (Figure 8B; One-Way ANOVA F(3, 8) = 2.262, p = .158) or dendrite length (Figure 9B; One-Way ANOVA F(3, 8) = 2.092, p = .180). There was no significant difference between gender, or interaction between gender and treatment on cell body size (Figure 5C; Univariate Analysis of Variance F(1, 16) = .067, p = .799 [gender]; F(3,16) = .920, p = .454 [interaction]), dendritic branching (Figure 6C; Univariate Analysis of Variance F(1, 16) = 3.424, p = .083 [gender]; F(3,16) = 1.384, p = .284 [interaction]), spine density

(Figure 7C; Univariate Analysis of Variance F(1, 16) = 1.146, p = .301 [gender]; F(3,16) = .175, p = .912 [interaction]), total number of spines (Figure 8C; Univariate Analysis of Variance F(1, 16) = .430, p = .521 [gender]; F(3,16) = 2.896, p = .067 [interaction]), or dendrite length (Figure 9C; Univariate Analysis of Variance F(1, 16) = .500, p = .489 [gender]; F(3,16) = 1.185, p = .347 [interaction]).

Plasma Corticosterone Level

There was no significant effect of treatment on plasma corticosterone levels in either females (Figure 10A; One-Way ANOVA F (3, 21) = .319, p = .811) or males (Factor 10B; One-Way ANOVA F(3, 20) = 2.147, p = .126). There was no significant difference between gender, or interaction between gender and treatment (Figure 10C; Univariate Analysis of Variance F(1, 40) = .418, p = .741 [gender]; F(3,40) = 1.041, p = .385 [interaction]).

DISCUSSION

The present thesis evaluated changes in morphological structures of the hippocampus of male and female juvenile rats following daily exposure to phthalates from PND 16 to PND 22, and possible mechanisms associated with these changes. It was hypothesized that exposure to phthalates during this time would result in abnormal remodeling and impairments in plasticity in the hippocampus and that these changes would be associated with an increase in HPA axis activation. Previous studies have shown similarities in changes to the developing hippocampus following phthalate exposure (Smith et al., 2011, Smith and Holahan, 2014) and stress exposure (Woolley, et al., 1990, reviewed by McEwen and Margarinos, 2001, Watanabe, 1992). The study by Smith, et al. (2011) demonstrated that acute exposure to phthalates (DEHP) disrupted hippocampal connectivity in the CA3 subregion in male rats but not females while the CA1 region did not show any disrupted connectivity in either males or females. In the present thesis, DEHP treatment during a sensitive period of neural development caused structural abnormalities in certain hippocampal areas. Specifically, DEHP-treated males showed a decrease in the ratio of the size of the DCX stained area of the basal dendrites over the size of the DCX stained area of the apical dendrites in the CA3 region at the middle and highest doses compared to controls with a trend towards a dose dependent decrease in the lower dose of DEHP. This replicates the findings of Smith, et al. (2011). Using the Golgi stain, females showed a decrease in the dendrite length of the granule cells of the DG at the lowest dose of DEHP with recovery at the higher doses. Females also showed a decrease in number of granule cell spines in the DG at the highest dose of DEHP.

The results from this study demonstrated a decrease in the ratio of the size of the DCX stained area of the basal dendrites over the size of the DCX stained area of the apical dendrites of

the CA3 in males, which is consistent with previous studies showing an impairment in the structural organization of the male CA3 following DEHP exposure (Smith and Holahan, 2014). In the current study, this deficiency was shown to be significantly different from controls at the middle and highest dose of DEHP (similar to Smith, et al., 2011), and there was a trend towards a dose dependent reduction in the ratio at the lowest dosage of DEHP. As DCX stains immature neurons (Seib and Martin-Villalba, 2014), these results indicate that there were fewer axonal projections from developing (or immature) granule cells to the basal dendrite postsynaptic targets in CA3 in the DEHP-treated male rats. This could be due to the immature neurons not being able to develop or not sending axons to the appropriate destinations, and therefore dying off with exposure to DEHP. The basal dendrites, which are located in the stratum oriens lamina, are the location of inputs from the other fields of the hippocampus as well as the DG (Amaral and Witter, 1989). Reductions in the number of axonal inputs from the DG can lead to impairment of information transmission within the hippocampus (Holahan et al., 2010, Holahan and Routtenberg, 2011). According to the lamellar hypothesis, the connections in the hippocampus are mostly unidirectional and activated in succession (Amaral and Witter, 1989). Following a reduction in the number of connections, there may be some loss of function within the hippocampus network, which may lead to deficits in memory or cognitive function. Reductions in transmission of electrical currents due to the lack of appropriate axonal inputs to these basal dendrites following DEHP exposure can possibly lead to memory impairments. These changes were evident in the male brains exposed to DEHP, not in the female brains, suggesting some possible protection against these effects in the females.

No differences were noted in the darkness of the stained area of the CA3 in either males or females following DEHP treatment. This measurement was done to determine the difference

in intensity in the DCX stained area of the basal dendrites over the intensity of the DCX stained area of the apical dendrites of the CA3. Although there were differences noted in the males for the size of the area, no differences were noted in the intensity of the stained area. This is possibly due to branching and length of the apical dendrites, or the size of the cell bodies being affected rather than the number of the cells itself. If there were to be a similar number of cells present following DEHP treatment compared to control, the darkness of the area may remain similar to control, with the size of the area itself being smaller following treatment. Previous studies however have found no differences in cell body size or dendritic branching in the CA3 following DEHP treatment (Smith et al., 2011). Morphological studies of the CA3 should be done to determine if the length and branching of these apical dendrites is affected by DEHP treatment at the doses that were used in this study.

Consistent with a previous study by Smith et al. (2011) of DEHP treatment during the period of rat brain development, no differences were noted in DCX staining of the DG. Counts were done on the number of particles, reflecting cell bodies stained with DCX, as well as measurements of the darkness of the staining of the DG dendrites compared to an area just exterior to the DG, to determine the intensity of staining. Both measurements were fairly consistent across treatment groups and compared to control, indicating no effect of DEHP on the DG in this study. This would be expected as previous studies performed with DEHP exposure during the same period showed no differences in the DG (Smith et al., 2011, Smith and Holahan, 2014), as well as no changes in the DG following stress exposure (Woolley, et al., 1990, reviewed by McEwen and Margarinos, 2001, Watanabe, 1992). Previous studies have suggested region and sex specific alterations in the hippocampus following acute exposure to DEHP, with no effect of treatment on granule cell morphology in the DG (Smith et al., 2011, Smith and

Holahan, 2014). Consistent with previous findings, there were no significant changes in the cell body size, dendrite branching, or spine density of either male or female rats.

The period of development in the DG starts at E10, and continues until the first week postnatally (Bayer, 1980). The DG of the hippocampus could therefore be almost fully developed during the period when the injections were done. It is therefore possible that the lack of finding of any major changes in the DG of either males or females exposed to DEHP is due to it being past the sensitive time period of development. Further studies should look at earlier time points of injections, potentially during the prenatal period or embryonic development, to determine the effect of DEHP exposure during this sensitive period of DG development.

The current study did show some region and gender specific changes in the DG. In females, there was a trend towards a smaller number of dendritic spines on the granule cells compared to control at all doses of DEHP, with a significant reduction at the highest dose. Males showed a decrease in the number of dendritic spines at the lowest and middle DEHP doses compared to controls and what appeared to be recovery at the highest dose. A similar trend was found in the dendrite length of the granule cells with a significant reduction in dendrite length compared to controls at the lowest DEHP dose in females and possible recovery at the higher dose. The reduced spine number and dendrite length is suggestive of a detriment to development of the granule cells, with stunted growth leading to underdeveloped neurons. The granule cells of the DG are the first main input into the hippocampus (Amaral and Witter, 1989). If there are underdeveloped neurons in this area, the input into the DG may be impacted, which could then lead to memory formation deficits.

Dendritic spines in the DG develop on the basal dendrites by PND 5, and are fully developed by PND 10 with an increase in density until PND 25 (Seress and Pokom, 1980). Therefore, it was hypothesized that exposure to DEHP during this period of increasing spine density would negatively impact the density of dendritic spines. However, these results were not found in the current study. It is possible that DEHP at these levels does not have an effect on dendritic spine density per se but rather, the microstructure of the spines could be affected, as the spine type (e.g., mushroom, thin, stubby) in this study was not determined. Spine structure plasticity is necessary for LTP, and changes in the types of spines can lead to more efficient transmission of electrical current (Bosch and Hayahi, 2012). It may be worthwhile to examine the type of spine present on the dendrites to determine if there were differing types found between the treated and control groups of rats, which would be thought to have effects on the efficiency of neural communication.

Estimates of human DEHP exposure are extremely varied between studies. The present work used an overestimate of doses that humans would be exposed to, but for a shorter period of time. In terms of human exposure, there is a much more chronic timeframe, occurring over the whole life time, yet at potentially lower exposure levels. It is important to understand what the impact of these lower, yet longer exposures levels may have on development.

This study did show some structural deficits in some measurements of the hippocampus, which were apparent only at the lower DEHP doses. This is important to the understanding of changes that may occur following phthalate exposure in humans, as some of these changes may occur only following low doses. Previous studies have only looked into the higher DEHP doses and the hippocampal development disruptions that occurred (Smith et al., 2011, Smith and Holahan, 2014). It is possible that these changes are only evident at lower doses of DEHP, as

would be experienced during human development. At higher doses, the body may recognize the toxicant and take preventive actions against its effects, which would explain how these changes in dendritic length and number of spines in both the males and females are only evident at the lower doses, with recovery at the higher doses. Further studies might examine lower doses for longer periods of time to see if there are more widespread changes in hippocampus morphology which would more closely mimic daily exposure in humans.

The present thesis also examined whether neurogenesis in the DG was affected by DEHP exposure, as the DG is one of only two areas where neurogenesis occurs at high rates (Erikkson et al., 1998). This was to be determined by staining with BrdU, following daily injections of BrdU during the same time period as injections of the DEHP. BrdU becomes incorporated into the DNA of neurons as they are developing (Erikkson et al., 1998). The staining however was unquantifiable; therefore it was not possible to determine whether phthalate exposure had effects on neurogenesis in this study.

One of the goals of this thesis was to investigate potential mechanisms that might be responsible for changes that occur in the hippocampus following DEHP exposure. It was hypothesized that HPA axis activation would be a possible mechanism for the hippocampal changes following DEHP exposure. It was hypothesized that DEHP would act as a stressor on the body, leading to HPA axis activation and a downstream increase in corticosterone release. It was hypothesized that there would be a DEHP dose-dependent increase in corticosterone levels, which would result in the deficits in hippocampal morphology. This was proposed due to similarities between previous studies of changes in the hippocampus following DEHP exposure (Smith et al., 2011, Smith and Holahan, 2014) and studies of changes following stress, showing impairments in the CA3 (Woolley, et al., 1990, reviewed by McEwen and Margarinos, 2001). In

the present thesis, there was no significant difference between treatment groups in either males or females on corticosterone levels compared to controls. There was however a large standard error noted in all treatment groups, suggesting large variability across the rats. It is possible that the method of euthanasia accounted for some of this variability, as the rats were taken individually from the mother rats, and not returned, while being introduced to a room with scents that could be stressful to them and could quickly increase their stress and therefore corticosterone levels. It is also possible that performing two daily injections, with injecting both the DEHP and the BrdU, on the animals increased their HPA axis activity more than would be expected following a single injection per day with only injecting the DEHP. Further studies would aim to remove this variability. A potential solution to the scents could be to perform the euthanasia in a better air filtered room. This could hopefully lead to a decrease in variability and allow for more accurate assessment of possible mechanisms involved in DEHP-associated hippocampal disruptions.

Previous studies have aimed to explain the differences in male and female vulnerability to DEHP exposure (Xu et al., 2015, Lyche et al. 2009). It is possible that the HPA axis does not significantly contribute to the changes in hippocampal structure, as suggested by the current thesis, and the alterations in morphology between males and females may be due to the well-established anti-androgenic properties of DEHP (Akingbemi et al., 2001, Gray et al., 2000, Andrade et al., 2006). Normal physiological levels of many different hormones are key to hippocampal development and organization and changes in these hormones may have effects on normal development (Cooke and Woolley, 2004). Previous studies have demonstrated a decrease in serum testosterone levels and aromatase enzyme activity in males following DEHP treatment (Akingbemi et al., 2001, Andrade et al., 2006) and a decrease in estradiol and progesterone levels

in females following DEHP treatment (Davis et al., 1994a, 1994b, Lovekamp-Swan and Davis, 2001). Further studies should be done to measure these specific hormones following DEHP treatment to determine how they might contribute to hippocampal developmental changes.

Another possible explanation for the differences between male and female vulnerability to DEHP exposure are changes in metabolism of the phthalate following exposure. Once ingested, gut lipases convert DEHP to MEHP (Tickner et al., 2001). This conversion may occur at different rates between males and females, leading to differences that are seen in hippocampus development in previous studies (Smith et al.,2011, Smith and Holahan, 2014). Work in the current thesis was based on DEHP intraperitoneal injections rather than oral administration. . It is possible that due to the DEHP being injected rather than ingested, the conversion to MEHP may not have occurred. The conversion is performed by gut lipases, and therefore, this conversion may be skipped by the injectable route (Tickner et al., 2011, Koch et al., 2005). This may lead to differences in hippocampal changes that would occur from ingestion, as previous studies have reported gender specific changes following ingestion of the phthalates (Gray and Gangolli, 1986).

As there were some gender and region specific changes in hippocampal development discovered, it would be useful for further studies to be performed using behavioural tests. As there was some evidence of changes in hippocampal structure following treatment with DEHP, it would be interesting to determine whether this translates to impairments in memory formation, with possible differences between males and females. These rats could be subjected to tests on the water maze following DEHP treatment. The hippocampus is known to be crucial for spatial memory formation (Morris, 1984, Khazal-Nazzal, 2013), and this test could allow the determination of whether there are impairments in spatial memory following treatment. This

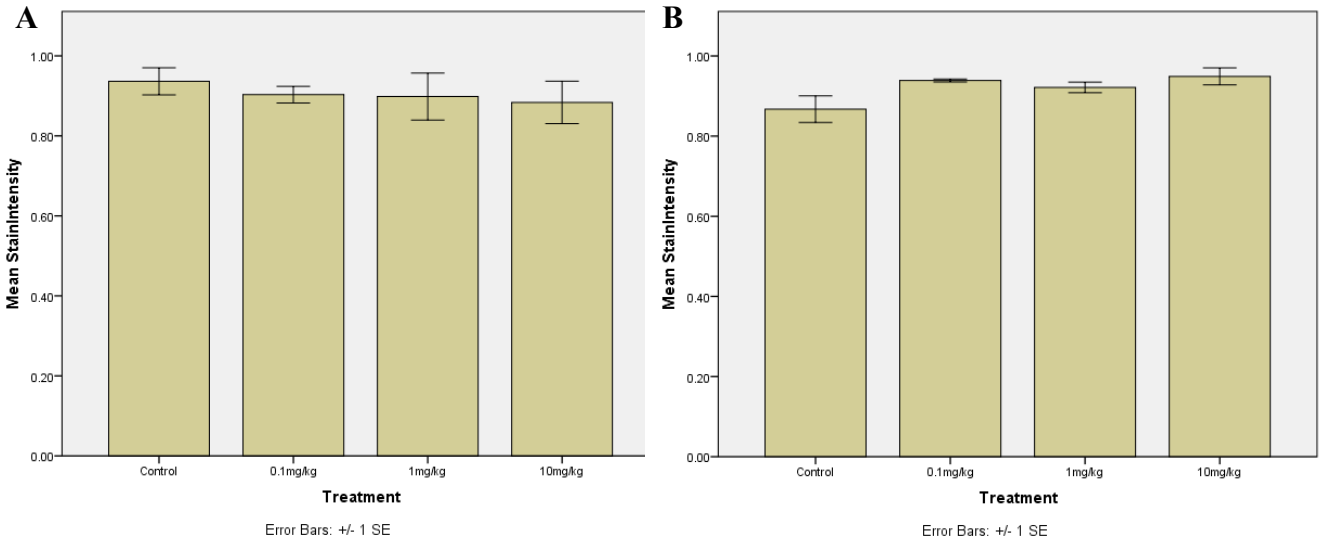
would allow for more insight into whether the hippocampal changes that were evident in this study led to direct changes in memory formation.

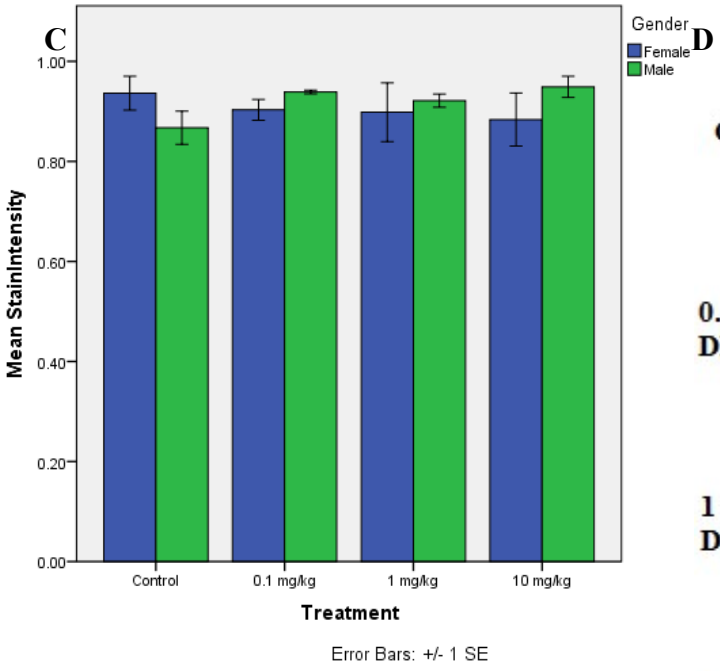
Further studies should also be conducted evaluating injections in the juvenile rats with a later time point of euthanasia. This study was done by euthanizing the animals immediately following the final injections, however it would be beneficial to determine some longer term effects on hippocampal development following juvenile DEHP exposure. It has been found in previous studies that DEHP has effects on some hormone levels in the exposed rats (Akingbemi et al., 2001; Andrade et al., 2006, Davis et al., 1994a; 1994b; Lovekamp-Swan and Davis, 2001), and that these hormones are important for regulation of development of the hippocampus (Cooke and Woolley, 2004). It is important to determine whether early life exposure to phthalates may affect neurodevelopment, with the changes in the hippocampus development becoming apparent following puberty of the animal as hormonal levels change (Atanassova et al., 2000, Dohler and Wuttke, 1975).

Conclusion

Phthalate exposure is almost inevitable in our environment, with exposure levels being significantly higher in children than in adults (Lyche et al., 2009). This prompts the necessities of performing studies to determine the effects of phthalate exposure during crucial developmental periods. This study on acute DEHP exposure during a crucial period of hippocampal development of the rat brain showed gender and region specific effects on development. The CA3 of males showed a decrease in the size of the area of basal dendrites at the middle and highest dose of DEHP treatment, potentially leading to synaptic transmission impairments. The

DG of females showed a decrease in number of spines at the highest dose, and a decrease in the dendrite length at only the lowest dose of DEHP treatment, with recovery at higher doses, leading to the need for further studies of the effects of DEHP at lower doses, more similar to human exposure levels. Further studies should be conducted to determine the mechanisms underlying these changes.


A
Mean StainIntensity
1.00
0.80
0.60
0.40
0.20
0.00
Control
0.1mg/kg
1mg/kg
10mg/kg
Treatment
Error Bars: +/- 1 SE
B
Mean StainIntensity
1.00
0.80
0.60
0.40
0.20
0.00
Control
0.1mg/kg
1mg/kg
10mg/kg
Treatment
Error Bars: +/- 1 SE


C
Gender
Female
Male
Mean StainIntensity
1.00
0.80
0.60
0.40
0.20
0.00
Control
0.1 mg/kg
1 mg/kg
10 mg/kg
Treatment
Error Bars: +/- 1 SE

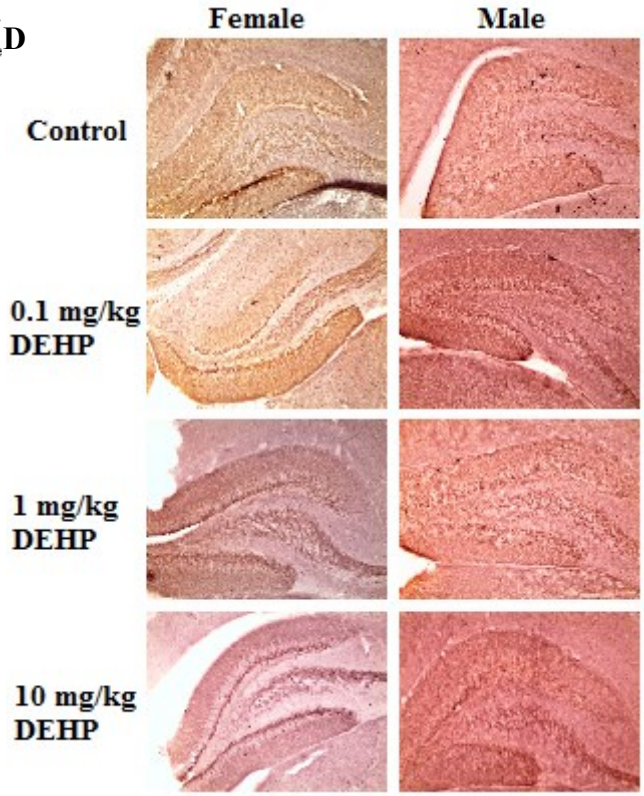
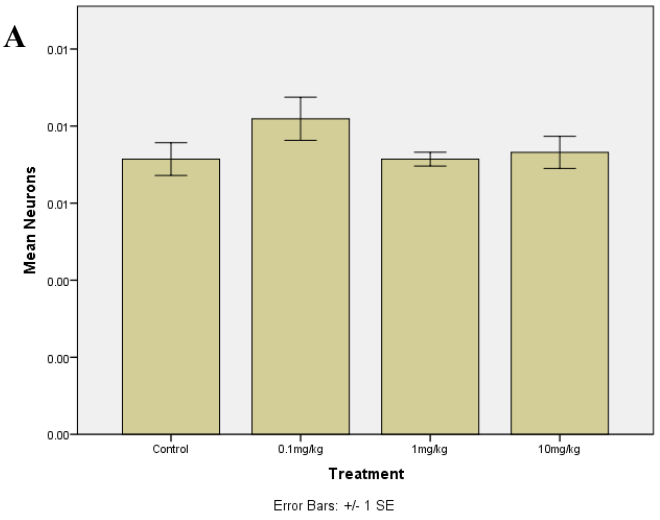

D
Female
Male
Control
0.1 mg/kg DEHP
1 mg/kg DEHP
10 mg/kg DEHP

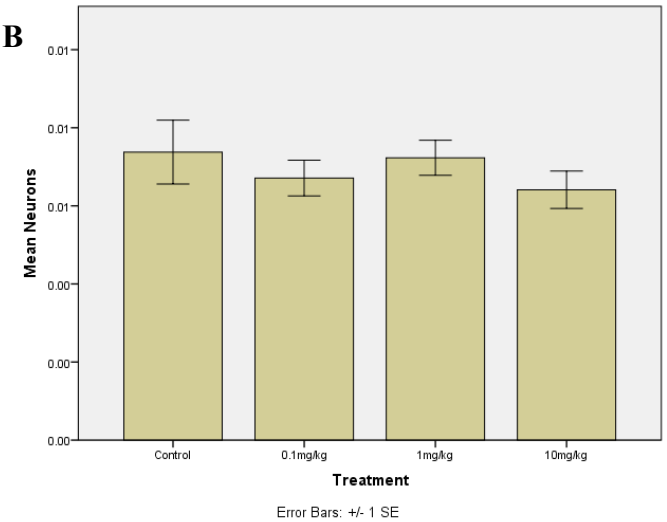
Figure 1: Intensity of DCX staining in the DG following acute DEHP treatment in juvenile rats

Average intensity of DCX staining (+/- standard error) in the DG of female rats (A), male rats (B), and combined (C) following acute DEHP treatment of 0(females: n=4; males n=4), 0.1 mg/kg (females: n=4; males n=4), 1 mg/kg (females: n=4; males n=4) or 10 mg /kg (females: n=4; males n=3) daily for 7 days. Representative photomicrographs (D) of DCX staining in the DG (4X). No differences were observed between groups, $p > .05$.

A


Mean Neurons
0.01
0.01
0.01
0.00
0.00
0.00
Control
0.1mg/kg
1mg/kg
10mg/kg
Treatment
Error Bars: +/- 1 SE

B


Mean Neurons
0.01
0.01
0.01
0.00
0.00
0.00
Control
0.1mg/kg
1mg/kg
10mg/kg
Treatment
Error Bars: +/- 1 SE

C

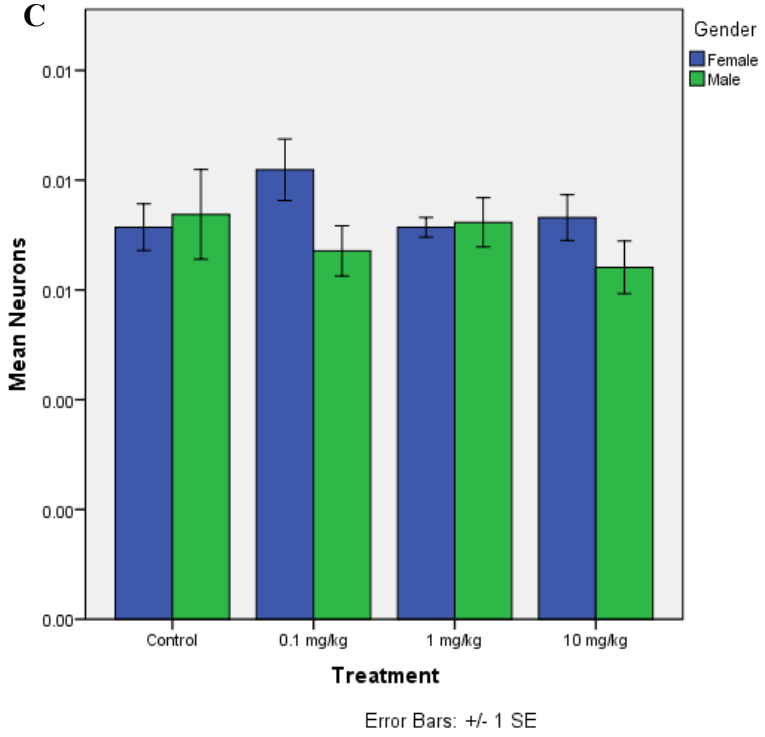
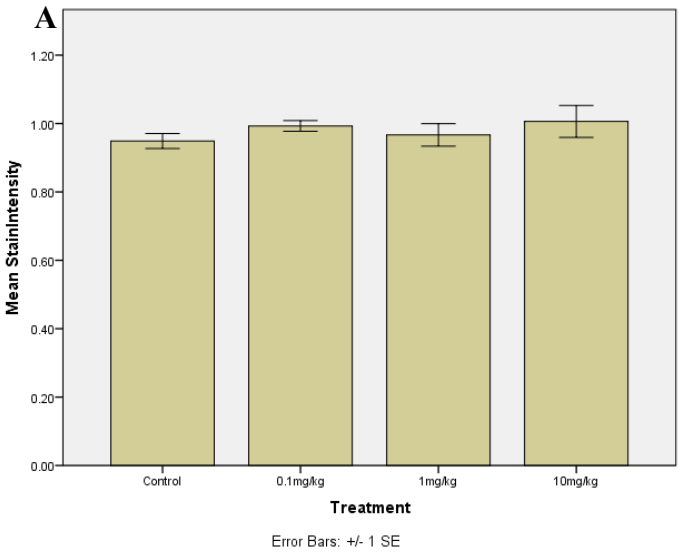
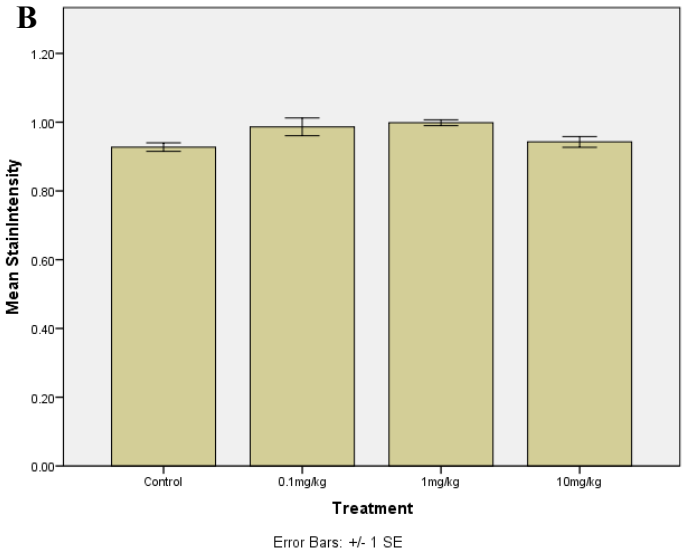
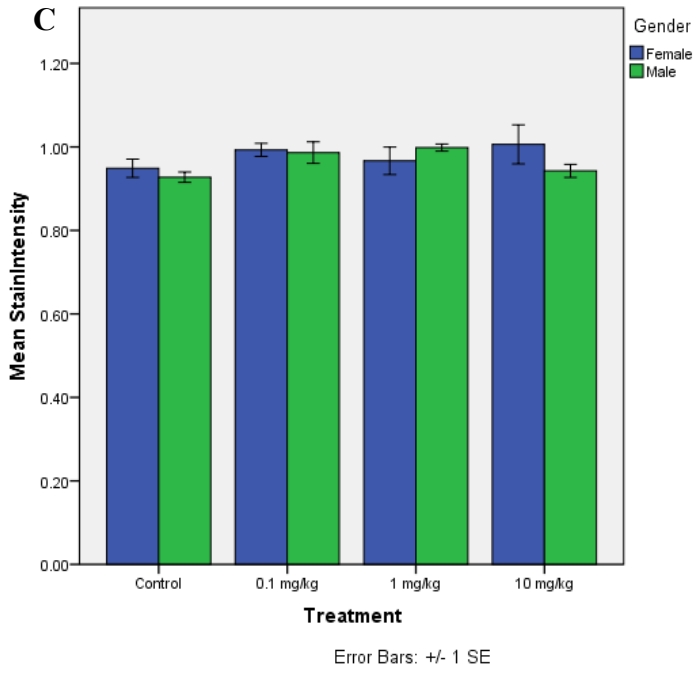

Gender
Female
Male
Mean Neurons
0.01
0.01
0.01
0.00
0.00
0.00
Control
0.1 mg/kg
1 mg/kg
10 mg/kg
Treatment
Error Bars: +/- 1 SE

Figure 2: Number of DCX stained neurons in the DG following acute DEHP treatment in juvenile rats

Average number of DCX stained neurons (+/- standard error) in the DG of female rats (A), male rats(B), and combined (C) following acute DEHP treatment of 0(females: n=4; males n=4), 0.1 mg/kg (females: n=4; males n=4), 1 mg/kg (females: n=4; males n=4) or 10 mg /kg (females: n=4; males n=3) daily for 7. No differences were observed between groups, $p > .05$.


A
Mean StainIntensity
1.20
1.00
0.80
0.60
0.40
0.20
0.00
Control
0.1mg/kg
1mg/kg
10mg/kg
Treatment
Error Bars: +/- 1 SE


B
Mean StainIntensity
1.20
1.00
0.80
0.60
0.40
0.20
0.00
Control
0.1mg/kg
1mg/kg
10mg/kg
Treatment
Error Bars: +/- 1 SE


C
Gender
Female
Male
Mean StainIntensity
1.20
1.00
0.80
0.60
0.40
0.20
0.00
Control
0.1 mg/kg
1 mg/kg
10 mg/kg
Treatment
Error Bars: +/- 1 SE

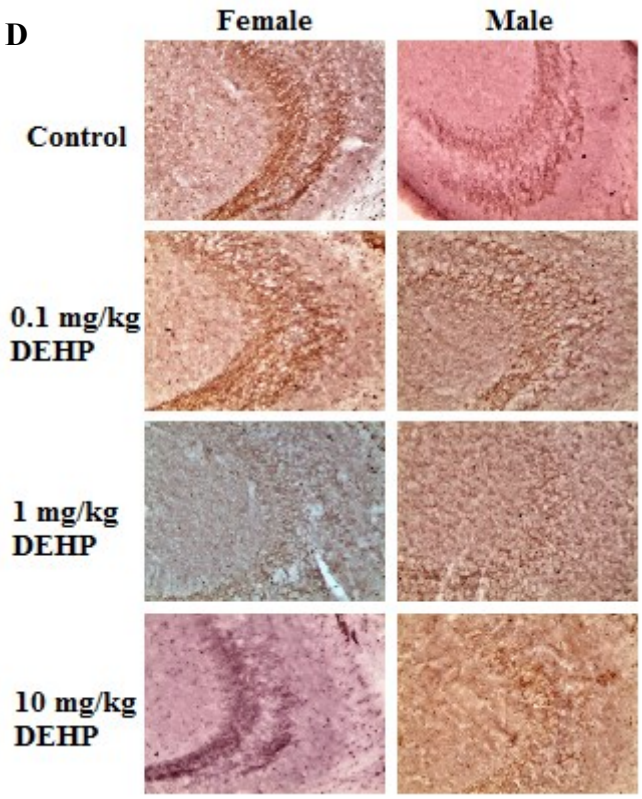
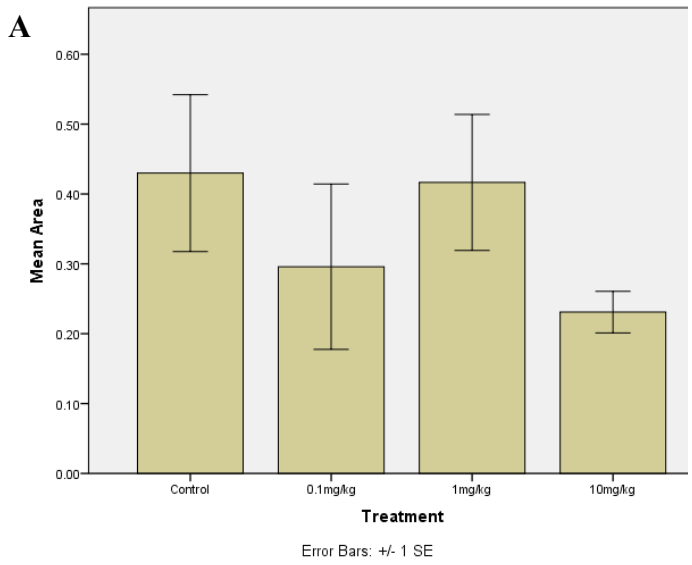
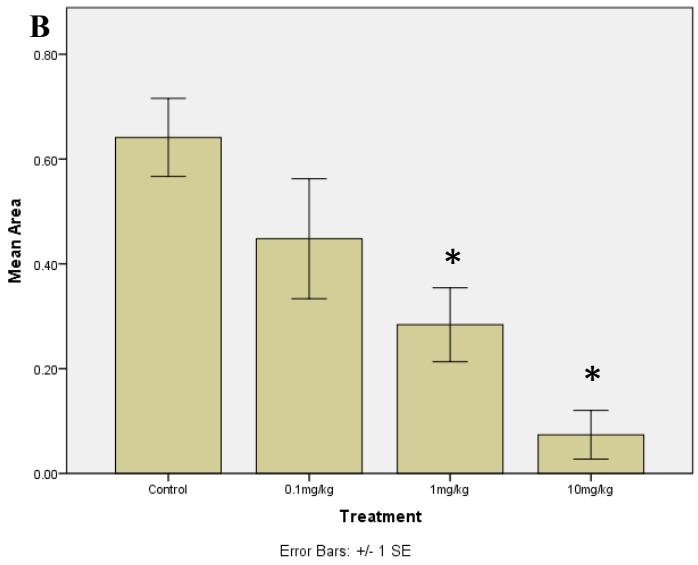

D
Female
Male
Control
0.1 mg/kg DEHP
1 mg/kg DEHP
10 mg/kg DEHP

Figure 3: Intensity of DCX staining in the CA3 following acute DEHP treatment in juvenile rats

Average intensity of DCX staining (+/- standard error) in the CA3 of female rats(A), male rats(B), and combined (C) following acute DEHP treatment of of 0(females: n=4; males n=4), 0.1 mg/kg (females: n=4; males n=4), 1 mg/kg (females: n=4; males n=4) or 10 mg /kg (females: n=4; males n=3) daily for 7. Representative photomicrographs (D) of DCX staining in the CA3 (10X). No differences were observed between groups, $p > .05$.


A
Mean Area
0.60
0.50
0.40
0.30
0.20
0.10
0.00
Control
0.1mg/kg
1mg/kg
10mg/kg
Treatment
Error Bars: +/- 1 SE


B
Mean Area
0.80
0.60
0.40
0.20
0.00
*
*
Control
0.1mg/kg
1mg/kg
10mg/kg
Treatment
Error Bars: +/- 1 SE

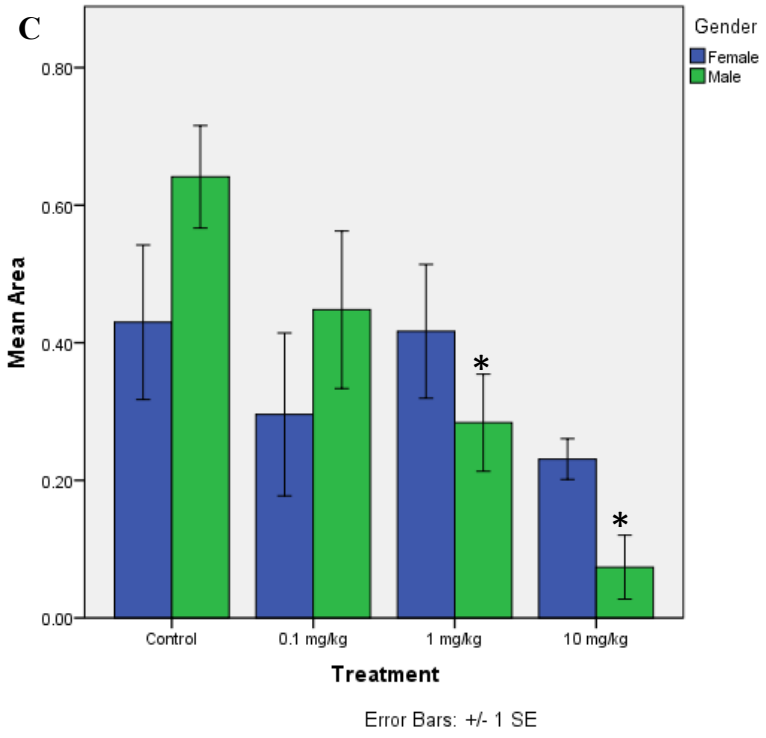
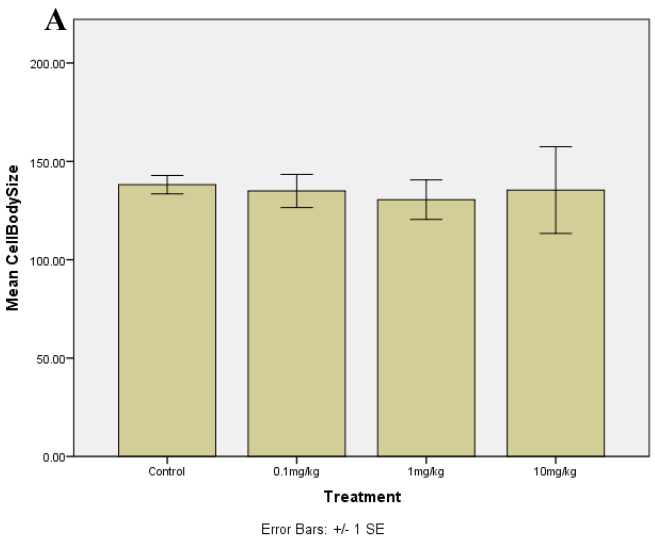
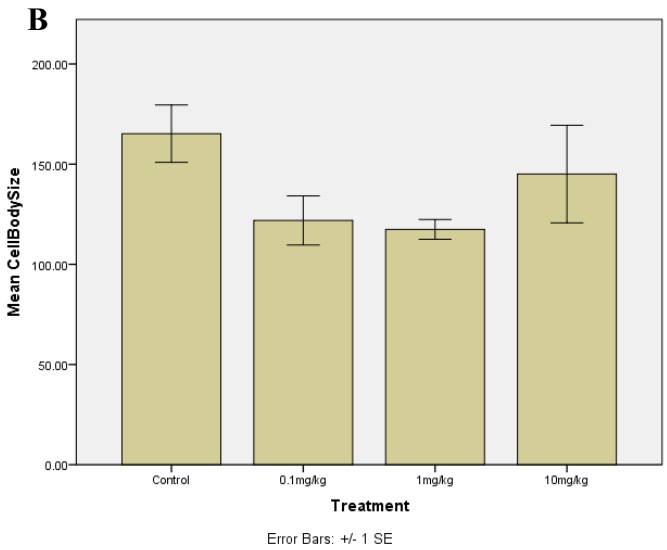

C
Gender
Female
Male
Mean Area
0.80
0.60
0.40
0.20
0.00
*
*
Control
0.1 mg/kg
1 mg/kg
10 mg/kg
Treatment
Error Bars: +/- 1 SE

Figure 4: Area of DCX staining in the CA3 following acute DEHP treatment in juvenile rats

Average area of DCX stained area (+/- standard error) in the CA3 of female rats (A), male rats(B), and combined (C) following acute DEHP treatment of of 0(females: n=4; males n=4), 0.1 mg/kg (females: n=4; males n=4), 1 mg/kg (females: n=4; males n=4) or 10 mg /kg (females: n=4; males n=3) daily for 7. No differences were observed between groups in females, $p > .05$. * = $p < 0.05$.


A
Mean CellBodySize
200.00
150.00
100.00
50.00
0.00
Control
0.1mg/kg
1mg/kg
10mg/kg
Treatment
Error Bars: +/- 1 SE


B
Mean CellBodySize
200.00
150.00
100.00
50.00
0.00
Control
0.1mg/kg
1mg/kg
10mg/kg
Treatment
Error Bars: +/- 1 SE

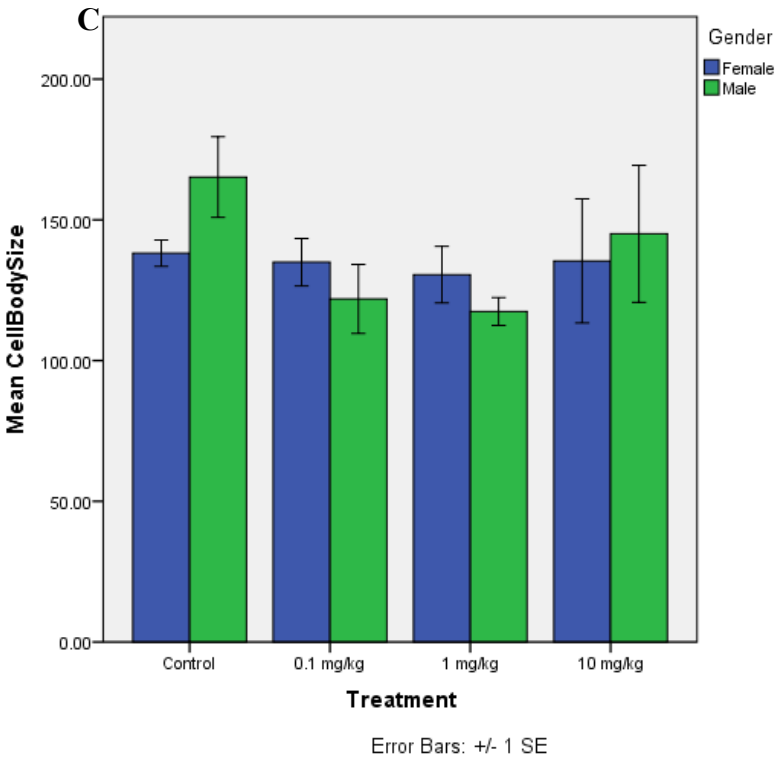
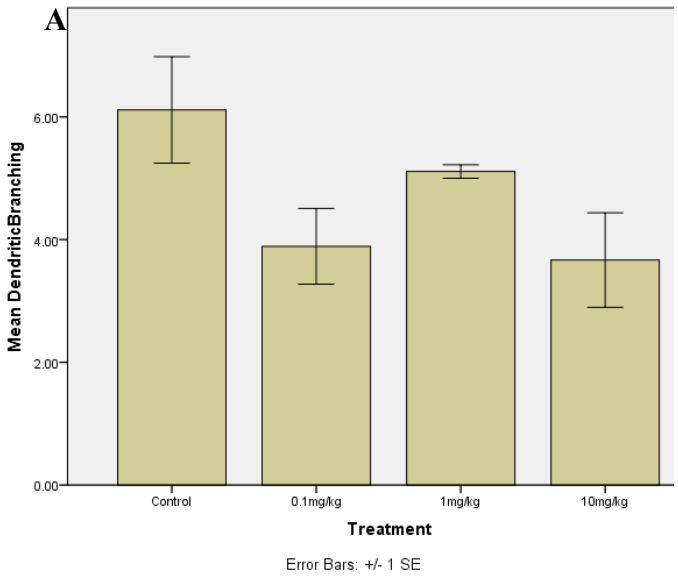
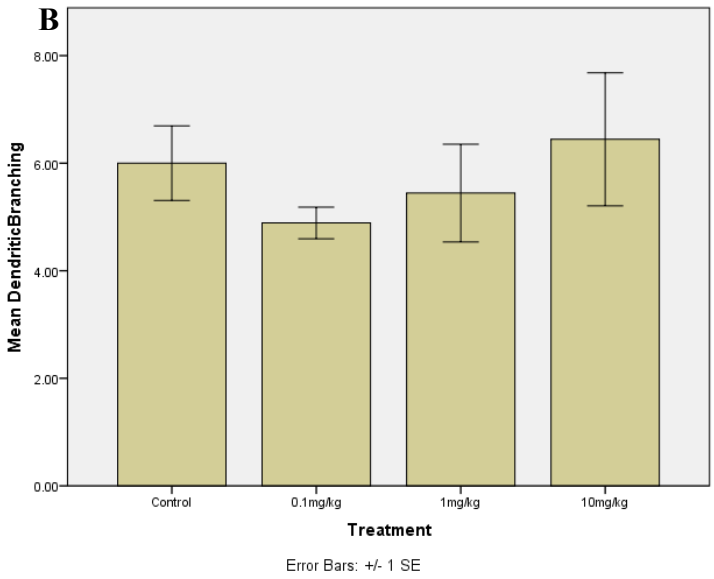

C
Gender
Female
Male
Mean CellBodySize
200.00
150.00
100.00
50.00
0.00
Control
0.1 mg/kg
1 mg/kg
10 mg/kg
Treatment
Error Bars: +/- 1 SE

Figure 5: Cell body size of granule cells in the DG following acute DEHP treatment in juvenile rats

Average cell body size (+/- standard error) of granule cells in the DG of female rats (A), male rats (B), and combined (C) following acute DEHP treatment of 0, 0.1 mg/kg, 1 mg/kg or 10 mg /kg daily for 7 days (n=3 per gender). No differences were observed between groups, $p > .05$.


A
Mean DendriticBranching
6.00
4.00
2.00
0.00
Control
0.1mg/kg
1mg/kg
10mg/kg
Treatment
Error Bars: +/- 1 SE


B
Mean DendriticBranching
8.00
6.00
4.00
2.00
0.00
Control
0.1mg/kg
1mg/kg
10mg/kg
Treatment
Error Bars: +/- 1 SE

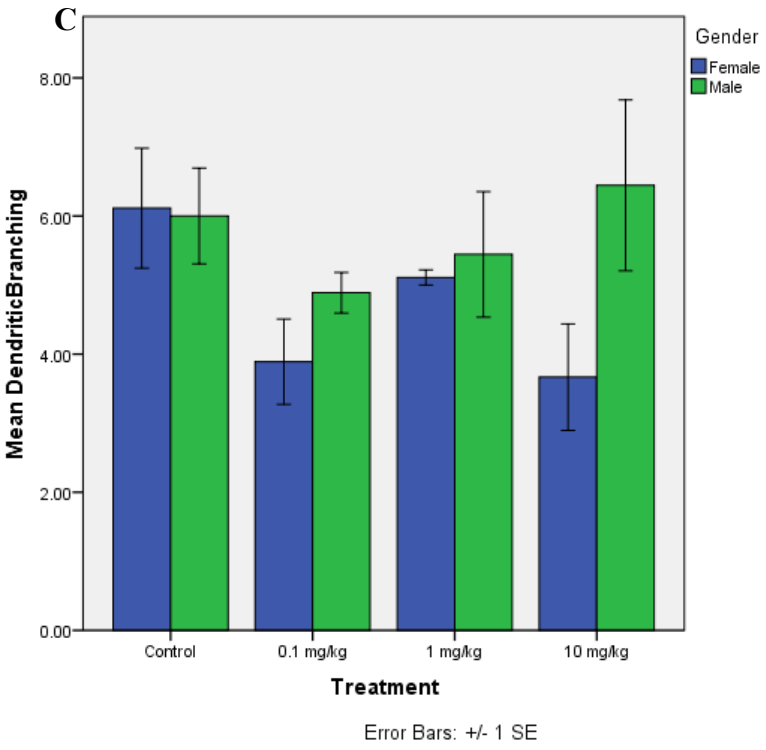
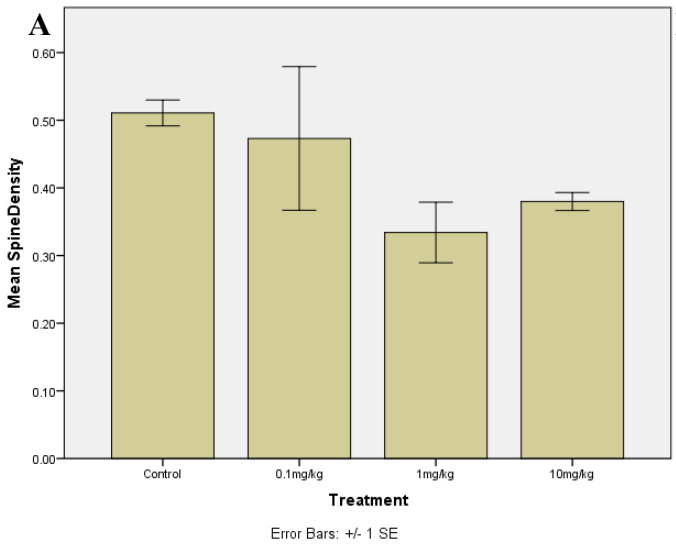

C
Gender
Female
Male
Mean DendriticBranching
8.00
6.00
4.00
2.00
0.00
Control
0.1 mg/kg
1 mg/kg
10 mg/kg
Treatment
Error Bars: +/- 1 SE

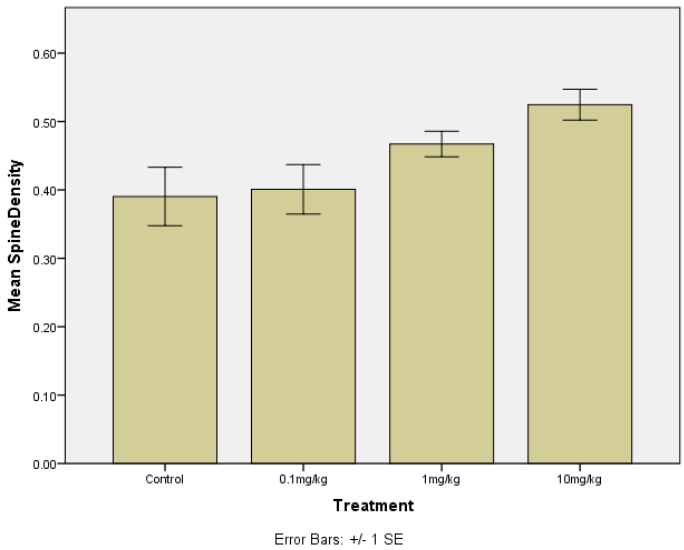
Figure 6: Dendritic branching of granule cells in the DG following acute DEHP treatment in juvenile rats

Average dendritic branching (+/- standard error) of granule cells in the DG of female rats (A), male rats (B), and combined (C) following acute DEHP treatment of 0, 0.1 mg/kg, 1 mg/kg or 10 mg /kg daily for 7 days (n=3 per gender). No differences were observed between groups, $p > .05$.

A


Mean SpineDensity
0.00
0.10
0.20
0.30
0.40
0.50
0.60
Control
0.1mg/kg
1mg/kg
10mg/kg
Treatment
Error Bars: +/- 1 SE

B


Mean SpineDensity
0.00
0.10
0.20
0.30
0.40
0.50
0.60
Control
0.1mg/kg
1mg/kg
10mg/kg
Treatment
Error Bars: +/- 1 SE

C

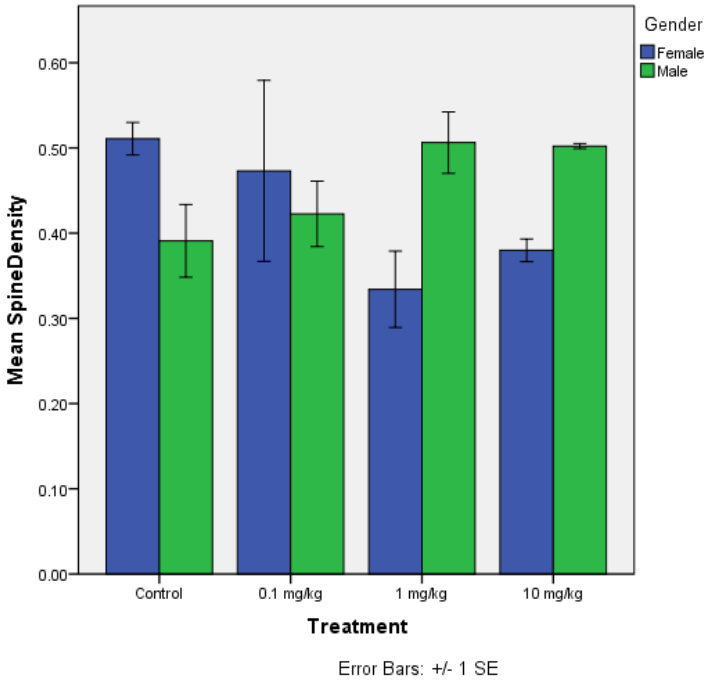
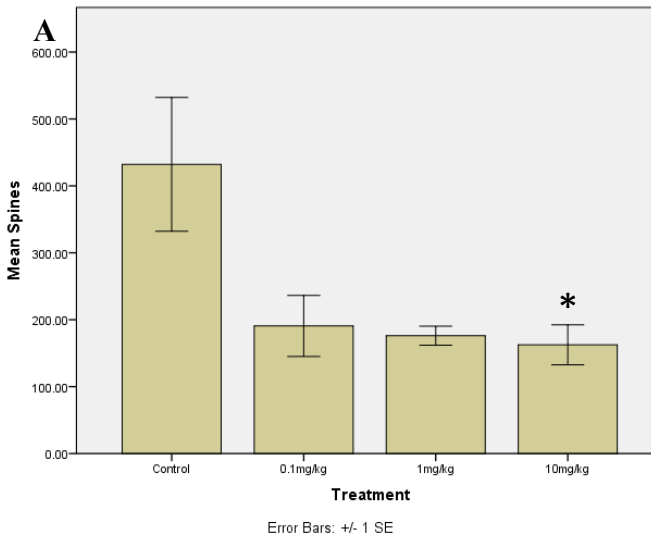
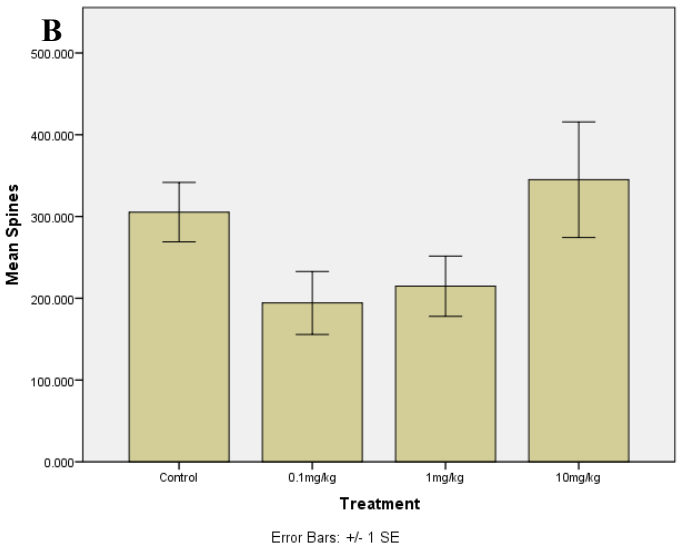

Gender
Female
Male
Mean SpineDensity
0.00
0.10
0.20
0.30
0.40
0.50
0.60
Control
0.1 mg/kg
1 mg/kg
10 mg/kg
Treatment
Error Bars: +/- 1 SE

Figure 7: Dendritic spine density of granule cells in the DG following acute DEHP treatment in juvenile rats

Average dendritic spine density (+/- standard error) of granule cells in the DG of female rats(A), male rats (B), and combined (C) following acute DEHP treatment of 0, 0.1 mg/kg, 1 mg/kg or 10 mg /kg daily for 7 days (n=3 per gender). No differences were observed between groups, $p > .05$.


A
600.00
500.00
400.00
300.00
200.00
100.00
0.00
Mean Spines
*
Control
0.1mg/kg
1mg/kg
10mg/kg
Treatment
Error Bars: +/- 1 SE


B
500.000
400.000
300.000
200.000
100.000
0.000
Mean Spines
Control
0.1mg/kg
1mg/kg
10mg/kg
Treatment
Error Bars: +/- 1 SE

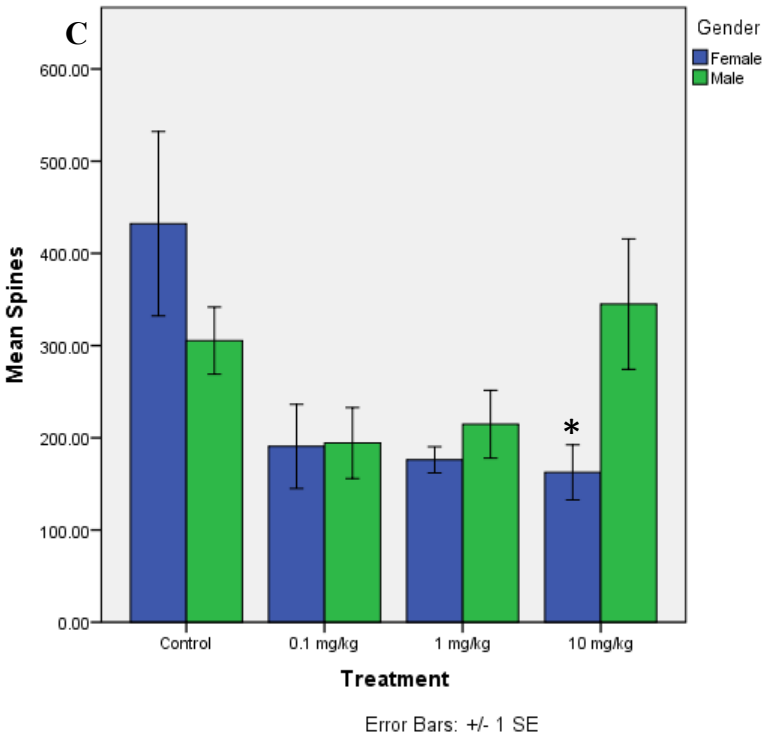
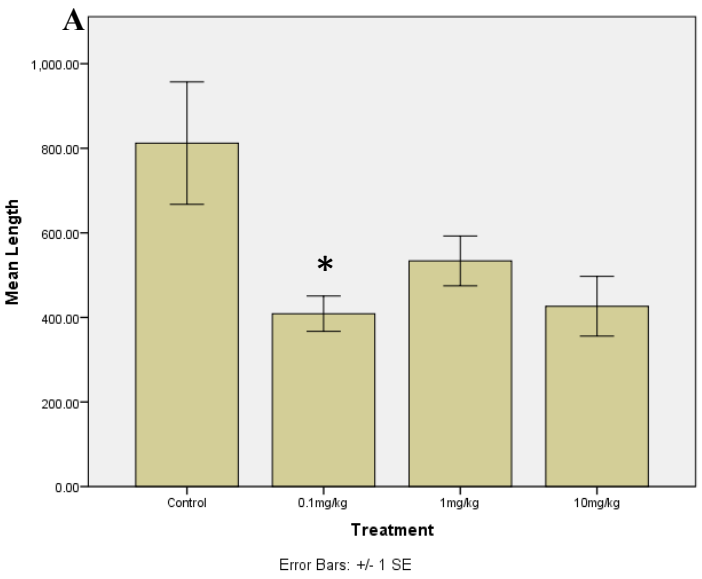
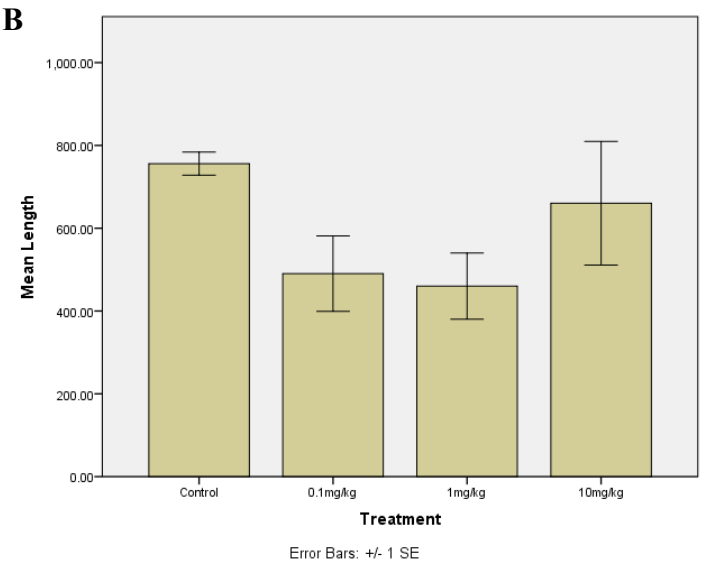

C
Gender
Female
Male
600.00
500.00
400.00
300.00
200.00
100.00
0.00
Mean Spines
*
Control
0.1 mg/kg
1 mg/kg
10 mg/kg
Treatment
Error Bars: +/- 1 SE

Figure 8: Number of spines of granule cells in the DG following acute DEHP treatment in juvenile rats

Average number of spines (+/- standard error) of granule cells in the DG of female rats(A), male rats (B), and combined (C) following acute DEHP treatment of 0, 0.1 mg/kg, 1 mg/kg or 10 mg /kg daily for 7 days (n=3 per gender). No differences were observed between groups in males, $p > .05$. * = $p < 0.05$.


A
1,000.00
800.00
600.00
400.00
200.00
0.00
Mean Length
*
Control
0.1mg/kg
1mg/kg
10mg/kg
Treatment
Error Bars: +/- 1 SE


B
1,000.00
800.00
600.00
400.00
200.00
0.00
Mean Length
Control
0.1mg/kg
1mg/kg
10mg/kg
Treatment
Error Bars: +/- 1 SE

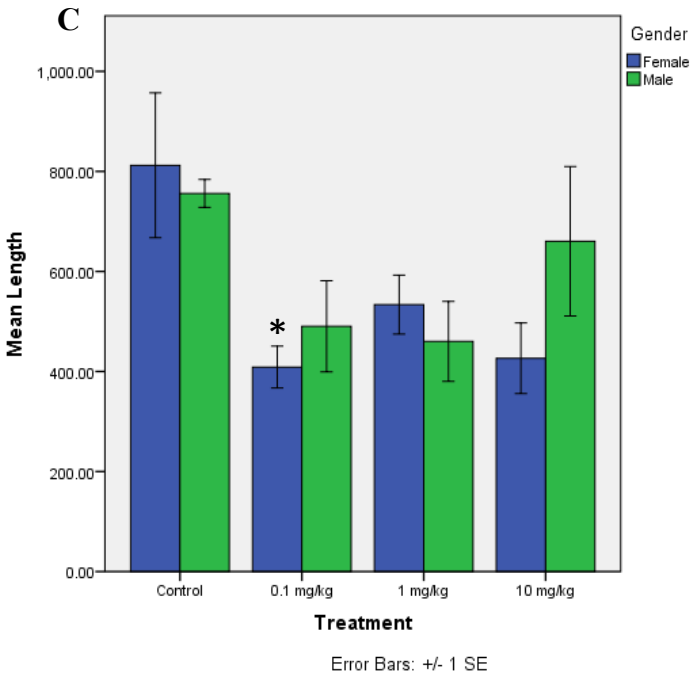
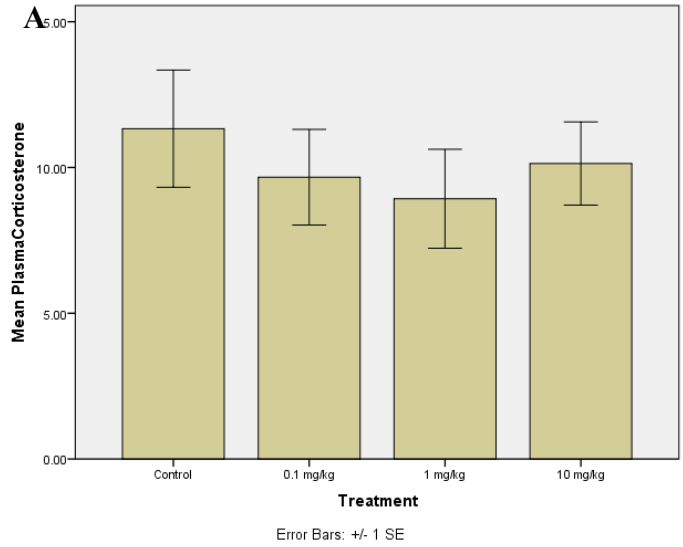

C
Gender
Female
Male
1,000.00
800.00
600.00
400.00
200.00
0.00
Mean Length
*
Control
0.1 mg/kg
1 mg/kg
10 mg/kg
Treatment
Error Bars: +/- 1 SE

Figure 9: Dendrite length of granule cells in the DG following acute DEHP treatment in juvenile rats

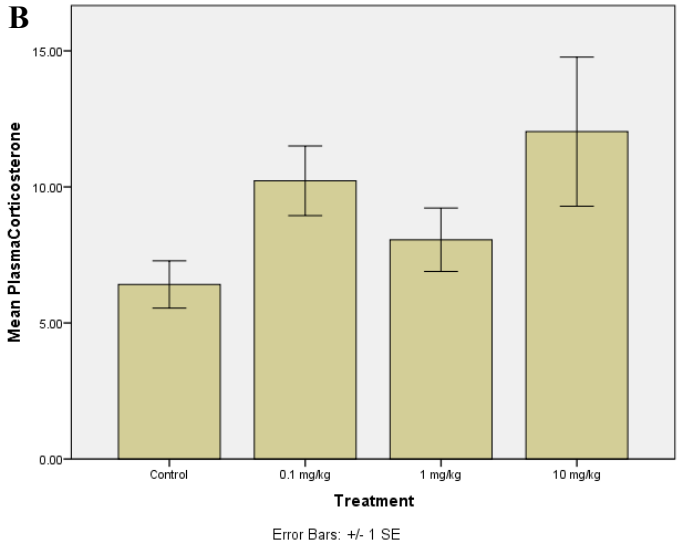
Average dendrite length (+/- standard error) of granule cells in the DG of female rats (A), male rats (B), and combined (C) following acute DEHP treatment of 0, 0.1 mg/kg, 1 mg/kg or 10 mg /kg daily for 7 days (n=3 per gender). No differences were observed between groups in males, $p > .05$. * = $p < 0.05$.

A



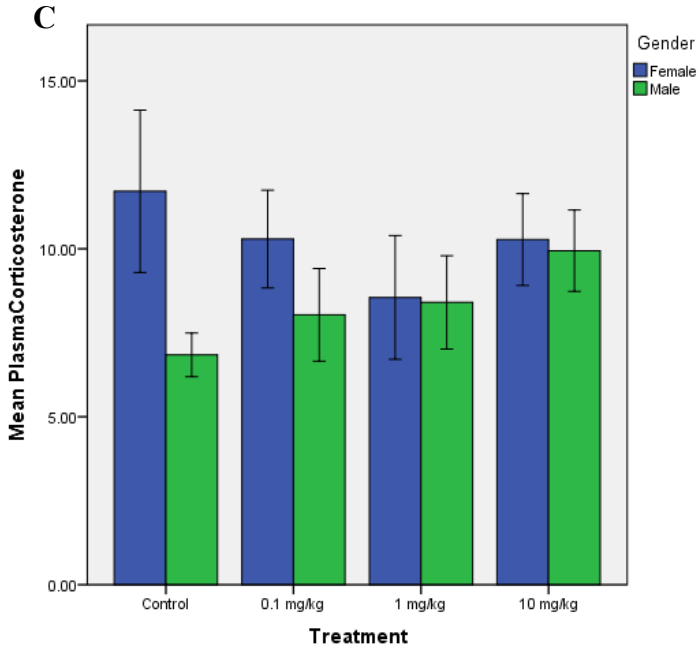
Error Bars: +/- 1 SE

B



Error Bars: +/- 1 SE

C



Error Bars: +/- 1 SE

Figure 10: Plasma corticosterone levels following acute DEHP treatment in juvenile rats

Average plasma corticosterone levels (ng/ml) (+/- standard error) of female rats (A), male rats (B), and combined (C) following acute DEHP treatment of of 0(females: n=6; males n=6), 0.1 mg/kg (females: n=7; males n=5), 1 mg/kg (females: n=5; males n=7) or 10 mg /kg (females: n=7; males n=6) daily for 7. No differences were observed between groups, $p > .05$.

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