PRENATAL ENDOCRINE DISRUPTOR AND ADULT ESTRADIOL EXPOSURES: SEX-SPECIFIC OUTCOMES ON NEUROBEHAVIORAL AND REPRODUCTIVE ENDPOINTS IN SPRAGUE-DAWLEY RATS

by

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ABSTRACT

The prevalence of neuropsychiatric disorders has risen globally at an alarming rate. The ubiquitous presence of endocrine disrupting chemicals (EDCs), which are capable of mimicking hormones in the body and disrupting endocrine system functioning, is suspected to contribute to declining mental health. Fetal programming with these chemicals, specifically, can reorganize developing systems in the body and predispose the fetus to adverse health outcomes throughout life. Additionally, changes in estrogen are also linked with increased susceptibility to mood disorders in women. We hypothesized that fetal programming of Sprague-Dawley rats with various EDCs, especially EDC mixtures, would sex- and dose-dependently alter stress-related behaviors and neurobiological mechanisms. Chronic treatment with estradiol (E2) in female offspring would exacerbate these effects. Our results confirm that male and female offspring behaviors are differentially affected as a result of prenatal EDC exposure. In male offspring, exposure to multiple EDCs reduced their ability to defend themselves from an aversive stimulus in an active manner. Di-(2-ethylhexyl) phthalate (DEHP) treatment at a low dose induced a passive behavioral phenotype in male offspring, whereas high-dose (HD) DEHP treatment

feminized anxiety-like behavior. Female offspring showed altered anxiety-like and defensive behaviors in response to prenatal EDCs at low doses, particularly to the bisphenol A (BPA) + DEHP mixture. Within the brain, EDC-exposed male offspring had marked deficits in dopamine levels in the paraventricular hypothalamic nucleus, which was correlated with their defensive behaviors. Female offspring with DEHP (HD) treatment alone or in combination with BPA showed a persistent hyperactivity of monoaminergic activity in the brain. In healthy control female offspring, E2 treatment in adulthood increased unconditioned anxiety and reduced active coping abilities. In EDC-exposed females, contrastingly, E2 treatment often reversed or abolished the effects on behavior and brain neurotransmitters observed in sham counterparts, implying that EDCs interact with E2 to alter neurobehavior. Finally, we also discovered that prenatal exposure to BPA analogues has deleterious effects on reproductive parameters in both dams and offspring, rendering them unsafe alternatives to BPA. These findings raise concerns about EDC mixtures and low-dose EDC exposures in male and female offspring, and call for increased regulation of these chemicals.

INDEX WORDS: Endocrine disrupting chemicals, prenatal, bisphenols, phthalates, estradiol, behavior, neurotransmitters

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CHAPTER 1

INTRODUCTION

1.1. Endocrine Disrupting Chemicals – Background

Endocrine disrupting chemicals (EDCs) are compounds that alter the functions of the endocrine system and affect health outcomes in an organism and/or its progeny [1]. The Endocrine Society defines an EDC as "an exogenous chemical, or mixture of chemicals, that interferes with any aspect of hormone action" [2]. These chemicals are ubiquitous in the environment and are widely used as plasticizers, pesticides, fungicides, antibacterials, and fire retardants. This concoction of chemicals that humans are regularly exposed to has been repeatedly associated with abnormalities related to the reproductive [3], metabolic [4, 5], developmental [6, 7], and neurobehavioral [8-10] systems. The plasticizers known as bisphenol A (BPA) and di-(2-ethylhexyl) phthalate (DEHP) are among some of the most prevalent EDCs in the environment. Additionally, the BPA analogues named bisphenol S (BPS) and bisphenol F (BPF) are increasingly being used as alternatives to BPA in BPA-free products in an effort to limit and phase out BPA usage [11]. Yet, even more concerning is the possibility that EDCs such as BPA and DEHP exist in combination with one another in the environment, which may be more harmful than exposure to these EDCs individually [12, 13].

BPA is mainly used in the manufacture of polycarbonate plastics and epoxy resins, which are used in food and beverage packaging, metal food and beverage container linings, and even dental sealants and tooth coatings [14]. DEHP, on the other hand, is used in polyvinyl chloride

(PVC) plastics to make them soft and malleable [15]. This chemical can be found in a wide variety of products including medical devices, food and beverage packaging, children's toys, building and furniture materials, and cosmetics and personal care products [16]. BPS is commonly used in cleaning products and thermal paper products such as cashier's receipts [17]. BPF is found in epoxy resins [18] and is a contaminant in fresh and canned foods including vegetables, meats, and dairy products [19, 20]. In addition to their presence in consumer products such as food packaging, plastics, and personal care products [21], both BPF and BPS are also used in other applications such as structural adhesives, dental materials, electrical varnishes, and industrial applications including coatings, flooring, and pipe linings [11, 17]. These analogues are superior to BPA with respect to certain properties, including increased stability and resistance to heat (BPS) [22] and better resistance to solvents (BPF) [23], which partially explains their continued use in BPA-free products.

Brief History of BPA, DEHP, and BPA Analogues

BPA was originally synthesized in 1891, but was commercially used in epoxy resins and polycarbonate plastics beginning in the 1950s [24]. However, its estrogenic properties were discovered approximately a decade earlier in 1936, making it one of the oldest known synthetic compounds characterized by its endocrine-disrupting actions. The discovery of the estrogen mimicking properties of BPA was initially not considered problematic and further investigation into these properties only resumed in the 1990s, following the discovery of nuclear hormone receptors [25]. Moreover, DEHP was synthesized in 1933 and consequently, its use in PVC plastics began during that decade [26]. Phthalate production substantially grew and totaled over 1 billion pounds by the 1970s. The ability of bisphenols and phthalates to migrate or leach out of plastics was determined in the 1970s in the case of phthalates [27], and not until the 1990s for

BPA [28]. Although BPS was first synthesized in 1869 and was used as a type of dye [29], both BPS and BPF were introduced as substitutes for BPA in consumer products only in the 2000s, following the surge in restrictions on BPA production and use [29, 30].

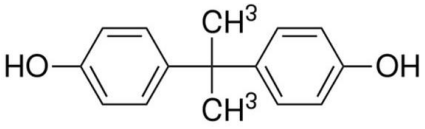
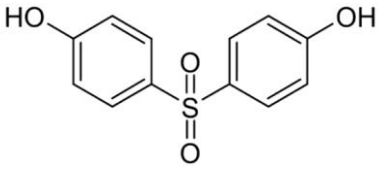
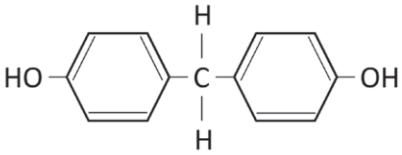
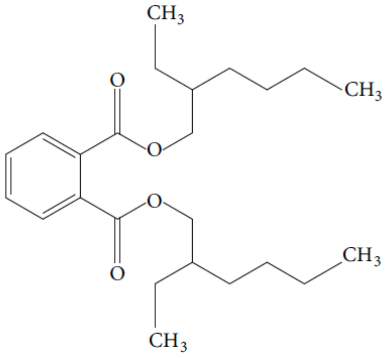
As a result of the vast number of studies confirming the adverse effects of BPA and DEHP on a wide range of endpoints, these EDCs have been tightly regulated over the past decade or so. Use of BPA in baby bottles was banned in Canada in 2008, in France in 2010, and in the European Union (EU) in 2011 [31]. In the US, the Food and Drug Administration (FDA) banned the use of BPA in baby bottles and sippy cups in 2012 and phased out BPA use in infant formula packaging the following year [32]. Canada also went on to prohibit the use of BPA in all food packaging and containers in 2010 [22], and France similarly banned the use of BPA in food and beverage packaging in 2015 [31]. Likewise, the EU essentially banned several phthalates found in children's toys and similar products, including DEHP, in 2005 [33]. This was followed by Canada proposing similar regulations in 2007, with a focus on DEHP. The following year, the United States also enacted legislation restricting the use of phthalates. The EU went on to ban phthalates such as DEHP in 2015 [34]. BPS and BPF, on the other hand, remain unregulated today with unrestricted use [31]. Yet, studies on the health consequences of BPS and BPF are growing, with increasing evidence linking the effects of these analogues with those of BPA itself [11]. This calls for increased regulation and updates to regulatory practices concerning BPA alternatives.

Basic Chemical Properties

BPA has a chemical formula of $C_{15}H_{16}O_2$ and a molecular weight of 228.29 g/mol [14]. It is a white solid and has a mild phenolic odor. The chemical formula for DEHP is $C_{24}H_{38}O_4$ and its molecular weight is equal to 390.56 g/mol [16]. Unlike BPA, DEHP is physically a colorless

and oily liquid with almost no odor. BPS and BPF are structural analogs of BPA [11]. BPS has a chemical formula of $C_{12}H_{10}O_4S$ and a molecular weight of 250.27 g/mol [22], whereas the chemical formula for BPF is $C_{13}H_{12}O_2$ and its molecular weight is 200.23 g/mol [30]. Similar to BPA, BPS is a white colorless solid [22]. The structures for these chemicals are shown in Table 1.1.

Table 1.1. Chemical structures of BPA, BPS, BPF, and DEHP.

EDC	Structure
Bisphenol A	
Bisphenol S	
Bisphenol F	
Di-(2-Ethylhexyl) Phthalate	

Note: BPA, bisphenol A; BPS, bisphenol S; BPF, bisphenol F; DEHP; di-(2-ethylhexyl) phthalate; EDC, endocrine disrupting chemical. BPA and BPS structures were adapted from [35], BPF structure was adapted from [36], and DEHP structure was adapted from [16].

Human Exposure Routes

The primary route of exposure of these particular EDCs in humans is via ingestion of contaminated food and beverages containing EDCs that have leached from the plastics [15, 37]. In the general population, exposure to BPA occurs most frequently through food; however, exposure can also occur through air, house dust, water, soil, and dermally in occupational settings via BPA in thermal paper products [14, 38]. Under heat exposure, in particular, free BPA – the biologically active, unconjugated form – can be released after ester bonds enabling BPA polymerization are broken [37]. Similar to BPA, DEHP exposure also occurs most often through food ingestion. Yet, the population with the most intense degree of exposure is hospital patients [15] since DEHP use is widespread in medical devices, including intravenous bags and tubing, blood bags, and dialysis bags [16]. Nevertheless, DEHP exposure can also occur through house dust, indoor air, soil, and watersheds [15]. BPS and BPF are also particularly prevalent in indoor dust, water, sediment, and sewage in the environment [39-42], with exposure routes primarily occurring dermally, orally, and via inhalation.

Consequently, these chemicals have been detected in a range of human samples, including urine [43-45], serum [46-48], hair [49], and saliva [50-52]. Alarmingly, bisphenols have been detected in urine and serum samples in more than half of the general population [53], with BPA being present in >90% of people [54]. Phthalate metabolites, particularly those of DEHP, have additionally been identified in >90% of human urine and hair samples [49]. All of this underscores the wide prevalence of these EDCs in the environment and the inevitability of exposure to them.

Mechanisms of Action and Dose-Response Characteristics

BPA is commonly classified as a "weak estrogen" [55], since it has the ability to bind to the two classical nuclear estrogen receptor (ER) subtypes, ERα and ERβ; however, the ability of BPA to bind to these receptors is 1000-2000 fold less than 17β-estradiol (E2), the most active form of estrogen in the body [56, 57]. BPA can act as an estrogen mimetic by serving as an agonist or antagonist of ERα and ERβ [58], thereby affecting various physiological and cellular processes. BPA also demonstrates high binding affinity for the rapid acting membrane-associated ER known as GPR30 [59]. Other receptor targets of BPA include androgen receptors (ARs) [60, 61], estrogen-related receptors [62], thyroid hormone receptors [63, 64], and peroxisome proliferator-activated receptors (PPARs) [65]. DEHP, on the contrary, is primarily known for its anti-androgenic effects [66-69], with similar binding affinity for ARs as testosterone [70], although it can also be anti-estrogenic [68]. In addition, DEHP can also interact with PPARs [71], mineralocorticoid receptors [72], and aryl hydrocarbon receptors [73].

The hormonal activities of BPS and BPF are increasingly being studied to compare their potencies with those of BPA. Compared to E2, BPS has weaker estrogenic potency in nuclear receptor models [74, 75], but has equivalent or greater potency in membrane receptor models [76, 77]. BPS can also show both androgenic [78] and anti-androgenic [75] activities. BPF has been reported to show estrogenic activity and binding affinities for ERs, while also demonstrating anti-androgenic activity [11, 75, 79, 80]. Furthermore, BPS and BPF have estrogenic potencies that are in the same order of magnitude as BPA, with BPF potentially showing higher potency than BPA [11]. Finally, BPS and BPF also demonstrate similar potencies as BPA in other hormonal activities, including anti-estrogenic, androgenic, and anti-androgenic activities [11].

Toxicological risk assessment studies have historically used high doses of chemicals under the assumption of a linear dose response curve – the higher the dose, the more toxic the effects – also known as "the dose makes the poison" [81]. Multiple EDCs, including BPA and phthalates, have challenged this assumption because they exhibit unique toxicological properties consisting of low-dose effects and nonmonotonic dose response curves (NMDRCs) [81, 82]. A low dose is defined as a dose of a chemical occurring in the range of typical human exposures with the ability to produce biological changes (the environmentally relevant dose), or any dose below the lowest observed adverse effect level (LOAEL) [81, 82]. Similarly, NMDRCs refer to a nonlinear relationship between the dose and the effects observed, in which the slope of the curve changes sign [81]. Low-dose effects often accompany NMDRCs, and both EDCs as well as endogenous hormones demonstrate these attributes [81]. These distinctive properties of BPA and DEHP make them more challenging to characterize, and highlight the importance of incorporating these aspects into EDC studies.

Maternal-Fetal Transfer of EDCs

Multiple factors influence the potential for adverse effects to occur in response to endocrine disruption, a major one being the timing of EDC exposure [83, 84]. The prenatal period is a critical window of development [84] during which exposure to exogenous compounds – including EDCs – can impact fetal development. EDC exposure during the prenatal period can interfere with the normal development of sexually dimorphic systems and induce irreversible effects on neurodevelopment, resulting in life-long health consequences [85, 86]. This concept was first introduced by David Barker, who demonstrated in the 1980s that environmental factors such as poor nutrition during gestation could increase the risk for developing diseases, including cardiovascular and metabolic diseases, later in life [87]. Today, this concept is known as the

Developmental Origins of Health and Disease (DOHaD) [88] and also, more simply, as fetal programming [89].

In pregnant females, EDCs such as BPA and DEHP can readily cross the placental barrier [90, 91] and directly impact the growing fetus, leading to "organizational" effects in the brain – effects arising from exposures during organ development that result in permanent changes in the affected systems [92]. The fetus is especially vulnerable during this sensitive period, and has limited capacity to metabolize and process exogenous hormones [93]. Evidence of EDC transfer from the mother to the fetus can be found in numerous studies confirming the presence of BPA and DEHP in maternal serum and urine, amniotic fluid, placenta, breast milk, and fetal cord blood [94-96].

1.2. Developmental EDC Exposures and Behavioral Consequences

According to the DOHaD, even neuropsychiatric disorders can be traced back to environmental exposures that may have occurred during development [83]. Affective disorders, including depression and anxiety, are among the most common neuropsychiatric disorders in the United States and affect approximately 20 million adults [97]. These disorders are responsible for higher economic costs than diabetes or cancer, and are projected to account for approximately $16 trillion in global cumulative economic output loss by the year 2030 [98]. The prevalence of these neuropsychiatric disorders has risen globally and can be partially attributed to widespread EDC use and developmental EDC exposures [99, 100]. An intriguing feature about numerous neuropsychiatric disorders is the presence of a sex bias in their prevalence, since females show higher rates of anxiety and depression, whereas ADHD and autism are more prevalent in males [101-103]. More significantly, there appear to be sex differences in the windows of vulnerability to exogenous exposures within the developmental period, as male-

biased neuropsychiatric disorders tend to originate during early development, while the onset of female-biased disorders tends to be during adolescence and early adulthood [101, 102].

BPA and DEHP exposures, specifically during the highly sensitive *in utero* period, are associated with adverse outcomes on human behavior, particularly in children. Gestational BPA exposure in girls has been linked with increased anxiety, depression, hyperactivity, and poor emotional control [104], but decreased internalization problems [105]; similarly, boys show increased emotional reactivity [106], aggressive behavior [106-108], internalizing and externalizing problems [105, 107, 108], and anxiety and depression [107, 108]. Moreover, prenatal exposure to phthalates may result in increased inattention, aggression, externalizing problems [109, 110], and reduced mental and psychomotor development [111] in children, with worse symptoms in boys compared to girls in general. In addition, boys may also demonstrate reduced male-typical play behavior [112], underlining the anti-androgenic effects of phthalates.

Accordingly, studies also report consequences on behavior in non-human animals. The following is a review of studies involving prenatal or perinatal exposures to BPA or DEHP, with a focus on rodent studies and behaviors assessed in the experiments proposed in this dissertation. These behavioral facets include cognition, exploration, locomotor activity, and stress-related behaviors. These studies are also summarized in Table 1.2.

Effects of BPA or DEHP on Cognition

The estimated range of BPA exposure in humans is 0.4-5 µg/kg/day [113], which is well below the Environmental Protection Agency (EPA) recommended reference dose of 50 µg/kg/day [114] and the no-observed-adverse-effect-level (NOAEL) dose of 5 mg/kg/day [115]. Treatment with very low doses of BPA during the perinatal period has been associated with deficits in spatial learning and memory in rat offspring. A dose as low as 25 µg/kg of BPA is

capable of altering cognition in the Morris Water Maze (MWM) task in adult female rat offspring [116]. These females showed reduced swim speed and length in this task, displaying a masculinization of spatial learning. Furthermore, sex differences observed between control male and female offspring in the MWM were also eliminated at this dose of BPA. Another study examining low-dose perinatal BPA exposure at 40 µg/kg also found deficits in spatial working memory in the Y-maze test, but in both male and female adult rat offspring [117]. This test measures short term memory in the form of spontaneous alternation. Animals are allowed to explore all three arms of the maze, and working memory is assessed by their ability to remember previously visited arms [118].

Perinatal exposure to a higher BPA dose of 0.1 mg/kg is also correlated with significant cognitive impairments in both rat and mouse offspring. One study demonstrated that BPA-exposed adult male rat offspring exhibit delayed latency to enter the dark compartment in the Passive Avoidance test and a reduced percentage of correct avoidance responses in the Active Avoidance test [119]. Another study also determined altered cognition in adult male rat offspring exposed to this dose of BPA, but in MWM spatial working memory abilities [120]. Moreover, pubertal mouse offspring perinatally exposed to 0.1 mg/kg (100 µg/kg) of BPA have also been shown to exhibit deficits in object recognition in the Novel Object Recognition (NOR) test, whereas they show impaired Y-maze alternation at both 100 and 500 µg/kg of BPA [121]. Although these studies overall support a sex-specific cognitive impact on male offspring treated with this dose of BPA, female offspring were excluded from evaluation in the Negishi et al. study [119] and data from male and female offspring were pooled in the Tian et al. study [121]. It is essential to include female offspring in developmental EDC studies to create a complete picture of the adverse effects of these chemicals.

Even higher doses of BPA, or those near the current NOAEL dose of 5 mg/kg/day [115], have additionally been shown to adversely impact cognition. Adult rat offspring perinatally treated with 1.5 mg/kg of BPA show abolished sex differences in the Passive Avoidance test relative to control offspring, in which robust differences are observed between male and female offspring [122]. On the other hand, male mouse offspring with exposure to 5 or 50 mg/kg of BPA display impairments in spatial learning in the Barnes Maze test, demonstrating a feminization of cognitive abilities with high-dose BPA [123]. Similarly, adult male and female mouse offspring with gestational BPA treatment at 5 mg/kg do not exhibit the high levels of object recognition shown by control offspring, confirming altered recognition memory at this dose as well [124]. Nevertheless, one study found no changes in MWM spatial memory in adult male or female rats with perinatal exposure to BPA doses between 5 µg/kg - 5 mg/kg [125], underscoring the inconsistencies that can arise in behavioral studies using different animal strains, exposure periods, and ages of offspring at evaluation.

On the other hand, male offspring with perinatal DEHP exposure appear to be more severely impacted in terms of cognition compared to their female counterparts. Even a dose as low as 5 µg/kg of DEHP during the perinatal period has been demonstrated to adversely affect spatial memory in the MWM in adult male rat offspring [126]. This dose of DEHP lies within the range of the estimated daily intake in adult humans (0.5-25 µg/kg/day) [127], and is below the EPA reference dose of 20 µg/kg/day for DEHP [128]. Safarpour et al. also confirmed in the same study that male offspring exposed to slightly higher DEHP doses of 40 and 400 µg/kg show deficits in MWM spatial memory as well, while their female counterparts showed MWM deficits at higher doses of 400 µg/kg and 300 mg/kg [126]. Finally, both male and female offspring demonstrated impaired Y-maze spontaneous alternation at 300 mg/kg of DEHP in this study. Y-

maze working memory deficits in male offspring gestationally treated with 200 µg/kg of DEHP have also been replicated in another study [129].

The established NOAEL dose for DEHP is 4.8 mg/kg/day [130]. Treatment with similar DEHP doses or those higher than the NOAEL dose has certainly been associated with negative cognitive effects. Juvenile male rats with gestational exposure to 10 mg/kg of DEHP show impaired MWM learning and spatial memory [131], whereas adult male rats treated with the same dose of DEHP during gestation also display deficits in working memory in the T-maze delayed non-match-to-sample task [132]. Interestingly, adult mouse offspring of dams treated with 30 mg/kg DEHP during the preconception, pregnancy, and lactational stages exhibited no cognitive or anxiety-related behavioral changes, including in MWM spatial memory [133]. However, juvenile or adult mouse and rat offspring, particularly males, exhibit impairments in various cognitive tests, including the MWM and NOR, at doses (200-750 mg/kg) significantly higher than the NOAEL [126, 129, 131, 134]. Zhao et al. further demonstrated that DEHP exposure during GD 13-17, as opposed to GD 6-12, leads to more severe impairments in male offspring in the MWM [134]. Yet, female offspring exposed to DEHP in the earlier gestational period showed cognitive impairments, highlighting the importance of the EDC exposure period.

Effects of BPA or DEHP on Exploration and Locomotor Activity

Perinatal treatment with a dose as low as 10 µg/kg of BPA can sex-specifically alter exploratory activity in mouse offspring. One study showed that both perinatal exposure (from GD 11 – PND 9) or lactational exposure (via cross-fostering pups) to this dose of BPA can reduce novelty exploration in the novelty preference test, but only in juvenile, not adult, female mouse offspring [135]. The authors replicated this particular finding again in another study examining 10 µg/kg BPA treatment solely during the perinatal period [136]. Gestational

exposure to this dose of BPA has also been demonstrated to affect exploration in the Open Field Test (OFT) in adult male mouse offspring [137]. These offspring display increased rearing, a form of rodent exploratory behavior in which the rodent momentarily stands on its hind legs to explore its surroundings [138], but reduced overall exploration time in the OFT.

Yet another study determined that a slightly higher dose of gestational BPA (15 µg/kg) altogether eliminates sex differences in OFT rearing behavior in adult rat offspring [139]. While control females had increased rearing relative to their male counterparts, the rearing frequency and duration of BPA male and female offspring was not significantly different from one another. However, BPA males did show increased rearing duration compared to control males, similar to the findings of Ponzi et al [137]. Likewise, a relatively higher dose of 30 µg/kg of perinatal BPA has also been demonstrated to increase OFT rearing in adult male rat offspring, as well as abolish sex differences in rearing in BPA offspring [140], confirming that this effect persists at different doses. More interestingly, 20 µg/kg of BPA administered to mouse dams during the preconception and gestational periods is capable of increasing locomotor activity in the OFT in pubertal offspring three generations later, particularly in males [141]. Finally, in terms of low-dose effects, 40 µg/kg of BPA may also lead to reduced exploration in the Y-maze task in adult female rats with perinatal exposure [117].

Higher BPA doses produce more varied effects on offspring activity and exploration. Farabollini et al. found that perinatal exposure from GD 14 – PND 6 to 400 µg/kg of BPA diminishes locomotor activity levels in the Elevated Plus Maze (EPM) in adult female rat offspring [142]. Furthermore, using sophisticated factor analyses, the authors were able to determine that BPA exposure also reduced the overall motivation to explore in both female and male offspring. However, adult female rat offspring treated during a wider range of development

(GD 7 – PND 14) and with a range of BPA doses (50, 500, or 5000 µg/kg) show increased EPM mobility [125]. Perinatal BPA treatment at 0.1 mg/kg, intriguingly, has been shown to either increase distance traveled and rearing in the OFT in adult male rats [120], or produce no effects on spontaneous activity in rats [119].

Next, when gestationally exposed to 2, 20, or 200 µg/kg of BPA, male and female mouse offspring show opposing effects on OFT activity – female offspring show decreased ambulation, but males show the reverse with increasing dose [143]. Gestational exposure to a higher dose of 5 mg/kg of BPA can reduce motor coordination and balance in the rotarod test as well as locomotor activity in the OFT in both male and female mouse offspring [124]. Finally, adult mouse offspring with BPA treatment at 5 or 50 mg/kg during preconception and throughout gestation and lactation may show decreased EPM exploratory activity, but only in males [123].

There have only been a few studies to date that have assessed exploratory and locomotor activity in offspring with perinatal DEHP exposure. Thus far, studies examining perinatal DEHP treatment have used doses of 10, 50, or 200 mg/kg during GD 7 – PND 21 and tested mouse offspring behavior either at puberty or adulthood. In pubertal females, perinatal DEHP was found to reduce rearing in the OFT at the low and high doses, but lower ambulation at the middle dose [144, 145]. In contrast, adult females displayed lower ambulation at the low and high doses and did not show changes in rearing, highlighting the significance of offspring age at evaluation. Additional studies are necessary to understand the behavioral effects of DEHP on offspring exploration and motor activity, particularly at low doses.

Effects of BPA or DEHP on Stress-Related Behaviors

Animal models of stress-related behaviors aim to embody core features of human neuropsychiatric disorders, including anxiety and depression. Common stress-related behavioral

paradigms measure unconditioned responses, in which the animal typically has to deal with an approach-avoidance conflict, or conditioned responses, which involve learned fear responses [146, 147]. An approach-avoidance conflict relates to an animal's natural tendency to explore or approach a novel environment, which competes with its innate desire to avoid the aversive aspects, including brightly lit areas or openness [148]. Anxiety- or depression-related behavioral paradigms therefore assess the behaviors presented by the animal as a result of these opposing drives. Some of the most common rodent behavioral tests used in prior studies to assess the effects of BPA or DEHP on stress-related behaviors are the OFT, EPM, Light/Dark Box (LDB), and Forced Swim Test (FST). The OFT and EPM are also used in the studies proposed in this dissertation to measure anxiety-like behaviors, in addition to the Shock Probe Defensive Burying (SPDB) test. While the OFT and EPM measure different types of unconditioned or unlearned anxiety-like behaviors, the SPDB measures conditioned fear responses [147].

BPA exerts dose- and sex-dependent effects on stress-related behaviors based on the behavioral paradigm. Perinatal BPA exposure at a very low dose of 250 ng/kg increases OFT anxiety-like behavior in juvenile and adult male mouse offspring, but female offspring are unaffected at this dose [149]. Interestingly, perinatal BPA treatment at 2 µg/kg can increase anxiety levels in the OFT and LDB as well as FST immobility in adult male rat offspring [150], but can also decrease anxiety-like behavior in female counterparts [151]. The increased anxiety- and depression-like behaviors in male offspring are further accompanied by elevated CORT and ACTH [150], whereas the corresponding females show reduced levels of these hormones, as expected [151]. Moreover, similar effects are also observed in adult mouse offspring with gestational BPA exposure at 2-200 µg/kg, but this study further reveals that anxiogenic effects increase in female offspring but decrease in male offspring in the OFT with increasing dose

[143]. 5 µg/kg of BPA administered perinatally has been shown to elevate immobility in the FST only in adult male rat offspring, as well as eliminate sex differences in both the EPM and FST [125]. An increase in FST immobility was also determined in male offspring exposed to 5000 µg/kg of BPA, and female offspring exposed to BPA at a dose of 50 µg/kg displayed an alleviation of anxiety in the EPM in this study.

Additionally, BPA exposure at 10 µg/kg can decrease OFT anxiety-like behavior in adult female mouse offspring that were exposed gestationally [137], but elevate OFT and EPM anxiety levels in adult female mice exposed during lactation via cross-fostering with different dams [135]. Anxiety-like behavior in the EPM, but not in the OFT, was also increased with perinatal 10 µg BPA treatment in this study [135] and also in the study by Gioiosa et al [136]. Ponzi et al. also indicated that sex differences were induced in the OFT in BPA offspring exposed to this dose, but were eliminated in the EPM [137]. A higher dose of BPA at 15 µg/kg during gestation, on the other hand, did not affect behavior in the EPM, but did lead to increased depressive-like behavior in the FST in adult male rats [139]. Intriguingly, prenatal exposure to 15 µg/kg of BPA can also increase the odor-avoidance response in adult rats, which implies heightened susceptibility to predator odor and increased anxiety-like behavior [152].

Anxiety-like behavior in the OFT is not affected with perinatal exposure to higher doses of 30 or 300 µg/kg of BPA, but these BPA doses can altogether eliminate sex differences in center time in the OFT [140]. Nevertheless, 40 µg/kg of perinatal BPA induces anxiogenic effects in female rat offspring in the OFT, EPM, and LDB [153] and also in the Y-maze task [117]; this is associated with increased levels of CORT and ACTH. Female offspring also demonstrate more pronounced depressive-like behavior in the FST with this dose of BPA administered perinatally, which is coupled with higher adrenal gland weights and elevated

CORT [154]. However, male rat offspring exposed to BPA at 40 µg/kg or a higher dose of 400 µg/kg prior to conception and perinatally display decreased EPM anxiety levels [142]. Yet, male offspring exposed to 40 µg/kg of BPA consistently show higher CORT levels following stress exposure [117, 154], suggesting an altered relationship between the hypothalamic-pituitary-adrenal (HPA) axis and stress-related behaviors in male offspring treated with this dose of BPA. Similar effects on anxiety-like behavior are observed in female and male mice treated with 50 µg/kg of BPA during preconception and throughout gestation and lactation [123].

Even higher BPA doses exert a variety of effects on stress-related behaviors depending on the rodent species. Adult male rats with perinatal BPA treatment at 100 µg/kg show elevated anxiety in the OFT [120], but this same dose of BPA can also lead to no alterations in anxiety-like behavior in male rats [119] or reduced OFT anxiety levels in pubertal mice [121]. 200 µg/kg of BPA, on the other hand, can increase EPM and light-dark box anxiety-like behavior in pubertal female mouse offspring that have been ovariectomized [155]. Yet, perinatal BPA at 500 µg/kg can also reduce anxiety in the EPM in pubertal mice [121], complicating the effects of higher BPA doses. However, studies using doses near the BPA NOAEL dose of 5 mg/kg generally support elevated anxiety- and depression-like behaviors in both rat and mouse offspring, regardless of sex (0.4-5 mg/kg of BPA) [123, 124, 156, 157]. A dose as high as 50 mg/kg of BPA is also correlated with increased anxiety levels in male and female mouse offspring, specifically in the EPM [123, 158]. Finally, one study also determined that adult female rat offspring with perinatal BPA treatment at 50 mg/kg exhibit reduced intake of saccharin solution in the sucrose preference test, indicative of increased anhedonia and depression-like behavior [159], but show no changes in EPM behavior or motor activity [116].

Similar to BPA, perinatal exposure to DEHP also exerts dose-dependent effects on stress-related behaviors in offspring. Very low doses have been reported to increase anxiety-like behavior in the EPM in both male and female pubertal mouse offspring with perinatal exposure to DEHP at 5 and 40 μg/kg [160]. Furthermore, this effect is persistent three generations later, but only in male offspring at 5 and 400 μg/kg of DEHP. Other studies using higher perinatal DEHP doses have found effects that depend on both the dose administered and age of offspring at evaluation. Pubertal female mouse offspring show elevated EPM anxiety at 10 and 200 mg/kg but lower EPM anxiety at the middle dose administered of 50 mg/kg [145]. On the contrary, adult female offspring exhibit increased anxiety levels in the EPM and OFT at 50 and 200 mg/kg [144, 145]. Male offspring, on the other hand, are more severely impacted at puberty – they demonstrate increased anxiety-like behavior in multiple behavioral tests at 10 and 50 mg/kg, and also show increased FST immobility at 50 mg [145]. Increased immobility in the FST is also observed at 10 mg in female mice and at 200 mg in male mice, regardless of age.

Relatively higher DEHP doses administered gestationally have also been associated with increased anxiety-like behavior in both the EPM at 750 mg/kg and in the OFT at doses between 200 μg/kg and 750 mg/kg in adult male mice [129]. However, adult female mouse offspring in the third generation (F3) following gestational DEHP treatment at 750 mg/kg show ameliorated anxiety levels in the EPM, whereas their male counterparts are unaffected [161]. In terms of HPA axis activity, one study demonstrated that gestational exposure to DEHP at 150 mg/kg induces transgenerational effects in F3 female mouse offspring at puberty, wherein they display a trend for reduced CORT levels at baseline but significantly lower CORT after stress exposure, and no changes in behavior [162]. Nevertheless, the behavioral effects of perinatal DEHP are

quite inconsistent because several studies report no changes in OFT or EPM behavior at doses between 10 and 200 mg/kg in both rat [132, 163] and mouse [133, 134] offspring.

Table 1.2. Behavioral effects of male and female offspring with pre- or peri-natal exposure to BPA or DEHP at various doses.

Exposure protocol	Offspring species and age at evaluation	Neurobehavioral effects	Reference
BPA			
1 mg/L/day BPA GD 6 - PND 40 Orally via water	Rats Juvenile or Adult (PND 24-28 or PND 60-70)	↑ anxiety in EPM and LDB in BPA juveniles (♂ & ♀ pooled) Loss of sexual dimorphism in EPM behavior in BPA adults	[156]
40 or 400 µg BPA/kg/day Prior to mating until PND 21 (40µg) or GD 14 - PND 6 (400µg) Oral administration	Rats Adulthood (PND 80-87)	BPA ♀: ↓ EPM activity (400µg), ↓ motivation to explore BPA ♂: ↓ EPM anxiety (both doses), ↓ motivation to explore	[142]
10 µg BPA/kg/day GD 11 - PND 9 OR during lactation via cross-fostering Orally via diet	Mice Juvenile or Adult (PND 28-30 or PND 70)	Juvenile BPA ♀: ↓ novelty exploration in novelty preference test (pre- & postnatal BPA) Adult BPA ♀: ↑ OFT anxiety (postnatal BPA), ↑ EPM anxiety (pre- & postnatal BPA)	[135]
40 µg BPA/kg/day Through gestation and lactation Oral administration	Rats Adulthood (PND 40-50)	BPA ♀: ↑ anxiety in OFT, EPM, & LDB; ↑ CORT & ACTH	[153]
100 or 500 µg BPA/kg/day GD 7 - PND 36 Oral administration	Mice Puberty (PND 35)	BPA offspring (♂ & ♀ pooled): ↓ OFT anxiety (100µg), ↓ EPM anxiety (500µg), ↓ Y-maze alternation/working memory (both doses), ↓ NOR object recognition (100µg)	[121]
Est. 15 µg BPA/kg/day GD 14 - birth Orally via drinking water	Rats Adulthood (Weeks 6-9)	BPA eliminated sex differences in OFT exploratory behavior BPA eliminated sex differences in FST struggling and ↑ FST immobility in ♂ offspring No changes in EPM or passive avoidance test	[139]
2 µg BPA/kg/day GD 10 - PND 7 Oral administration	Rats Adulthood (PND 80)	BPA ↑ anxiety in OFT & LDB and ↑ FST immobility; ↑ pre- and post-stress CORT & ACTH in ♂ offspring	[150]

10 µg BPA/kg/day GD 11 - 18 Orally via diet	Mice Adulthood (PND 60)	BPA ♀: ↓ OFT anxiety, ↓ EPM center time BPA ♂: ↑ OFT rearing, ↓ OFT exploration time Sex differences induced in BPA offspring in OFT behaviors Sex differences eliminated in BPA offspring in EPM anxiety-like behavior	[137]
5 mg BPA/kg/day GD 9 - 16 Miniosmotic pump into peritoneal cavity	Mice Adulthood (18 months)	BPA ♀ & ♂ offspring had ↓ rotarod motor coordination and balance, ↓ activity but ↑ anxiety in OFT, & altered NOR recognition memory	[124]
~1.5 mg BPA/kg/day Through pregnancy and lactation Orally via drinking water	Rats Adulthood (PND 42)	Sex differences were eliminated in BPA offspring in the OFT and passive avoidance test; no changes observed in these behaviors compared to controls	[122]
~30 or 300 µg BPA/kg/day Through pregnancy and lactation Orally via drinking water	Rats Adulthood (PND 42)	BPA ♂ (30µg): ↑ OFT rearing Sex differences eliminated in 30µg BPA offspring in OFT rearing and anxiety Sex differences eliminated in 300µg BPA offspring in all OFT behaviors	[140]
0.1 mg BPA/kg/day GD 3 - PND 20 Oral gavage	Rats Adulthood (Weeks 8-15)	BPA ♂: ↑ cognitive deficits No changes in spontaneous motor activity No changes in OFT or EPM	[119]
15 µg BPA/kg/day GD 13 - birth Orally via drinking water	Rats Adulthood (Weeks 10-14)	BPA offspring (♂ & ♀ pooled): ↑ odor-avoidance response	[152]
50µg, 5 or 50mg BPA/kg/day Prior to mating and through gestation and lactation Orally via diet	Mice Adulthood (~PND 60)	5 mg & 50 mg BPA ♂: ↓ Barnes Maze spatial learning, ↓ EPM exploratory behavior, and ↑ EPM anxiety 50 µg BPA ♀: ↑ EPM anxiety 50 µg BPA ♂: ↓ EPM anxiety	[123]
50 mg BPA/kg/day Prior to mating and through gestation and lactation Orally via diet	Mice Puberty (PND 22-30)	BPA ♂ raised by biological or foster dams: ↑ EPM anxiety BPA ♀ raised by foster dams: ↑ EPM anxiety	[158]
40 µg BPA/kg/day Through gestation and lactation Orally via diet	Rats Adulthood (PND 46)	BPA ♀: ↑ FST depressive-like behavior, ↑ adrenal weights, and ↑ basal plasma CORT BPA ♂: ↑ post-stress CORT	[154]
2, 20, or 200 µg BPA/kg/day GD 0-19 Oral administration	Mice Adulthood (PND 30-70)	BPA ♀: ↑ OFT anxiety and ↓ activity as dose ↑ BPA ♂: ↓ OFT anxiety and ↑ activity as dose ↑	[143]
Est. 20 µg BPA/kg/day 7-10 days prior to mating and throughout gestation Orally via diet	Mice Puberty (PND 21-24)	BPA ♀ & ♂: ↑ OFT activity in F3 generation, particularly in ♂	[141]

10 µg BPA/kg/day GD 11 - PND 7 Orally via diet	Mice Puberty or Adulthood (PND 28-30 or PND 70)	BPA ♀: ↓ novelty exploration in novelty preference test as juveniles and ↑ EPM anxiety in adulthood BPA reduced or reversed sex differences	[136]
2 or 200 µg BPA/kg/day GD 3 - PND 21 Oral gavage	Mice (OVX) Puberty (~PND 42)	200 µg BPA ♀: ↑ anxiety in EPM and LDB	[155]
40 µg BPA/kg/day Through gestation and lactation Orally via diet	Rats Adulthood (PND 46)	BPA ♀: ↓ exploration & ↑ anxiety in the Y-maze task, and ↑ basal & post-stress CORT BPA ♂: ↑ post-stress CORT BPA ♂ and ♀: ↓ Y maze spatial memory	[117]
0.1 mg/L/day BPA GD 20 - PND 21 Orally via water	Rats Adulthood (Weeks 7-9)	BPA ♂: ↑ OFT activity, rearing, and anxiety; ↓ spatial memory in MWM	[120]
250 ng BPA/kg/day GD 10 - PND 20 Subcutaneous injections	Mice Puberty & Adulthood (PND 28 & PND 56)	BPA juvenile and adult ♂: ↑ OFT anxiety BPA adult ♂: ↑ DA levels in HC & MED, and ↓ DOPAC/DA ratio in HC, AMYG, MED BPA ♀: no changes	[149]
5, 50, 500, or 5000µg BPA/kg/day GD 7 - PND 14 Oral administration	Rats Adulthood (PND 90-150)	50, 500, & 5000µg BPA ♀: ↑ EPM activity 50µg BPA ♀: ↓ EPM anxiety 5 & 5000µg BPA ♂: ↑ FST immobility 5µg BPA eliminated sex differences in EPM and FST No changes in MWM	[125]
25 or 250µg, 5 or 50mg BPA/kg/day GD 7 - PND 22 Oral gavage	Rats Adulthood (3-7 months)	25µg BPA ♀: masculinization of MWM spatial learning and elimination of sex differences at this dose in MWM 50mg BPA ♀: ↓ sweetened water intake in sucrose preference test No changes in EPM or motor activity	[116]
500 µg BPA/kg/day GD 0 - PNW 3 Oral administration	Mice Adulthood (PNW 11-15)	BPA ♀: ↑ NE in somatosensory cortex, hypothalamus, and thalamus; ↑ GABA and Glu in most regions BPA ♂: ↑ NE in somatosensory cortex, ↓ GABA and ↑ Glu in the brain	[164]
0.4 or 4 mg BPA/kg/day Gestational (GD 7-20) or lactational (PND 1-14) Oral administration	Mice Adulthood (PND 56-60)	Gestational BPA ♀: ↑ anxiety in LDB, EPM, & mirrored maze at both doses; ↑ OFT grooming (4mg); ↑ FST immobility (both doses) Lactational BPA ♀: ↑ anxiety in EPM & LDB at both doses; ↑ FST immobility (4mg) Gestational BPA ♂: ↑ anxiety in EPM & LDB at both doses; ↑ FST immobility (both doses) Lactational BPA ♂: ↑ anxiety in EPM & ↑ FST immobility at 4mg	[157]

2 µg BPA/kg/day GD 10 - PND 7 Subcutaneous injections	Rats Adulthood (PND 80)	BPA ♀: ↓ anxiety in OFT and LDB; ↓ post-stress CORT and ACTH BPA ♂: ↑ FST immobility; ↑ basal and post-stress CORT and ACTH	[150]
DEHP			
10 mg DEHP/kg/day GD 14-21 Oral gavage	Rats Adulthood (PND 56-63)	No changes in EPM behavior	[163]
5, 40, 400µg, 300mg DEHP/kg/day GD 0 - PND 21 Oral gavage	Rats Adulthood (~PND 60)	DEHP ♂: ↓ MWM spatial memory (all doses), ↓ Y-maze spontaneous alternation (300mg) DEHP ♀: ↓ MWM spatial memory (400µg, 300mg), ↓ Y-maze spontaneous alternation (300mg)	[126]
200 mg DEHP/kg/day GD 6-12 (G1) or GD 13-17 (G2) Oral gavage	Mice Puberty & Adulthood (PND 42 & 56)	DEHP ♂: ↓ MWM spatial memory (esp. in G2) DEHP ♀: ↓ MWM spatial memory (G1) No changes in OFT or EPM behavior	[134]
10 mg DEHP/kg/day GD 14-21 Oral gavage	Rats Adulthood (PND 57-66)	DEHP ↓ working memory in the T-maze delayed non-match-to-sample task in ♂ offspring No changes in OFT behavior	[132]
30 mg DEHP/kg/day Prior to mating and through pregnancy & lactation Oral administration	Mice Adulthood (PND 56)	No changes in OFT, EPM, or MWM behavior in DEHP offspring	[133]
10 or 750 mg DEHP/kg/day GD 12-21 Oral gavage	Rats Juvenile (PND 8)	DEHP impaired learning and spatial memory in MWM in ♂ offspring	[131]
5, 40, or 400 µg DEHP/kg/day Gestation - PND 10 Orally via diet	Mice Puberty (PND 28-35)	DEHP F1 ♀ & ♂: ↑ EPM anxiety (5 & 40 µg) DEHP F3 ♂: ↑ EPM anxiety (5 & 400 µg)	[160]
10, 50, or 200 mg DEHP/kg/day GD 7 - PND 21 Oral administration	Mice Puberty or Adulthood (PND 42 or PND 84)	Pubertal DEHP ♀: ↓ OFT rearing & ↑ EPM anxiety (10 & 200mg), ↓ OFT ambulation & ↓ EPM anxiety (50mg) Adult DEHP ♀: ↓ OFT ambulation (10 & 200mg), ↑ OFT anxiety (50 & 200mg), ↑ EPM anxiety (50mg) Pubertal DEHP ♂: ↑ anxiety in EPM, LDB, mirrored chamber (10 & 50mg), ↑ FST immobility (50mg) No EPM changes in adult DEHP ♂ or ♀ (10 & 200mg) ↑ FST immobility in pubertal and adult ♀ (10mg) & ♂ (200mg)	[145]
150 or 200 mg DEHP/kg/day GD 7-14 Oral gavage	Mice (F3 generation) Puberty (PND 25-42)	DEHP ♀ (150mg): ↓ CORT after restraint stress No EPM behavioral changes in DEHP ♀ or ♂ offspring	[162]

10, 50, or 200 mg DEHP/kg/day GD 7 - PND 21 Oral administration	Mice Puberty or Adulthood (PND 42 or PND 84)	Pubertal DEHP ♀: ↓ OFT ambulation (50mg), ↓ OFT rearing (10 & 200mg) Adult DEHP ♀: ↓ OFT ambulation (10 & 200mg), ↑ OFT anxiety (50 & 200mg) Pubertal DEHP ♂: ↓ MWM spatial memory (50mg)	[144]
200µg, 500 or 750mg DEHP/kg/day GD 11 - birth Oral administration	Mice Adulthood (16-18 months)	DEHP ♂: ↑ OFT anxiety (all doses), ↑ EPM anxiety (750mg), ↓ Y-maze spatial memory (200µg), ↓ NOR object recognition (500 & 750mg)	[129]
20 or 200µg, 500 or 750mg DEHP/kg/day GD 10.5 - birth Oral administration	Mice (F3 generation) Adulthood (PND 90-100)	DEHP ♀: ↓ EPM anxiety (750mg) No EPM behavioral changes in DEHP ♂ offspring	[161]

Note: BPA, bisphenol A; DEHP, diethylhexyl phthalate; GD, gestational day; PND, postnatal day; EPM, elevated plus maze; LDB, light/dark box test; OFT, open field test; CORT, corticosterone; ACTH, adrenocorticotropic hormone; NOR, novel object recognition test; FST, forced swim test; MWM, Morris water maze; HC, hippocampus; MED, medulla; AMYG, amygdala; DA, dopamine; DOPAC, 3,4-dihydroxyphenylacetic acid; NE, norepinephrine; GABA, gamma-aminobutyric acid; Glu, glutamate.

1.3. Study Aims

To summarize, EDC exposures are concerning due to their adverse effects on behavioral and developmental endpoints as outlined above, they have non-monotonic dose responses and lead to health consequences even at low doses, and these chemicals are ubiquitous in everyday consumer products, making their exposures unavoidable in the human population. Furthermore, women are twice as likely as men to develop mood and anxiety disorders [101], suggesting the involvement of estrogens in the development of these disorders. Hormonal exposures occurring in adulthood lead to "activational" effects [165], which are generally reversible but may nonetheless be harmful; hence, it is worth examining if they contribute to the development of anxiety disorders. Therefore, the experiments proposed in this dissertation were conducted to examine the following hypotheses:

1. Fetal programming with BPA, DEHP, or a combination of the two alters anxiety-like behaviors and HPA axis activity in a sex- and dose-dependent manner in rats.

2. Male and female rat offspring exhibit distinct profiles of monoaminergic activity in stress-regulating brain regions as a result of fetal programming with EDCs.
3. Exogenous estradiol treatment in adulthood exacerbates neurobehavioral effects that were induced by prenatal EDC exposures in female rat offspring.
4. Fetal programming with BPA analogues BPS and BPF sex-specifically alters developmental and reproductive endpoints in a manner that is unique from, and potentially more severe than, BPA itself.

The proposed experiments follow a basic experimental protocol: Pregnant Sprague-Dawley dams were orally administered various EDCs – BPA, low-dose or high-dose DEHP, BPA + DEHP in combination, BPS, or BPF – during gestational days 6-21. The female and male offspring treated *in utero* with BPA, DEHP, or their combination were evaluated for behavioral, hormonal, and brain neurotransmitter changes in adulthood. The female and male offspring treated *in utero* with BPA, BPS, or BPF were tracked for their growth trajectories and assessed for developmental and reproductive outcomes. Finally, the dams treated with BPA, BPS, or BPF were also examined for a variety of gestational parameters.

CHAPTER 2

PRENATAL EXPOSURE TO BISPHENOL A AND/OR DIETHYLHEXYL PHTHALATE ALTERS STRESS RESPONSES IN RATS IN A SEX- AND DOSE-DEPENDENT MANNER [1]

[1] Kaimal et al. Submitted to *Chemosphere*, 02/15/22.

2.1. ABSTRACT

Prenatal exposures to bisphenol A (B) or diethylhexyl phthalate (D) are correlated with adverse behavioral outcomes but the effects of combinations of these chemicals are unclear. The aim of this study was to determine the dose-dependent effects of prenatal exposure to B, D, or a combination of the two on male and female behavior. Pregnant Sprague-Dawley rats were orally dosed with vehicle, B (5 µg/kg body weight (BW)/day), low-dose (LD) D (5 µg/kg BW/day), high-dose (HD) D (7.5 mg/kg BW/day), a combination of B and LD-D (B+D (LD)), or a combination of B and HD-D (B+D (HD)) on gestational days 6-21. The offspring were subjected to the Open Field Test (OFT), Elevated Plus Maze (EPM), and Shock Probe Defensive Burying test (SPDB) in adulthood. Body and certain organ weights were collected at sacrifice. Corticosterone (CORT) was measured in the serum. Female EDC-exposed offspring showed anxiolytic effects in the OFT, while male offspring were unaffected. D (HD) male offspring demonstrated a feminization of behavior in the EPM. Most EDC-exposed male offspring buried less in the SPDB, whereas D (LD) males and females and B+D (LD) females displayed increased immobility, indicating maladaptive alterations in defensive behaviors. This was coupled with a trend for elevated CORT levels in D (LD) male, although this group had lower adrenal gland weights. Moreover, females treated with B or D (HD) and their combination had blunted CORT levels. Prenatal exposure to BPA, DEHP, or a mixture of the two affects behavior, CORT levels, and adrenal gland weights in a sex- and dose-dependent manner.

2.2. INTRODUCTION

The global rise in the prevalence of neuropsychiatric disorders can be partially attributed to the widespread use of endocrine disrupting chemicals (EDCs) [99, 100]. These environmental contaminants have been repeatedly associated with reproductive [3], metabolic [5], and developmental [6] abnormalities. Some of the most prevalent EDCs in the environment are the plasticizers bisphenol A (BPA) and diethylhexyl phthalate (DEHP), which are typically found in water bottles and food can linings [14], medical devices [15], and personal care products [16]. Humans are primarily exposed to these particular EDCs via ingestion of contaminated food and beverages containing BPA and/or DEHP that has leached from the plastics [15, 37].

In pregnant females, these chemicals can readily cross the placental barrier and affect fetal brain development *in utero* [90, 91]. Additionally, there appears to be a sex bias in the prevalence of multiple neuropsychiatric disorders [101-103]. It is highly likely that sex differences in the risk for developing these disorders may stem from exposures to EDCs *in utero*, a sensitive critical period of fetal development [166].

Exposure to BPA during the *in utero* and early postnatal stages exerts a variety of sex-specific effects on stress-related behaviors and hormones, including corticosterone (CORT). Low-dose BPA exposure (2-200 µg/kg/day) induces anxiogenic effects and reduces locomotor activity in the Open Field Test (OFT) in female mouse offspring; however, the reverse is observed in males [143]. These effects are compounded with increasing doses, suggesting a dose-dependent response. Furthermore, perinatal treatment with 5 µg/kg of BPA eliminates sex differences in rats in the Elevated Plus Maze (EPM), without affecting learning and memory in the Morris Water Maze [125]. In addition, studies report increased basal and post-stress levels of CORT accompanied by increased anxiety-like behavior in female rat offspring with perinatal

BPA exposure (40 µg/kg/day) [117]. BPA-exposed male offspring, on the other hand, display an even higher post-stress CORT response than females. However, treatment with a lower BPA dose (2 µg/kg/day) can induce anxiolytic effects in female offspring without affecting CORT levels before or after stress exposure [151]. Yet their BPA-exposed male counterparts have persistently heightened pre- and post-stress CORT concentrations.

Perinatal DEHP appears to have a non-monotonic dose response on exploratory and locomotor activity. Low DEHP doses (5-400 µg/kg/day) are associated with increased home cage exploration in mouse offspring of both sexes [160], whereas higher doses (10-200 mg/kg/day) lead to decreased locomotor activity in the OFT in female mice [145]. Anxiogenic effects in the OFT and EPM are additionally observed in both male and female mice following low-dose (5 and 40 µg/kg) [160], as well as high-dose (50-750 mg/kg) [129, 144, 145] perinatal DEHP exposure. Moreover, male offspring appear to show increased dose-dependent deficits in learning and memory following prenatal DEHP exposure [129, 131]. Although no changes were observed in CORT in male offspring with high-dose DEHP exposure [167], prenatal treatment with 150 mg/kg of DEHP has been shown to transgenerationally reduce CORT concentrations in female mouse offspring instead [162]. Thus, it appears that alterations in CORT levels in EDC-exposed offspring vary with the type of EDC and the dose used.

An especially problematic aspect of these contaminants is that, in reality, they exist in combination with one another in the environment – as mixtures. Determining the mechanisms of action underlying EDC mixtures is a relatively novel area of research, but studies agree that EDC mixture effects are more elusive and vary from those of individual EDCs [12, 13]. EDC mixtures, including BPA and DEHP combinations, are associated with a variety of consequences on the metabolic [168, 169], reproductive [170-172], and cardiovascular [173] systems in male

and female rodents. Yet, there is an alarming lack of data on sex-specific outcomes on behavior following exposures to BPA and DEHP mixtures.

The goal of the present study was to assess sex differences in stress-related behaviors, cognition, and the hypothalamic-pituitary-adrenal (HPA) axis following prenatal BPA or DEHP exposure individually or in combination at low and high doses. Our hypothesis was that prenatal EDC exposure, particularly in combination, would induce sex-specific and dose-dependent alterations in behavioral responses to stress that are mediated by the HPA axis.

2.3. MATERIALS AND METHODS

Animals

Adult female Sprague-Dawley rats were purchased from Envigo (Indianapolis, IN) and housed in rooms that were light- (12:12 light-dark cycle) and temperature-controlled (23.2 ± 2°C, 50 ± 20% relative humidity) at the University of Georgia. Food and water were provided *ad libitum*. The rats were fed Pico Lab Rodent Diet 20 (LabDiet). Animals were housed in polycarbonate cages with corn cob bedding. Bisphenol exposures from the environment (cages, water bottles etc.) were not controlled for since all animals were maintained in the same environment. Prior to mating, each of the female breeders underwent vaginal cytology for 10 consecutive days to track their individual estrous cycles. Once in proestrus, a female was randomly assigned a male by generating random numbers using the standard = RAND() function in Microsoft Excel, and the two were co-housed for one day. The presence of a vaginal plug was used to confirm the occurrence of mating. Gestational day (GD) 0 represented the day of copulation. Experimental protocols were in compliance with the National Institutes of Health's

Guide for the Care and Use of Laboratory Animals approved by the Institutional Animal Care and Use Committee (IACUC) at the University of Georgia.

Chemicals

BPA (Lot MKBH2096V; Catalog No. 239658) and DEHP (Lot BCBR8079V; Catalog No. 36735) were purchased from Sigma Aldrich (St. Louis, MO). Stock solutions were made in dimethylsulfoxide (DMSO) to obtain complete dissolution. Doses were calculated daily based on BW and mixed with 20µl PBS for oral dosing. Daily oral dosing occurred from GD 6-21. The vehicle or EDC treatments were discharged into the oral cavity using a micropipette to avoid any local irritation to the gastrointestinal tract and potential stress to the pregnant dam. The BPA dose was selected because it is significantly lower than the Environmental Protection Agency (EPA) recommended no-observed-adverse-effect-level (NOAEL) dose of 5 mg/kg/day [115] and it is also 10-fold below the tolerable daily intake (TDI) dose of 50 µg/kg/day [114]. The high dose of DEHP was selected since it is higher than the established NOAEL dose of 4.8 mg/kg/day [130], whereas the low DEHP dose is significantly lower than this. The low dose of DEHP used in this study lies within the range of the typical daily intake of this chemical in adult humans (0.5-25 µg/kg/day) [127], but is well below the EPA reference dose of 20 µg/kg/day [128].

Experimental Design

The experimental design is demonstrated in Figure 2.1. The dam was considered the experimental unit. Each dam was randomly assigned to one of 6 different treatment groups: control (10 µl Phosphate Buffered Saline or PBS; n=6), BPA (5 µg/kg BW/day; n=7), low-dose (LD) DEHP (5 µg/kg BW/day; n=6), high-dose (HD) DEHP (7.5 mg/kg BW/day; n=6), a combination of BPA and LD DEHP (5 µg/kg/day of BPA + 5 µg/kg/day of DEHP; n=6), and a combination of BPA and HD DEHP (5 µg/kg/day of BPA + 7.5 mg/kg/day of DEHP; n=7).

Generation of random assignment numbers was completed using the standard = RAND() function in Microsoft Excel. These are displayed in Table 2.1.

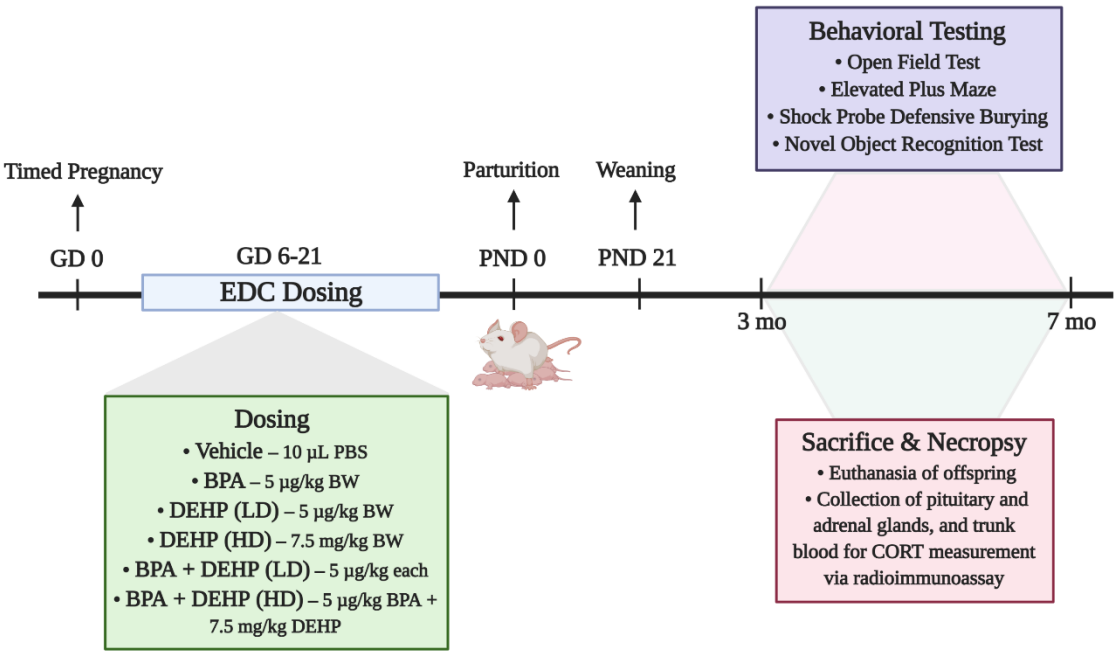


Figure 2.1. Summary of the experimental design of the study. Pregnant Sprague-Dawley dams were orally dosed daily from gestational days (GD) 6-21 with vehicle (Control) (10 µL PBS; *n*=6), BPA (5 µg/kg/day; *n*=7), low-dose (LD) DEHP (5 µg/kg/day; *n*=6), high-dose (HD) DEHP (7.5 mg/kg/day; *n*=6), a mixture of BPA + LD DEHP (5 µg/kg/day of BPA + 5 µg/kg/day of DEHP; n=6), or a mixture of BPA + HD DEHP (5 µg/kg/day of BPA + 7.5 mg/kg/day of DEHP; n=7). Male and female offspring underwent behavioral testing in adulthood. Offspring were euthanized immediately following testing. Pituitary and adrenal glands were dissected and weighed upon euthanasia. Trunk blood was collected for the measurement of serum CORT using RIA. Experimental design schematic was created using Biorender.com.

Note: EDC, endocrine disrupting chemical; PBS, Phosphate Buffered Saline; BPA, Bisphenol A; DEHP, diethylhexyl phthalate; BW, body weight; LD, low-dose; HD, high-dose; CORT, corticosterone.

The adult male and female offspring of the treated dams were administered a battery of behavioral tests including the Open Field Test (OFT), Elevated Plus Maze (EPM), and Shock Probe Defensive Burying (SPDB). The Novel Object Recognition test (NOR) was also administered, but only to the animals in the LD group since the laboratory equipment was not available at the time of the HD animal experimentation. The order for the tests was OFT, EPM, SPDB, followed by NOR. All behavioral testing occurred during the dark cycle. 4 rats were tested per day on average and the rats were administered each test once. A total of 73 animals were used for this study (control-males n = 6, control-females n = 6, BPA-males n = 6, BPA-females n = 7, DEHP (LD)-males n = 6, DEHP (LD)-females n = 6, DEHP (HD)-males n = 6, DEHP (HD)-females n = 6, BPA+DEHP (LD)-males n = 5, BPA+DEHP (LD)-females n = 6, BPA+DEHP (HD)-males n = 7, BPA+DEHP (HD)-females n = 6) (see Table 2.1).

The animals' behaviors in each test were video recorded by a direct overhead webcam (Microsoft), and all videos were manually scored by experimenters unaware of the treatment groups. Rats were habituated to the testing areas for 5 minutes prior to testing. All boxes and equipment were disinfected between every trial. Vaginal smears were obtained from all female rats, for 2-10 days prior to behavioral testing to ensure that females were tested when they were in estrus.

Open Field Test (OFT)

Each animal was placed in a transparent plexiglass test chamber (43.3 cm long x 43.3 cm wide x 30.5 cm high) consisting of a center zone and a perimeter zone [174]. The perimeter zone

was the region inside the box measuring 0-9” from the wall and the area considered the center zone measured 9-35” from the wall. At the beginning of the testing session, all rats were placed in the lower left corner of the box facing the opposite wall. The animal was allowed to freely explore the box for 10 minutes. OFT behaviors were automatically recorded using Activity Monitor software (Med Associates, Fairfax, VT, USA) on a desktop computer to automate behavioral testing and provide unbiased analyses of data. The following measures were recorded during the testing session: number of entries and time spent in the center and perimeter zones, frequency and time spent rearing, as well as distance traveled, and time spent ambulating within the box.

Elevated Plus Maze (EPM)

The testing apparatus consisted of a wooden maze painted black matte with two pairs of arms set perpendicular to each other and placed 50 cm above the floor. The arms extended from a central platform (9 x 9 cm) and formed a pair of open arms (45 x 9 cm) and a pair of closed arms (45 x 9 x 38 cm) [175]. The open arms were not bound by walls and the closed arms were enclosed by high walls and no ceilings. To begin the testing session, the animal was placed on the central platform facing an open arm opposite the experimenter. The number of entries and time spent in each arm and crosses through the central platform were recorded. Entry into an arm was considered as the presence of all four feet of the rat in the arm. After the rat was returned to its home cage at the end of the testing session, the number of fecal pellets in the maze was manually recorded.

Shock Probe Defensive Burying (SPDB)

Animals were placed in a covered clear polycarbonate cage (20 x 40 x 20 cm) containing bedding at the beginning of the testing session. An electrified probe extended 6 cm into the cage

and 2 cm above the bedding [175]. The experimenter administered a mild shock of 3 mA DC (E13-08, Coulbourn Instruments, Allentown, PA) to the animal after initial contact with the probe. The intensity of the animal's response to the shock, or shock reactivity, on a scale of 1-4 was manually recorded. Following this, the frequency and time spent engaging in the following behaviors were measured during the 10-minute testing session: burying, immobility, rearing, exploring the shock-probe, and grooming. After returning the rat to its home cage at the end of the session, the height of the highest point of the bedding was measured and manually recorded.

Novel Object Recognition Test (NOR)

The test chambers had opaque walls with no ceiling (52 x 35 x 32.5 cm, Sterlite). A variety of objects that varied in size (maximum size: 10.5 cm high x 18.5 cm wide), shape, and material (plastic, glass, metal, and ceramic) were used. These particular objects were selected because they were previously tested for object preference bias by Simone et al. [175], and no significant differences were found in bias or exploration of these objects. All objects were glued to a small jar that was fixed in place by screwing the jar to a lid within the box. The test consisted of the following procedure: (1) The rat was allowed to freely explore two randomized identical objects for five minutes during the familiarization phase (T1). Randomization was completed using a computer based random number generator. (2) The rat was returned to its home cage with food and water for 45 minutes during the retention phase. (3) The rat was returned to the test chamber for 3 minutes for the test phase (T2), in which one of the identical objects from T1 was replaced with a novel object. The other identical T1 object was replaced with a duplicate object. The novel object and locations of the objects in T2 were counterbalanced across all rats.

For the T1 phase, the average exploration time was measured, and the discrimination index (DI) was calculated. Exploration was defined as sniffing the object within 2 cm from the edge or touching the object. Climbing on/over or rearing on the objects were not counted as part of the exploration time. DI in this phase was defined as the difference in exploration time between the right object and left object divided by the total exploration time of the right and left objects [176]. For the T2 phase, we calculated both the DI and the recognition index (RI). DI in this phase was defined as the difference in exploration time between the novel object and familiar object divided by the total exploration time of the novel and familiar objects [177]. Finally, the RI was considered the main index of novel object recognition in our study. It was defined as the percent of time spent exploring the novel object relative to the total amount of time exploring both the novel and familiar objects [178, 179].

Tissue Collection and Preparation

Immediately following behavioral testing, female offspring in estrus (as confirmed by vaginal cytology) and male offspring were euthanized by rapid decapitation. Pituitary glands and adrenal glands were dissected, weighed, and stored at -80°C for further processing.

Corticosterone Measurement

Following the sacrifice of the rats, trunk blood was collected, centrifuged and serum was separated and stored at -80°C. Serum corticosterone levels were measured in duplicate using a double antibody radioimmunoassay (MP Biomedicals, Santa Ana, CA; SKU:07120121), according to the manufacturer's protocol. Values were expressed as ng/ml.

Statistical Analysis

Prism 9.0.0 (GraphPad, Inc.) software was used to perform statistical analyses. Behavioral data, organ weights, and corticosterone levels were analyzed separately by sex using

one-way ANOVA to identify EDC exposure effects. Male and female measures were also analyzed together by two-way ANOVA to determine main effects of sex, followed by uncorrected Fischer's LSD post-hoc analyses. Statistical differences between control and EDC groups in behavioral parameters were measured using uncorrected Fischer's LSD post-hoc test. Differences between control and EDC groups in body weights, organ weights, and corticosterone levels were analyzed using Tukey's multiple comparisons post hoc test. Values that were statistical outliers were excluded from all of the analyses. P-value < 0.05 was considered to indicate a statistically significant difference. Data was expressed as mean ± standard error of mean (SEM).

2.4. RESULTS

Open Field Test

No significant differences were observed in distance traveled (Figure 2.2 E and F) or time spent (Table 2.2) ambulating within the chamber in males or females from any of the groups. A significant main effect of sex was observed in rearing frequency (mean ± SEM) (Table 2.2). DEHP (HD) females (114.0 ± 29.7) displayed a marked increase in rearing compared to their male counterparts (53.2 ± 2.7; $p = 0.002$), suggesting a possible anxiety-like response in DEHP (HD) females. No additional differences were found in this measure, or in rearing time (Table 2.2).

Figure 2.2 and Table 2.2 depict the results from the OFT. There was a significant effect of EDC exposure in the time spent in the center zone (%, mean ± SEM; $p = 0.0221$), but only in female offspring (Figure 2.2A). BPA ($p = 0.0633$) and B+D (LD) ($p = 0.0519$) females showed near-significant trends for increased time in the center zone, suggesting anxiolytic effects. A

main effect of sex was also observed, with control (p = 0.0327) and B+D (HD) (p = 0.0518) females having decreased center time compared to their male counterparts (Table 2.2), possibly indicating that these females are more anxiety-prone than the corresponding males.

Additionally, DEHP (LD) (p = 0.0227), DEHP (HD) (p = 0.0042), and B+D (HD) (p = 0.0255) female offspring displayed a reduction in entries into the perimeter zone than control females (Figure 2.2C), whereas male offspring were unaffected in this measure (Figure 2.2D). This is also a potential indicator of reduced anxiety levels in these females. A main effect of sex was additionally determined in perimeter zone entries, with significant differences found only within the BPA group (p = 0.0281) (Table 2.2). Finally, there was no effect of EDC exposure in the amount of time males or females spent in the perimeter zone. However, a main effect of sex was observed in this measure (Table 2.2). BPA (p = 0.0267) and B+D (HD) (p = 0.0063) females spent more time in the periphery (118.3% and 128.6% more, respectively) compared to their male counterparts, indicating higher levels of anxiety.

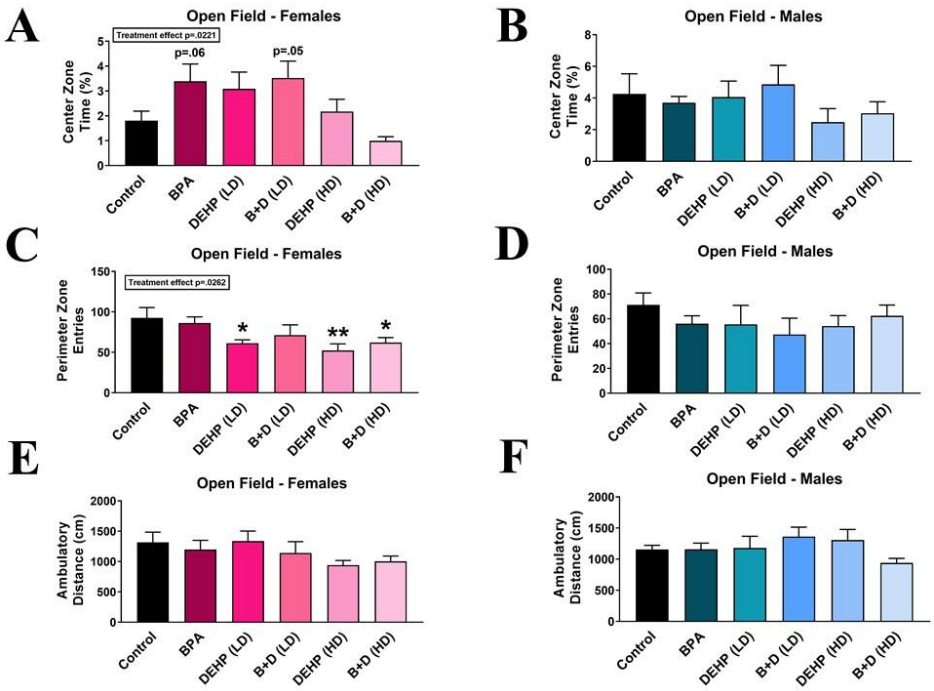


Figure 2.2. Behavioral effects of prenatal exposure to vehicle or EDCs in female and male rat offspring in the open field test (OFT). (*A*) Center zone time in female offspring, (*B*) center zone time in male offspring, (*C*) perimeter zone entries in female offspring, (*D*) perimeter zone entries in male offspring, (*E*) locomotor activity in female offspring, and (*F*) locomotor activity in male offspring. Behavioral data was collected from adult male and female offspring prenatally exposed to EDCs as described in Fig 1.1. Data were analyzed by one-way ANOVA, followed by Fisher's LSD post hoc test. $^{*}p<0.05$, $^{**}p<0.01$ comparison between control and EDC-exposed female offspring. Error bars represent the standard error of the mean (SEM).

Elevated Plus Maze

We did not observe any significant effects of EDC exposure on exploration in the EPM in either sex (Figure 2.3 E and F). However, a main effect of sex was discovered in the amount of time spent in the central platform of the EPM (Table 2.2). Females in a majority of the groups spent significantly more time in the center. Control ($p = 0.0010$), BPA ($p = 0.0069$), DEHP (HD) ($p = 0.0217$), and B+D (HD) ($p = 0.0079$) females exhibited robust increases (142.4%, 96.5%, 134.3%, 120.0%, respectively) in center time than their male counterparts, which may be interpreted as increased risk assessment of the maze.

DEHP (HD) male offspring, on the other hand, had increased ratios (mean ± SEM) of open-to-total (OTT) arm time (0.6 ± 0.1) (Figure 2.3A) and OTT entries (0.5 ± 0.1) (Figure 2.3C) compared to control males (OTT time: 0.3 ± 0.1; OTT entries: 0.4 ± 0.1), suggesting that DEHP (HD) males had reduced anxiety. A similar effect was observed in control ($p = 0.0477$) and BPA ($p = 0.0002$) females, with them having considerably higher ratios for OTT arm time (64.3% and 195.2%, respectively) compared to their male counterparts. BPA females also demonstrated a higher ratio of OTT arm entries than BPA males (females: 0.6 ± 0.1; males: 0.3 ±

0.0; $p = 0.0007$). The corollary was true in the time spent in the closed arms with females in the control ($p = 0.0197$) and BPA ($p = 0.0001$) groups spending less time compared to the males (Table 2.2), confirming reduced anxiety in these two groups. Additionally, B+D (HD) females also spent less time in the closed arms (40.7 ± 7.3; $p = 0.0506$) than their male counterparts (60.5 ± 2.7). Further, the reduced OTT arm time and entries in DEHP (HD) males resembled that in control females (OTT time: 0.6 ± 0.1; OTT entries: 0.5 ± 0.1), suggesting a possible feminization of behavior in these males.

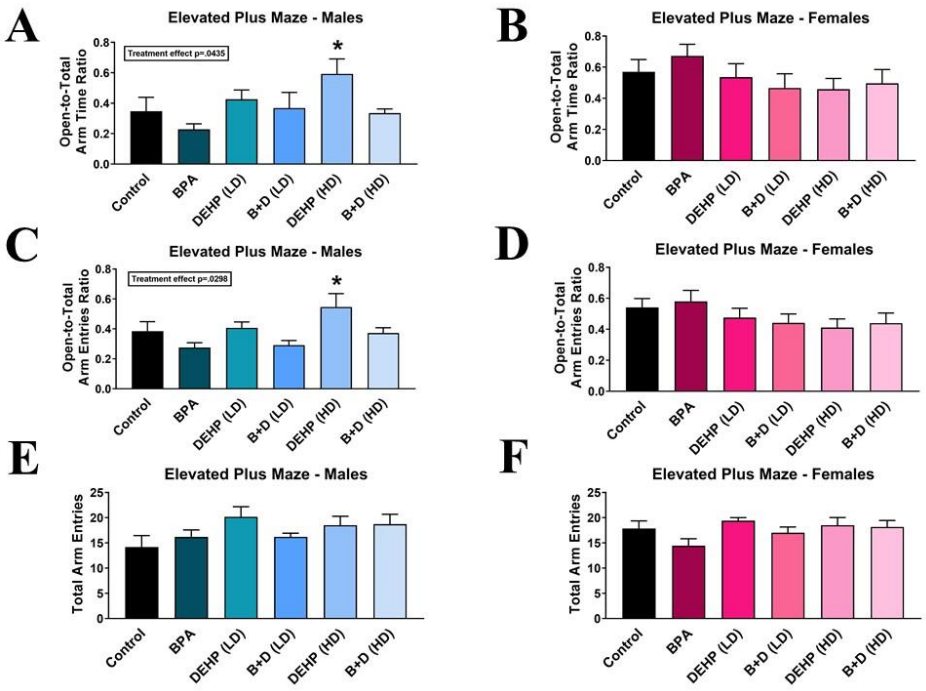


Figure 2.3. Behavioral effects of prenatal exposure to vehicle or EDCs in adult male and female rat offspring in the elevated plus maze (EPM). (*A*) Time spent in open arms relative to total arms in male offspring, (*B*) time spent in open arms relative to total arms in female offspring, (*C*) number of entries into open arms relative to total arms in male offspring, (*D*) number of entries into open arms relative to total arms in female offspring, (*E*) total exploration

of the maze in male offspring, and (*F*) total exploration of the maze in female offspring. Data were analyzed by one-way ANOVA, followed by Fisher's LSD post hoc test. $^{*}p<0.05$, comparison between control and EDC-exposed male offspring. Error bars represent the standard error of the mean (SEM).

Shock Probe Defensive Burying

Figures 2.4 A-D display our results from the SPDB test. A majority of the EDC-treated male offspring demonstrated robust reductions in the amount of time spent burying (%, mean ± SEM) (Figure 2.4A). Compared to control males (36.9 ± 12.4), males prenatally exposed to BPA (14.8 ± 8.5; $p = 0.0518$), DEHP (LD) (0.3 ± 0.3; $p = 0.0034$), DEHP (HD) (1.8 ± 1.0; $p = 0.0046$), and B+D (HD) (13.7 ± 5.1; $p = 0.0353$) spent significantly less time burying the probe, which indicates a decrease in active coping. DEHP (LD) males (37.4 ± 13.2; $p = 0.0027$) instead spent substantially more time immobile than control males (4.2 ± 1.6) (Figure 2.4C), which is suggestive of passive coping [180]. Similar to DEHP (LD) males, DEHP (LD) ($p = 0.01$) and B+D (LD) ($p = 0.0134$) females had over 600% increases in immobility time compared to control females (Figure 2.4D), indicating that they were prone to passive coping as well. No other differences were observed in burying or immobility frequency, rearing frequency or time, probe exploration frequency or time, or grooming frequency or time in male or female offspring (Table 2.2).

In addition to these behavioral parameters, a significant EDC effect was observed in female shock reactivity (mean ± SEM; $p = 0.0086$) (Table 2.2). Females prenatally treated with BPA (1.1 ± 0.1; $p = 0.0089$), DEHP (LD) (1.0 ± 0.0; $p = 0.0018$), B+D (LD) (1.0 ± 0.0; $p = 0.0018$), and B+D (HD) (1.0 ± 0.0; $p = 0.0018$) displayed moderately decreased shock reactivity in comparison with control females (1.7 ± 0.2), suggesting a dampening of fear responses and

reduced physical reactivity to shock stress. No such differences were observed in males, nor were there differences in bedding height after EDC exposure in both male and female offspring (Table 2.2).

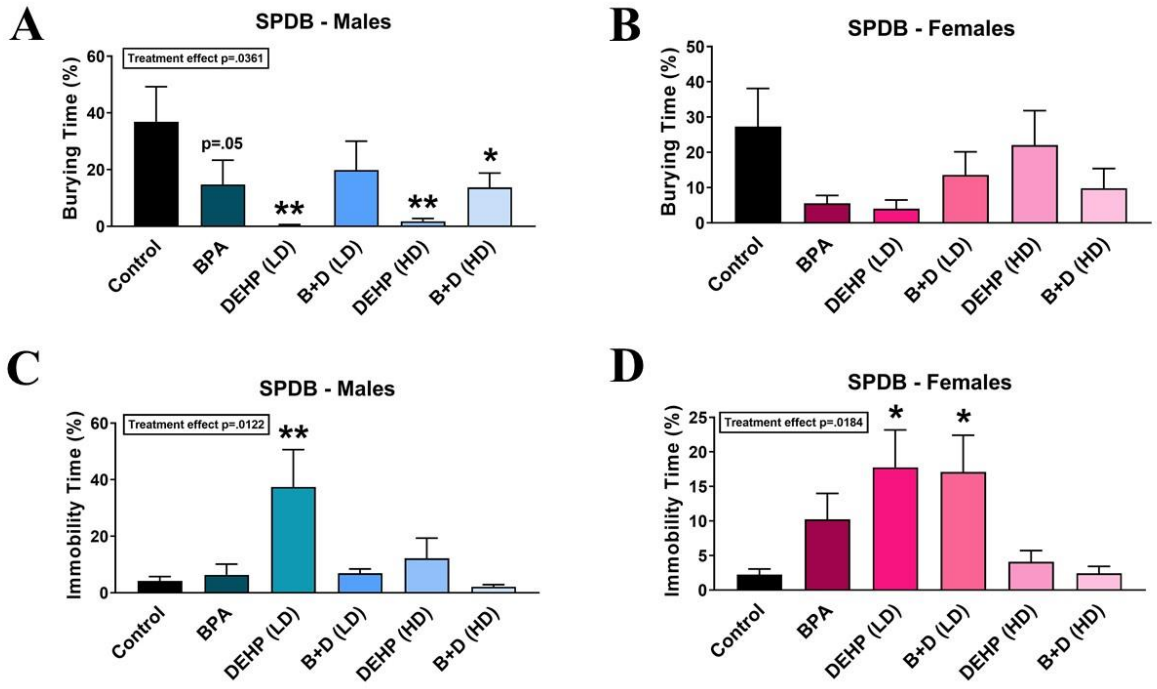


Figure 2.4. Behavioral effects of prenatal exposure to vehicle or EDCs in adult male and female rat offspring in the shock probe defensive burying (SPDB) test. (*A*) Amount of time spent burying in male offspring, (*B*) amount of time spent burying in female offspring, (*C*) amount of time spent immobile in male offspring, and (*D*) amount of time spent immobile in female offspring. Data were analyzed by one-way ANOVA, followed by Fisher's LSD post hoc test. $^{*}p<0.05$, $^{**}p<0.01$, comparison between control and EDC-exposed offspring. Error bars represent the standard error of the mean (SEM).

Novel Object Recognition Test

The results from the NOR are displayed in Figure 2.5 and Table 2.2. No significant differences were observed in the EDC-exposed offspring in any of the NOR measures.

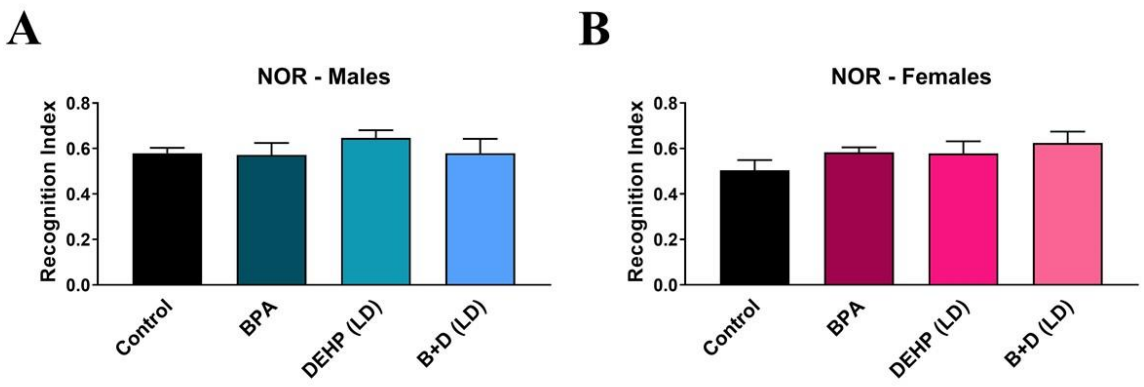


Figure 2.5. Behavioral effects of prenatal exposure to vehicle or EDCs in adult male and female rat offspring in the novel object recognition (NOR) test. (*A*) Object recognition in male offspring and (*B*) object recognition in female offspring. Data were analyzed by one-way ANOVA, followed by Fisher's LSD post hoc test. Error bars represent the standard error of the mean (SEM).

Corticosterone Levels

There was a significant EDC exposure effect in CORT levels in both male offspring (p = 0.0113) (Figure 2.6A) and female offspring (p = 0.0030) (Figure 2.6B). Although no significant differences were found within the males, we did observe a near-significant elevation in CORT levels in DEHP (LD) males compared to control males (p = 0.0720). In contrast, BPA (p = 0.0391) and DEHP (HD) (p = 0.0285) exposure reduced CORT levels in females compared to controls, and B+D (HD) females showed a similar trend for lower CORT (p = 0.0780). Finally, a main effect of sex was apparent in CORT levels (Table 2.3). Control (p < 0.0001), DEHP (LD)

(p = 0.0250), and B+D (LD) (p = 0.0166) female offspring displayed significantly higher CORT concentrations compared to their male counterparts. No sex differences were observed in the rest of the treatment groups.

Body and Organ Weights

There was a significant EDC exposure effect (p = 0.0232) in adrenal gland relative weights, with DEHP (LD) (p = 0.0362) and B+D (LD) (p = 0.0267) male offspring having lower weights than corresponding controls (Figure 2.6C). In contrast, no significant EDC effects were found in female adrenal gland weights (Figure 2.6D). Furthermore, there were no differences in male or female body weights (BWs) or pituitary gland relative weights due to EDC treatment (Table 2.3). However, males had higher body weights and lower pituitary gland relative weights, compared to the female offspring. Moreover, females exposed to both low and high doses of DEHP (p = 0.0269 and 0.0234 respectively) and B+D (p = 0.0114 and <0.0001 respectively) had significantly higher adrenal weights than their male counterparts.

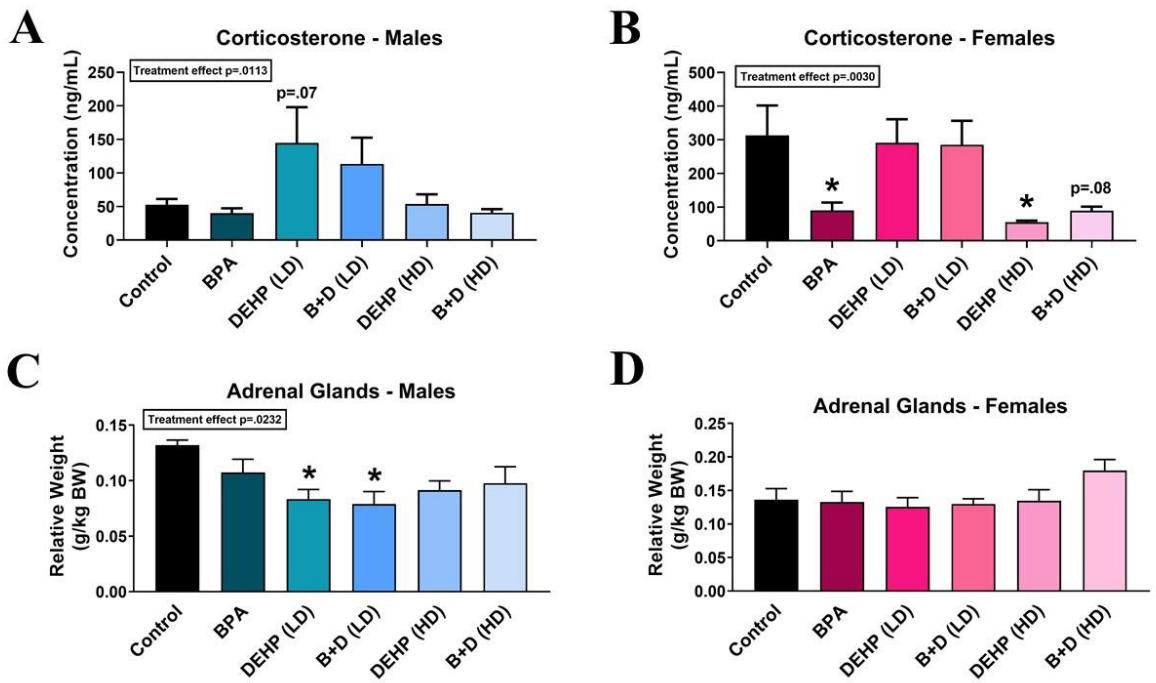


Figure 2.6. Hypothalamic-pituitary-adrenal (HPA) axis activity in adult male and female offspring prenatally exposed to EDCs or mixtures. (*A*) Serum corticosterone (CORT) levels (ng/mL) measured in male offspring, (*B*) serum CORT levels (ng/mL) measured in female offspring, (*C*) adrenal gland relative weights (g/kg BW) in male offspring, and (*D*) adrenal gland relative weights (g/kg BW) in female offspring. Data were analyzed by one-way ANOVA, followed by Tukey's multiple comparisons test. $^{*}p<0.05$, comparison between control and EDC-exposed male offspring. Error bars represent the standard error of the mean (SEM).

2.5. DISCUSSION

In this study, we aimed to determine sex differences in the adverse behavioral and cognitive effects resulting from prenatal EDC exposure. The results of our study revealed a myriad of effects on stress-related behaviors, exploration, and locomotion. In control offspring, we observed anxiogenic effects in females compared to males in the OFT, which assesses exploration of a novel environment [181]. These results are consistent with the findings of Verstraeten et al. (2019) [182], in which control females showed anxiogenic effects in the OFT. We did not discover any effects on locomotor or exploratory activity. On the contrary, our control females displayed decreased anxiety-like behavior relative to their male counterparts in the EPM, which represents a riskier environment and evaluates unconditioned anxiety [183]. These results are in line with previous studies, which have demonstrated that females show significantly more open arm entries [182] and time [184] in the EPM. Additionally, anxiety-related behaviors in the EPM are independent of estrous cycle stage in females [184]. All of the female offspring in our study were tested in estrus, which may be perceived as a limitation due to

the comparisons with males. Yet, the aforementioned finding confirms that it is not a major confound.

The control females in our study also spent more time in the central platform of the EPM than their male counterparts. Center time in the EPM has been associated with decision making processes [185], and the increased center time observed in our control females may be interpreted as increased risk assessment of the open arms. Prior studies have found either a positive association [186] or no relationship [187] between risk assessment and anxiety-like behavior. Finally, control offspring did not show any robust differences in coping strategies in the SPDB. The males appeared to prefer burying, and although the females also buried quite extensively, it was not significantly different from the males. Burying is an adaptive coping style since it embodies an active coping behavioral response to stress [188].

Since there is already a plethora of established research on BPA, we incorporated BPA in this study as a positive control and therefore only tested the effects of a single low dose. BPA-treated female offspring displayed a near-significant increase in time spent in the center zone in the OFT, indicative of decreased anxiety-like behavior. This was coupled with reduced CORT concentrations in BPA females. Although these results contrast with some studies [117, 143], they are partially consistent with one study that found decreased OFT anxiety-like behavior in females, but no changes in serum CORT following perinatal exposure to 2 µg/kg of BPA [151]. Our CORT results imply a hypoactivity of the HPA axis, which may underlie the antianxiety effect observed in BPA female offspring.

BPA males, on the other hand, spent less time burying in the SPDB. Although the ANOVA effect for burying time was non-significant in females ($p = 0.1369$), post-hoc analyses did reveal a significant reduction in burying time for BPA females as well ($p = 0.0284$). A

decrease in burying represents a maladaptive reduction in active coping mechanisms [189]; however, it may also be interpreted as a decrease in anxiety-like behavior [175, 190]. Additionally, the BPA females in our study further exhibited altered defensive behaviors in the form of reduced shock reactivity in the SPDB. These effects taken together suggest that prenatal exposure to BPA at a low dose of 5 µg may lead to aberrant effects on species-specific defensive behaviors and fear responses.

BPA-exposed offspring showed similar sex differences to control offspring in the EPM. Both control and BPA females spent increased time in the open arms and the center, and less time in the closed arms relative to their male counterparts. Anxiogenic effects in the EPM are common in male offspring with prenatal BPA treatment [157]. Our results are also similar to those of Jones and Watson (2012), which showed similar effects in BPA-exposed males and females, but at a higher dose (50 µg/kg) [125]. Interestingly, 5 µg/kg of BPA abolished sex differences in the aforementioned study, in contrast with the results from our study.

Prenatal exposure to DEHP at low and high doses yielded varying effects in each of the behavioral tests. In the OFT, DEHP-treated female offspring exposed to both doses had fewer entries into the perimeter zone, but showed no changes in perimeter zone time. While a reduction of entries into the perimeter zone of the OFT may represent an anxiolytic-like effect due to reduced activity in the periphery, changes in exploration of the center zone in this test are typically used to measure anxiety-like behavior [184, 191]. DEHP females were unaffected in the center zone parameters, rendering unclear anxiety-related effects. However, DEHP (HD) females did have reduced CORT levels, similar to our BPA females, which may support an antianxiety effect since a linear relationship between anxiety and CORT is often observed [192, 193]. DEHP (HD) females additionally showed increased rearing relative to their male

counterparts. This effect is vastly different from that reported in previous studies, which have observed reduced rearing both in control female mice [194] as well as in perinatal DEHP-exposed (10 and 200 mg/kg) female mice [144, 145]. Hence, treatment with 7.5 mg/kg of DEHP *in utero* may reverse sex differences in exploratory behavior in exposed offspring.

DEHP (HD) males exhibited decreased anxiety-like behavior in the EPM, contrasting with studies that have observed anxiogenic effects in the EPM in males with perinatal low-dose (5-40 µg) and high-dose (10-50 mg) DEHP exposure [145, 160]. Quinnies et al. (2017), in particular, demonstrated that DEHP can dose-dependently alter EPM anxiety-like behavior in male offspring even in the third generation following exposure, implying transgenerational effects [160]. In addition, our DEHP (HD) male offspring behavior in the EPM resembled that of control females. The sex difference between HD males and females in the proportion of time spent in the open arms was also reversed compared to controls, and this particular effect was not noted in any other EDC group. This suggests a potential feminization of HD males in anxiety-like behavior. This is consistent with the anti-androgenic effects of DEHP commonly discerned in exposed males [66, 67, 69]. We can further confirm based on our results that this particular effect is not present at a low dose of 5 µg.

In the SPDB, we observed robust decreases in burying in LD and HD males in the DEHP-exposed groups. LD males and females also engaged in increased immobility, which was not reflected in HD offspring. In addition, we observed significantly reduced shock reactivity in LD, but not HD, females. These findings imply aberrant changes in defensive behaviors – reduced active coping in DEHP males, regardless of dose, and a definite shift to passive coping in DEHP (LD) male and female offspring. An increase in immobility reflects a preference for passive reactive coping mechanisms [189, 195], a maladaptive stress response that is often

observed in individuals with affective disorders, including depression [196, 197]. Immobility can also be interpreted as a measure of anxiety-like behavior [198].

Heightened immobility responses in the Forced Swim Test, an indicator of depressive-like behavior, have previously been reported in both male and female offspring perinatally treated with DEHP at high doses (10-200 mg/kg) [145]. Our study is the first to demonstrate an acute stress-induced passive behavioral phenotype in the SPDB in male and female offspring exposed to low-dose DEHP *in utero*. In addition, this effect was associated with a sex-specific trend for heightened CORT levels in male, but not female, offspring. This finding is consistent with prior studies that have found associations between increased CORT levels and freezing behavior [199, 200]. It is highly likely that our finding would have reached statistical significance with a larger sample size. Furthermore, our DEHP (LD) males also showed a reduction in adrenal gland relative weights. This is intriguing because adrenal hormones have been implicated in freezing behavior, with adrenalectomy impairing the duration of fear-induced freezing [201]. Hence, the lower adrenal weights in our DEHP (LD) males may have contributed to their behavioral and CORT outcomes. Additional experiments should examine mineralocorticoid and glucocorticoid receptor alterations for further insight.

The fact that DEHP (LD), as well as B+D (LD), female offspring had comparable levels of CORT to control females, yet displayed increased immobility than control females is significant. It suggests that females with low-dose EDC exposure that show more passive responses may be affected in other ways that do not involve changes in CORT; this needs to be explored further. One possible mechanism of action underlying these behavioral changes could be epigenetic modifications. Low-dose DEHP exposures have been associated with changes in

DNA methylation [202-204], which is one mechanism of epigenetic alterations, and these are linked with behavioral and reproductive dysfunctions.

Treatment with BPA and DEHP in combination altered the behavior of female offspring more than male offspring. B+D (LD) females exhibited anxiolytic effects in the OFT, but passive coping responses in the SPDB. B+D (HD) females showed unclear and slightly conflicting anxiety-like behavioral responses as well. In the OFT, they displayed significantly fewer perimeter zone entries, but they also spent the least amount of time in the center zone compared to other female offspring. However, this was not significantly different from control females. B+D (HD) females did not demonstrate any other effects, apart from a trend for lower CORT concentrations.

B+D (LD) males were not affected in any of the behavioral measures, but instead had lower adrenal gland relative weights, similar to DEHP (LD) males. This result is particularly striking because it suggests that B+D (LD) male offspring may be more impacted at the organ systems level. Ours is the first study to our knowledge to determine associations between B+D exposure and reductions in adrenal weights in male offspring. B+D (HD) males, on the other hand, did show behavioral alterations. They demonstrated significant reductions in active coping in the SPDB, with no accompanying alterations in CORT or adrenal weights. Therefore, the dose of B+D at which male offspring are exposed to determines the nature of the outcomes they will exhibit.

Interestingly, B+D exposure at the high dose generally produced similar behavioral effects to DEHP (HD) among female offspring, but led to similar effects as BPA offspring in the males. B+D at the low dose, on the other hand, revealed less clear cumulative effects of BPA and DEHP in combination. B+D (LD) females paralleled the anxiety-like behaviors of BPA females

in the OFT, but were comparable to DEHP (LD) females in defensive behaviors and CORT levels. Likewise, B+D (LD) males appeared to resemble BPA males in defensive behaviors, but were closer to DEHP (LD) males in adrenal gland weights and even CORT levels. Overall, the cumulative effects of BPA and DEHP on behavior are unclear but are definitely dependent on the behavioral parameter assessed. B+D exposure may produce similar effects to BPA or DEHP offspring in some cases, or even cancel out the independent effects of BPA and DEHP. Perinatal exposure to high-dose EDC mixtures, including BPA and DEHP, has been shown to counteract the independent effects of these chemicals on the brain [205]. Our results reveal that this effect can also be observed in behavior at low doses of B+D.

In addition to the behavioral measures, we obtained body weights as well as the weights of the pituitary and adrenal glands. We did not observe any effects on body weights as a result of EDC exposure; however, sex differences were discovered in every group, with male offspring weighing more than their female counterparts, as expected. Sex differences, but no effects of EDC exposure, were also identified in every group in pituitary gland relative weights. The females in these groups displayed significantly higher pituitary weights than their male counterparts. Moreover, our adrenal gland weight data demonstrated that sex differences not observed in control offspring were induced in the DEHP and B+D groups, regardless of dose. Female offspring in these groups had increased adrenal weights than their male counterparts.

To summarize, offspring with prenatal EDC exposures were dose- and sex-specifically affected in each of the behavioral measures assessed. BPA offspring were impacted in anxiety-like and defensive behaviors, as well as in CORT levels. DEHP (LD) offspring were more severely affected in terms of defensive behaviors, whereas DEHP (HD) offspring displayed aberrant anxiety-like behavior and altered CORT. Low dose B+D exposure modified anxiety-like

and defensive behaviors, but high-dose exposure affected defensive behaviors and CORT. Sex differences were induced in adrenal gland relative weights only in the DEHP and B+D groups at both doses. Furthermore, DEHP and B+D males showed a reduction in adrenal gland weights following low-dose exposure. These results raise significant health concerns, in particular due to the harmful or elusive effects at low doses. Future studies should focus on molecular mediators underlying these behavioral alterations, including neurotransmitter systems and hormone receptor dysregulation in the brain.

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Table 2.1. Sample sizes of control or EDC-treated dams, and their male and female offspring.

	Control	BPA	DEHP (5 µg)	DEHP (7.5 mg)	BPA+DEHP (5 µg)	BPA+DEHP (7.5 mg)
Dams	6	7	6	6	6	7
Offspring						
Males	6	6	6	6	5	7
Females	6	7	6	6	6	6

Note: BPA, bisphenol A; DEHP; diethylhexyl phthalate; EDC, endocrine disrupting chemical. Measures were obtained from dams and offspring treated with vehicle (Control), BPA (5 µg/Kg BW), low-dose DEHP (5 µg/Kg BW), high-dose DEHP (7.5 mg/Kg BW), BPA + low-dose DEHP (5 µg/Kg BW BPA + 5 µg/Kg BW DEHP), or BPA + high-dose DEHP (5 µg/Kg BW BPA + 7.5 mg/Kg BW DEHP).

Table 2.2. Behavioral data of male and female offspring following low-dose (5 µg) and high-dose (7.5 mg) prenatal EDC exposure.

Behavioral Parameter	Control		BPA (5 µg)		DEHP (5 µg)		BPA + 5 µg DEHP		DEHP (7.5 mg)		BPA + 7.5 mg DEHP		Sex Effect *p-value*
	Male	**Female**	**Male**	**Female**	**Male**	**Female**	**Male**	**Female**	**Male**	**Female**	**Male**	**Female**	
OFT													
Ambulation (% time)	19.9 ± 1.8	21.6 ± 2.8	21.7 ± 1.6	17.6 ± 2.4	19.3 ± 3.2	22.5 ± 2.7	20.7 ± 2.1	18.2 ± 3.4	23.1 ± 3.4	15.0 ± 1.3	17.7 ± 1.8	16.4 ± 1.6	NS
Rearing Frequency	69.3 ± 10.2	82.7 ± 14.9	67.3 ± 14.7	69.2 ± 1.4	69.2 ± 7.7	80.2 ± 4.7	71.6 ± 11.2	91.7 ± 10.7	53.2 ± 2.7^{++}	114.0 ± 29.7^{++}	60.9 ± 8.8	83.2 ± 6.6	*p*=.0054
Rearing (% time)	21.7 ± 3.4	21.6 ± 4.4	16.5 ± 3.0	25.6 ± 4.7	20.0 ± 2.9	24.0 ± 2.7	18.7 ± 3.6	24.7 ± 2.5	18.7 ± 1.6	23.1 ± 2.2	18.3 ± 4.1	16.4 ± 1.4	NS
Center zone (% time)	4.3 ± 1.3^{+}	1.8 ± 0.4^{+}	3.7 ± 0.4	3.4 ± 0.7	4.1 ± 1.0	3.1 ± 0.7	4.9 ± 1.2	3.5 ± 0.7	2.5 ± 0.9	2.2 ± 0.5	3.0 ± 0.7^{a}	1.0 ± 0.2^{a}	*p*=.0074
Center zone (# of entries)	40.8 ± 7.4	29.3 ± 5.6	32.0 ± 2.8	26.4 ± 5.5	52.8 ± 1.8	42.5 ± 11.1	50.6 ± 10.3	31.2 ± 7.6	31.6 ± 11.0	23.8 ± 0.4	28.3 ± 5.8	17.7 ± 4.6	NS
Perimeter zone (% time)	23.5 ± 5.0	39.6 ± 6.5	18.3 ± 1.6^{+}	36.9 ± 4.8^{+}	21.8 ± 5.8	26.9 ± 6.2	24.3 ± 9.2	36.6 ± 2.5	25.8 ± 8.5	32.2 ± 8.8	18.7 ± 3.5^{++}	42.8 ± 6.2^{++}	*p*=.0002
Perimeter zone (# of entries)	71.2 ± 9.7	92.5 ± 12.9	56.0 ± 6.4^{+}	86.3 ± 7.5^{+}	55.5 ± 15.4	61.3 ± 4.1	47.4 ± 13.2	71.3 ± 12.6	54.2 ± 8.5	52.3 ± 8.1	62.4 ± 8.7	62.0 ± 6.2	*p*=.0237
EPM													
Central platform (% time)	9.4 ± 1.9^{++} $^{+}$	22.9 ± 4.6^{+++}	10.8 ± 1.1^{++}	21.2 ± 3.6^{++}	13.2 ± 1.7	10.3 ± 1.5	11.6 ± 2.2	10.8 ± 2.2	7.1 ± 1.2^{+}	16.7 ± 4.7^{+}	8.5 ± 1.7^{++}	18.8 ± 2.4^{++}	*p*=.0001

Open-to-total arm entries ratio	0.4 ± 0.1	0.5 ± 0.1	0.3 ± 0.0^{+++}	0.6 ± 0.1^{+++}	0.4 ± 0.0	0.5 ± 0.1	0.3 ± 0.0	0.4 ± 0.1	0.5 ± 0.1	0.4 ± 0.1	0.4 ± 0.0	0.4 ± 0.1	*p*=.004 7
Open-to-total arm time ratio	0.3 ± 0.1^{+}	0.6 ± 0.1^{+}	0.2 ± 0.0^{+++}	0.7 ± 0.1^{+++}	0.4 ± 0.1	0.5 ± 0.1	0.4 ± 0.1	0.5 ± 0.1	0.6 ± 0.1	0.5 ± 0.1	0.3 ± 0.0	0.5 ± 0.1	*p*=.001 6
Closed arms (# of entries)	10.0 ± 0.6	8.8 ± 1.0	12.2 ± 2.3	7.4 ± 1.6	13.5 ± 1.4	11.5 ± 1.8	12.2 ± 1.9	10.3 ± 0.7	10.7 ± 2.7	12.0 ± 0.8	14.0 ± 1.4	12.0 ± 2.1	NS
Closed arms (% time)	58.7 ± 8.5^{+}	34.0 ± 8.0^{+}	68.2 ± 3.4^{+++}	24.8 ± 5.7^{+++}	49.9 ± 5.8	41.0 ± 7.4	56.2 ± 10.1	44.8 ± 9.2	37.1 ± 9.1	41.4 ± 7.5	60.5 ± 2.7^{a}	40.7 ± 7.3^{a}	*p*=.000 1
SPDB													
Burying (frequency)	33.3 ± 11.3	25.2 ± 6.8	20.5 ± 11.1	9.7 ± 3.2	0.6 ± 0.4	7.0 ± 3.5	24.4 ± 10.9	14.2 ± 7.2	3.0 ± 1.1	21.2 ± 7.5	22.1 ± 8.3	10.3 ± 5.4	NS
Immobility (frequency)	14.5 ± 4.5	12.7 ± 5.8	13.2 ± 3.5	17.7 ± 4.8	21.3 ± 1.6	24.7 ± 5.1	20.8 ± 2.9	19.5 ± 4.8	10.3 ± 4.3	10.8 ± 4.5	8.3 ± 2.6	9.8 ± 4.0	NS
Rearing (frequency)	9.3 ± 2.4	19.3 ± 4.8	11.0 ± 3.1	20.6 ± 4.3	6.2 ± 2.9	14.8 ± 3.9	12.8 ± 4.3	13.5 ± 5.5	18.7 ± 5.4	15.8 ± 4.0	18.3 ± 2.9	17.7 ± 4.0	NS
Rearing (% time)	4.5 ± 0.8	11.1 ± 3.7	8.8 ± 3.0	14.5 ± 4.5	6.3 ± 3.1	7.9 ± 2.2	5.7 ± 2.1	6.4 ± 2.3	10.6 ± 3.7	8.8 ± 2.9	9.1 ± 1.8	10.3 ± 2.9	NS
Probe exploration (frequency)	4.8 ± 1.8	14.3 ± 4.4	10.2 ± 4.5	13.4 ± 3.1	6.3 ± 3.1	13.3 ± 4.9	8.8 ± 3.6	9.8 ± 3.0	9.5 ± 3.6	8.2 ± 2.3	13.0 ± 2.9	9.2 ± 2.1	NS
Probe exploration (% time)	1.2 ± 0.2	7.7 ± 3.3	3.9 ± 2.2	9.7 ± 3.3	0.7 ± 0.5	5.3 ± 1.8	3.5 ± 1.7	2.4 ± 0.8	3.3 ± 1.6	4.3 ± 1.6	8.2 ± 3.2	5.1 ± 1.8	NS
Grooming (frequency)	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.4 ± 0.3	0.2 ± 0.2	0.5 ± 0.3	0.2 ± 0.2	0.5 ± 0.3	0.0 ± 0.0	0.0 ± 0.0	0.1 ± 0.1	0.2 ± 0.2	NS
Grooming (% time)	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.5 ± 0.3	0.4 ± 0.4	0.7 ± 0.6	0.1 ± 0.1	0.5 ± 0.3	0.0 ± 0.0	0.0 ± 0.0	0.1 ± 0.1	0.1 ± 0.1	NS
Shock reactivity	1.3 ± 0.2	1.7 ± 0.2	1.5 ± 0.2	1.1 ± 0.1**	1.3 ± 0.2	1.0 ± 0.0**	1.0 ± 0.0	1.0 ± 0.0**	1.5 ± 0.2	1.3 ± 0.2	1.1 ± 0.1	1.0 ± 0.0**	NS

Bedding height (cm)	7.8 ± 0.8	7.8 ± 0.7	6.3 ± 0.8	6.3 ± 0.6	6.1 ± 0.9	5.9 ± 0.4	7.1 ± 1.0	6.8 ± 0.6	5.5 ± 0.4	6.4 ± 0.6	5.8 ± 0.4	6.3 ± 0.5	NS
NOR													
T1 Average Exploration (% time)	23.4 ± 2.6	19.6 ± 4.6	24.7 ± 1.7	20.9 ± 1.2	16.0 ± 2.8	22.0 ± 1.6	24.3 ± 3.9	24.6 ± 2.5	N/A	N/A	N/A	N/A	NS
T1 Discrimination Index	-0.04 ± 0.1	-0.1 ± 0.1	-0.1 ± 0.1	-0.04 ± 0.2	-0.1 ± 0.1	-0.01 ± 0.1	0.1 ± 0.1	0.1 ± 0.0	N/A	N/A	N/A	N/A	NS
T2 Discrimination Index	0.2 ± 0.0	0.01 ± 0.1	0.1 ± 0.1	0.2 ± 0.0	0.3 ± 0.1	0.2 ± 0.1	0.2 ± 0.1	0.2 ± 0.1	N/A	N/A	N/A	N/A	NS

Note: EDC, endocrine disrupting chemicals; BPA, bisphenol A; DEHP, di-(2-ethylhexyl) phthalate; OFT, open field test; EPM, elevated plus maze; SPDB, shock probe defensive burying; NOR, novel object recognition test; NS, non-significant. Data are presented as mean ± SEM. ** $p < 0.01$, difference between control and EDC females, one-way ANOVA followed by uncorrected Fisher's LSD post hoc analyses. $^{+}$ $p < 0.05$, $^{++}$ $p < 0.01$, $^{+++}$ $p < 0.001$, difference between males and females of the same treatment group, two-way ANOVA followed by uncorrected Fisher's LSD post hoc analyses. ^{a} $p = 0.05$, difference between males and females of the same treatment group, two-way ANOVA followed by uncorrected Fisher's LSD post hoc analyses.

Table 2.3. Sex differences in body and organ weights of male and female offspring following low-dose (5 µg) and high-dose (7.5 mg) prenatal EDC exposure.

Parameter	Control		BPA (5 µg)		DEHP (5 µg)		BPA + 5 µg DEHP		DEHP (7.5 mg)		BPA + 7.5 mg DEHP		Sex Effect *p-value*
	Male	**Female**	**Male**	**Female**	**Male**	**Female**	**Male**	**Female**	**Male**	**Female**	**Male**	**Female**	
Corticosterone (ng/mL)	52.5 ± 8.9^{++++}	313.2 ± 88.7^{++++}	40.2 ± 7.1	90.2 ± 23.2	144.8 ± 53.3^{+}	290.9 ± 70.1^{+}	113.5 ± 39.1^{+}	285.1 ± 71.5^{+}	53.9 ± 14.4	55.0 ± 4.7	40.7 ± 5.5	89.3 ± 12.1	*p*<.0001
Body weights (g)	436.2 ± 13.8^{++++}	290.0 ± 4.6^{++++}	449.0 ± 7.7^{++++}	288.7 ± 2.6^{++++}	462.8 ± 12.2^{++++}	295.2 ± 4.2^{++++}	432.4 ± 17.1^{++++}	274.5 ± 8.8^{++++}	437.8 ± 11.5^{++++}	288.0 ± 4.8^{++++}	446.3 ± 4.3^{++++}	289.5 ± 9.6^{++++}	*p*<.0001
Pituitary gland relative weights (g/kg BW)	0.03 ± 0.00^{++++}	0.05 ± 0.00^{++++}	0.03 ± 0.00^{++++}	0.05 ± 0.00^{++++}	0.03 ± 0.00^{++++}	0.06 ± 0.00^{++++}	0.03 ± 0.00^{++++}	0.06 ± 0.00^{++++}	0.03 ± 0.00^{++++}	0.06 ± 0.00^{++++}	0.03 ± 0.00^{++++}	0.06 ± 0.01^{++++}	*p*<.0001
Adrenal gland relative weights (g/kg BW)	0.13 ± 0.01	0.14 ± 0.02	0.11 ± 0.01	0.13 ± 0.02	0.08 ± 0.01^{+}	0.13 ± 0.01^{+}	0.08 ± 0.01^{+}	0.13 ± 0.01^{+}	0.09 ± 0.01^{+}	0.14 ± 0.02^{+}	0.10 ± 0.02^{++++}	0.18 ± 0.02^{++++}	*p*<.0001

Note: EDC, endocrine disrupting chemicals; BPA, bisphenol A; DEHP, di-(2-ethylhexyl) phthalate; BW, body weight. $^{+}$ $p < 0.05$, $^{++++}$ $p < 0.0001$, difference between males and females of the same treatment group, two-way ANOVA followed by uncorrected Fisher's LSD post hoc analyses.

Table S2.1. Data from Chapter 2 figures. Data are presented as mean ± SEM.

	Control		BPA (5 µg)		DEHP (5 µg)		BPA + 5 µg DEHP		DEHP (7.5 mg)		BPA + 7.5 mg DEHP	
	Males	**Females**	**Males**	**Females**	**Males**	**Females**	**Males**	**Females**	**Males**	**Females**	**Males**	**Females**
Figure 2.2 - OFT												
Center zone time	4.3 ± 1.3	1.8 ± 0.4	3.7 ± 0.4	3.4 ± 0.7	4.1 ± 1.0	3.1 ± 0.7	4.9 ± 1.2	3.5 ± 0.7	2.5 ± 0.9	2.2 ± 0.5	3.0 ± 0.7	1.0 ± 0.2
Perimeter zone entries	71.2 ± 9.7	92.5 ± 12.9	56.0 ± 6.4	86.3 ± 7.5	55.5 ± 15.4	61.3 ± 4.1	47.4 ± 13.2	71.3 ± 12.6	54.2 ± 8.5	52.3 ± 8.1	62.4 ± 8.7	62.0 ± 6.2
Ambulatory distance	1156.4 ± 65.9	1316.8 ± 167.5	1158.0 ± 100.1	1196.8 ± 153.1	1179.8 ± 187.8	1337.3 ± 165.0	1361.9 ± 152.6	1140.9 ± 185.9	1308.2 ± 169.3	941.3 ± 76.0	939.7 ± 72.2	1004.5 ± 87.1
Figure 2.3 - EPM												
OTT arm time ratio	0.35 ± 0.09	0.57 ± 0.08	0.23 ± 0.04	0.67 ± 0.08	0.43 ± 0.06	0.54 ± 0.09	0.37 ± 0.10	0.47 ± 0.09	0.59 ± 0.10	0.46 ± 0.07	0.34 ± 0.03	0.50 ± 0.09
OTT arm entries ratio	0.39 ± 0.06	0.54 ± 0.06	0.28 ± 0.03	0.58 ± 0.07	0.41 ± 0.04	0.48 ± 0.06	0.29 ± 0.03	0.44 ± 0.06	0.55 ± 0.09	0.41 ± 0.06	0.37 ± 0.04	0.44 ± 0.07
Total arm entries	14.2 ± 2.3	17.8 ± 1.5	16.2 ± 1.4	14.4 ± 1.4	20.2 ± 2.0	19.4 ± 0.6	16.2 ± 0.7	17.0 ± 1.2	18.5 ± 1.8	18.5 ± 1.5	18.7 ± 2.0	18.2 ± 1.3
Figure 2.4 - SPDB												
Burying time	36.9 ± 12.4	27.3 ± 10.8	14.8 ± 8.5	5.5 ± 2.2	0.33 ± 0.28	4.0 ± 2.4	19.9 ± 10.2	13.6 ± 6.5	1.76 ± 1.04	22.0 ± 9.8	13.7 ± 5.1	9.8 ± 5.6
Immobility time	4.2 ± 1.6	2.2 ± 0.8	6.3 ± 3.8	10.2 ± 3.8	37.4 ± 13.2	17.8 ± 5.4	6.9 ± 1.5	17.1 ± 5.3	12.2 ± 7.2	4.1 ± 1.6	2.1 ± 0.7	2.4 ± 1.0
Figure 2.5 - NOR												
Recognition index	0.58 ± 0.02	0.50 ± 0.05	0.57 ± 0.05	0.58 ± 0.02	0.65 ± 0.03	0.58 ± 0.05	0.58 ± 0.06	0.62 ± 0.05	-	-	-	-

Figure 2.6 - HPA axis

CORT	52.5 ± 8.9	313.2 ± 88.7	40.2 ± 7.1	90.2 ± 23.2	144.8 ± 53.3	290.9 ± 70.1	113.5 ± 39.1	285.1 ± 71.5	53.9 ± 14.4	55.0 ± 4.7	40.7 ± 5.5	89.3 ± 12.1
Adrenal gland relative weights	0.13 ± 0.01	0.14 ± 0.02	0.11 ± 0.01	0.13 ± 0.02	0.08 ± 0.01	0.13 ± 0.01	0.08 ± 0.01	0.13 ± 0.01	0.09 ± 0.01	0.14 ± 0.02	0.10 ± 0.01	0.18 ± 0.02

CHAPTER 3

THE DOSE-DEPENDENT EFFECTS OF PRENATAL EXPOSURE TO BISPHENOL A AND/OR DIETHYLHEXYL PHTHALATE ON BRAIN MONOAMINERGIC ACTIVITY IN MALE AND FEMALE RAT OFFSPRING [2]

[2] Kaimal et al. To be submitted to *Environmental Pollution*.

3.1. ABSTRACT

Exposures to bisphenol A (B) or diethylhexyl phthalate (D) *in utero* affect neurotransmitter systems in the brain, but the mixture effects of these EDCs are understudied. The aim of this study was to determine the dose- and sex-dependent effects of prenatal exposure to B, D, or a combination of the two on brain monoaminergic activity. Pregnant Sprague-Dawley rats were orally dosed with vehicle, B (5 µg/kg body weight (BW)/day), low-dose (LD) D (5 µg/kg BW/day), high-dose (HD) D (7.5 mg/kg BW/day), a combination of B and LD-D (B+D (LD)), or a combination of B and HD-D (B+D (HD)) on gestational days 6-21. The offspring were sacrificed in adulthood and brains were collected. Specific brain areas were analyzed for levels of monoamines and their major metabolites using high performance liquid chromatography. In the paraventricular hypothalamic nucleus, there was a marked reduction in dopamine levels in male offspring from the B, D, and B+D (HD) groups, which correlated well with their shock probe defensive burying times. Neurotransmitter changes in the prefrontal cortex and hippocampus were significant in female offspring, with D (HD) females being affected the most, followed by the B+D groups. Only neurotransmitter turnover was affected in the basolateral amygdala in both sexes in the B+D groups. Prenatal exposure to B, D, or a mixture of the two alters monoaminergic activity in a brain region-specific, sex-specific, and dose-dependent manner, which could have implications for their behavioral and neuroendocrine effects.

3.2. INTRODUCTION

Exposure to endocrine disrupting chemicals (EDCs), particularly the ubiquitous plasticizers bisphenol A (BPA) and diethylhexyl phthalate (DEHP), has been correlated with a variety of neurobehavioral disorders including mood disorders, ADHD, and autism [99, 100]. An interesting feature about these disorders is the presence of a sex bias in their prevalence, with females showing higher rates of anxiety and depression, whereas an increased proportion of males have ADHD and autism [101-103]. EDC exposure during the *in utero* period is especially concerning as these chemicals can easily cross the placental barrier in pregnant women [90, 91], which can interfere with the development of sexually dimorphic systems and produce irreversible effects on fetal neurodevelopment [86, 206, 207]. Additionally, it is highly likely that EDCs such as BPA and DEHP may be present as mixtures in the environment, or in combination with one another. The effects of EDC mixtures are known to be more elusive and differ from those of individual EDCs [12, 13]. Currently, the extent to which EDC mixtures affect neurobiological mechanisms following prenatal exposure is unknown.

Monoamines including norepinephrine (NE), dopamine (DA), and serotonin (5-HT) have long been implicated in the development and progression of anxiety and mood disorders [208-210], but the impact of prenatal BPA and/or DEHP exposure on monoaminergic activity in the brain is unclear. Perinatal exposure to BPA can dose- and sex-dependently modify levels of NE, 5-HT and its metabolite 5-hydroxyindoleacetic acid (5-HIAA), as well as DA and its metabolite 3,4-dihydroxyphenylacetic acid (DOPAC) in brain areas including the forebrain and hypothalamus in male and female rodents [164, 211, 212]. Specifically, BPA exposure can interfere with DA and 5-HT synthesis in the prefrontal cortex (PFC) [213], and reduce 5-HT levels in the hippocampus (HC) [214] of adult male offspring. 6-month-old adult female

offspring show decreased DOPAC/DA ratio, or dopamine turnover, in the brain following perinatal treatment with BPA doses as low as 10 µg/kg [215]. Additionally, perinatal BPA exposure can disrupt sexual dimorphism of the noradrenergic system [137, 216].

On the other hand, much less is known about the repercussions of prenatal exposures to DEHP alone and BPA + DEHP combinations on neurotransmitter systems. Gestational DEHP at both low (20 µg/kg) and high doses (750 mg/kg) have been found to transgenerationally affect DA receptor expression levels in the amygdala in a sex-specific manner in mice offspring [161]. However, the potential changes to other neurotransmitter systems in offspring with *in utero* low-dose DEHP exposure are largely unexplored. A recent study discovered reductions in DA and 5-HT in male offspring following perinatal exposures to high-dose BPA (50 mg/kg) or DEHP (30 mg/kg), both independently and in combination with one another [217]. This was coupled with alterations in the reuptake and biotransformation mechanisms of these neurotransmitters. It will be beneficial to further determine the ways in which monoaminergic activity is affected following prenatal exposure to low doses of these individual EDCs, as well as their mixtures, to get a clearer picture of the impact of EDC exposure on the brain.

The goal of the present study was to create a profile of brain neurotransmitter and metabolite concentrations in adult male and female offspring with prenatal exposure to BPA, low-dose DEHP, or high-dose DEHP either individually or in combination. This study is a continuation of a prior study from our lab focusing on the behavioral effects of these exposures (Kaimal et al. (2022) – manuscript in review). Our hypothesis was that prenatal EDC exposure, particularly the combination of BPA+DEHP, would lead to sex-specific and dose-dependent alterations in monoaminergic activity.

3.3. MATERIALS AND METHODS

Animals

Adult female Sprague-Dawley rats obtained from Envigo (Indianapolis, IN) were housed at the University of Georgia (UGA). They were housed in light- (12:12 light-dark cycle) and temperature-controlled rooms (23.2 ± 2°C, 50 ± 20% relative humidity), food and water provided *ad libitum*. The rats were fed Pico Lab Rodent Diet 20 (LabDiet). All animals were housed in polycarbonate cages with corn cob bedding. Prior to mating, vaginal cytology was performed for 10 consecutive days on each of the female breeders to track their individual estrous cycles. When females were in proestrus, they were randomly assigned one male and were co-housed for one day. Presence of a vaginal plug confirmed that mating had occurred. Gestational day 0 represented the day of copulation. Experimental protocols followed the National Institutes of Health's *Guide for the Care and Use of Laboratory Animals* approved by the Institutional Animal Care and Use Committee (IACUC) at the University of Georgia.

EDC exposure paradigm

The experimental design is demonstrated in Figure 3.1. Daily oral dosing of dams with EDCs or vehicle began on gestational day 6. Each dam was randomly assigned to one of 6 different treatment groups: control (10 µl Phosphate Buffered Saline or PBS; n=7), BPA (5 µg/kg BW/day; n=7), low-dose DEHP (5 µg/kg BW/day; n=6), high-dose DEHP (7.5 mg/kg BW/day; n=6), a combination of BPA and low-dose DEHP (5 µg/kg/day of BPA + 5 µg/kg/day of DEHP; n=6), and a combination of BPA and high-dose DEHP (5 µg/kg/day of BPA + 7.5 mg/kg/day of DEHP; n=7) (see Table 3.1). The dam was considered the experimental unit. A total of 76 offspring were used for the neurotransmitter analyses in this study (control-males n = 7, control-females n = 7, BPA-males n = 7, BPA-females n = 7, DEHP (LD)-males n = 6, DEHP

(LD)-females *n* = 6, DEHP (HD)-males *n* = 6, DEHP (HD)-females *n* = 6, BPA+DEHP (LD)-males *n* = 5, BPA+DEHP (LD)-females *n* = 6, BPA+DEHP (HD)-males *n* = 7, BPA+DEHP (HD)-females *n* = 6) (see Table 3.1).

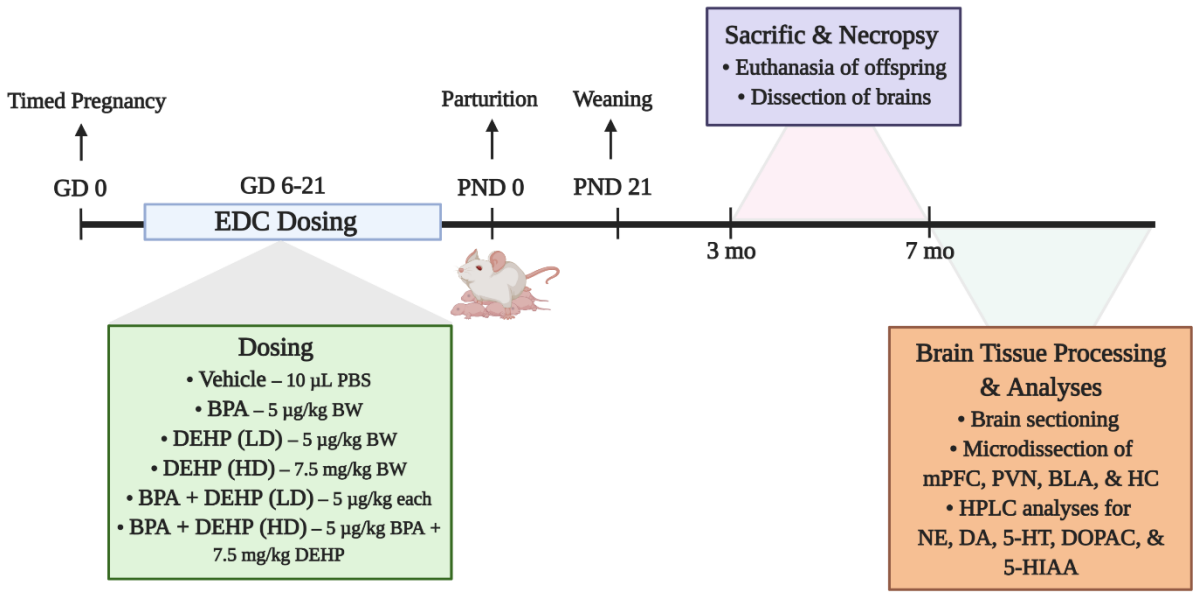

Figure 3.1. Summary of the experimental design of the study. Pregnant Sprague-Dawley dams were orally dosed daily from gestational days (GD) 6-21 with vehicle (Control) (10 µL PBS; *n*=7), BPA (5 µg/kg/day; *n*=7), low-dose (LD) DEHP (5 µg/kg/day; *n*=6), high-dose (HD) DEHP (7.5 mg/kg/day; *n*=6), a mixture of BPA + LD DEHP (5 µg/kg/day of BPA + 5 µg/kg/day of DEHP; n=6), or a mixture of BPA + HD DEHP (5 µg/kg/day of BPA + 7.5 mg/kg/day of DEHP; n=7). Male and female offspring were euthanized in adulthood. Brains were dissected, sectioned, and micro-dissected for mPFC, PVN, BLA, and HC tissues. Tissues were then

analyzed for monoamines and major metabolites. Experimental design schematic was created using Biorender.com.

Note: EDC, endocrine disrupting chemical; PBS, Phosphate Buffered Saline; BPA, Bisphenol A; DEHP, diethylhexyl phthalate; BW, body weight; LD, low-dose; HD, high-dose; mPFC, medial prefrontal cortex; PVN, paraventricular nucleus; BLA, basolateral amygdala; HC, hippocampus; HPLC, high performance liquid chromatography; NE, norepinephrine; DA, dopamine; 5-HT, serotonin; DOPAC, 3,4-dihydroxyphenylacetic acid; 5-HIAA, 5-hydroxyindoleacetic acid.

BPA (Catalog No. 239658; Lot MKBH2096V) and DEHP (Catalog No. 36735; Lot BCBR8079V) were obtained from Sigma Aldrich (St. Louis, MO). Stock solutions were made in dimethylsulfoxide (DMSO) to obtain complete dissolution. Doses were calculated daily based on BW and mixed with 20µl PBS for oral dosing, which occurred daily from gestational days 6-21. Dams were administered the vehicle or EDC treatment into the oral cavity using a micropipette to avoid causing local irritation to the gastrointestinal tract and potential stress to the pregnant dam. The BPA dose was selected because it is significantly lower than the Environmental Protection Agency (EPA) recommended no-observed-adverse-effect-level (NOAEL) dose of 5 mg/kg/day [115], as well as 10-fold below the tolerable daily intake (TDI) dose of 50 µg/kg/day [114]. The high dose of DEHP used in our study is higher than the established NOAEL dose of 4.8 mg/kg/day [130], whereas the low DEHP dose is significantly lower than this. Additionally, the low dose of DEHP used lies within the range of the typical daily intake of DEHP in adult humans (0.5-25 µg/kg/day) [127], and is well below the EPA reference dose of 20 µg/kg/day [128].

Tissue collection and preparation

Adult female offspring in estrus (as confirmed by vaginal cytology) and male offspring were euthanized by rapid decapitation. Brains were dissected and stored at -80°C for further processing.

Brain sectioning and microdissection

A cryostat (Slee, London, UK) maintained at -10°C was used to section brains at 300 µm thickness. Following this, the medial PFC (mPFC), paraventricular nucleus of the hypothalamus (PVN), basolateral amygdala (BLA), and ventral subdivision of the HC were micro-dissected on a cold stage using the Palkovits' microdissection procedure and a stereotaxic brain atlas for reference [218]. Microdissections were completed using a 500 µm diameter punch. All brain punches were stored at -80°C until further analyses.

Neurotransmitter analysis by HPLC-EC

HPLC-EC was used to analyze brain punches for NE, DA, DOPAC, 5-HT, and 5-HIAA as previously described [219]. Brain punches were briefly homogenized in 0.05 M perchloric acid on ice and an aliquot was used for protein estimation (MicroBCA assay, Pierce, Rockford, IL). The remaining homogenate was centrifuged at 18,000 × g for 8 min at 4°C. The supernatant was injected with an internal standard (dihydroxybenzylamine, 0.05 M) into the autoinjector for HPLC analysis. The HPLC-EC system comprised of a 5-µm ODS reverse phase C-18 column (Phenomenex, Torrance, CA), a SIL-20AC autoinjector, a CTO-20AC column oven (Shimadzu, Columbia, MD) maintained at 37°C and a LC-4C detector (Bioanalytical Systems, West Lafayette, IN). The flow rate of the mobile phase was maintained at 1.8 ml/min using a LC-20AD pump (Shimadzu, Columbia, MD). Chromatograms were analyzed for neurotransmitter concentrations using the Class VP software v 7.2 (Shimadzu, Columbia, MD). Neurotransmitter

concentrations in tissue samples were expressed as pg/µg of protein. Protein levels in tissue punches were measured using the micro bicinchoninic acid assay (Pierce, Rockford, IL). Samples were assayed in duplicate according to the manufacturer's protocol. Besides actual neurotransmitter values, turnover rates for DA and 5-HT were obtained by dividing the concentrations of the metabolites by the concentration of the parent neurotransmitter.

Statistical analysis

Prism 9.0.0 (GraphPad, Inc.) software was used to perform statistical analyses. A two-way ANOVA (EDC exposure × sex) was constructed for each neurotransmitter as the dependent variable, and a one-way ANOVA was also conducted for male PVN DA levels using EDC exposure as the dependent variable. Interaction effects between EDC exposure and sex were also assessed. Sex differences within EDC groups were measured using uncorrected Fisher's LSD post-hoc test. EDC effects within males and females separately were analyzed using Tukey's multiple comparisons post-hoc test. In addition, a simple linear regression was performed to determine any correlations between PVN DA levels and SPDB burying times in male offspring. P-value < 0.05 was considered to indicate a statistically significant difference. Data is expressed as mean ± standard error of mean (SEM).

3.4. RESULTS

Medial prefrontal cortex

In the mPFC (mean ± SEM; pg/µg protein), DEHP (HD) female offspring demonstrated robust increases in NE (27.9 ± 8.7; $p = 0.0210$) (Figure 3.2A), 5-HT (6.6 ± 2.0; $p = 0.0080$) (Figure 3.2B), and DA (30.4 ± 7.6; $p = 0.0020$) (Figure 3.2C) compared to control females. Sex

differences were also identified in all three monoamines within DEHP (HD) males and females ($p < 0.0001$), and within B+D (HD) males and females in DA only ($p = 0.0093$).

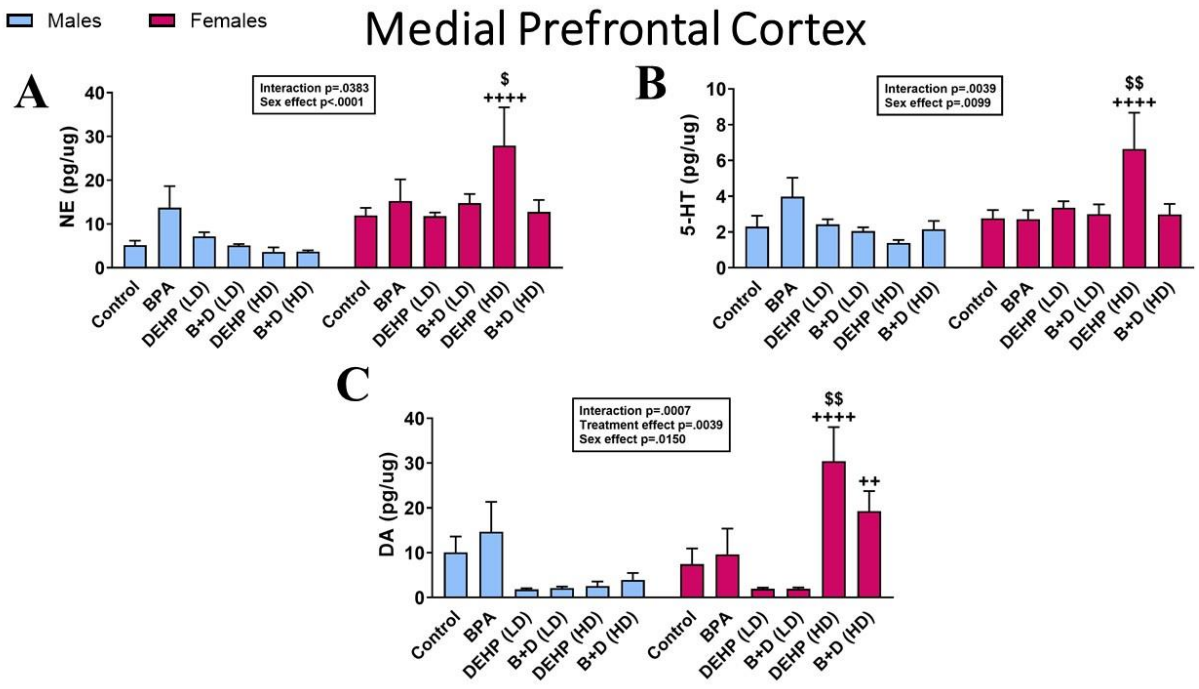

Figure 3.2. Effects of prenatal exposure to vehicle or EDCs on monoamine levels in the medial prefrontal cortex (mPFC) of female and male rat offspring. (*A*) Norepinephrine (NE), (*B*) serotonin (5-HT), and (*C*) dopamine (DA) concentrations (mean ± SEM; pg/µg protein) in the mPFC are shown in the figure. $^{\$}p<0.05$, $^{\$\$}p<0.01$, comparison between control and EDC-exposed female offspring. $^{++}p<0.01$, $^{+++}p<0.001$, $^{++++}p<0.0001$, comparison between males and females of the same treatment group. Error bars represent the standard error of the mean (SEM).

In terms of DOPAC/DA ratios (mean ± SEM), DEHP (LD) male offspring had significantly higher DA turnover (1.1 ± 0.2) compared to control male offspring (0.4 ± 0.1; $p = 0.0117$). Additionally, B+D (HD) male offspring had increased DOPAC/DA ratio relative to their female counterparts ($p = 0.0026$; Figure 3.3A). Serotonin turnover, as depicted by the 5-HIAA/5-HT ratio, was markedly increased in the mPFC in HD female offspring (Figure 3.3B).

Female offspring in the DEHP (HD) (19.2 ± 4.0; $p < 0.0001$) and B+D (HD) (13.1 ± 3.8; $p = 0.0140$) groups had significantly higher 5-HT turnover rates compared to control females (3.5 ± 0.9) and also their male counterparts (DEHP (HD): $p < 0.0001$; B+D (HD): $p = 0.0004$) (Figure 3.3 and Table 3.2).

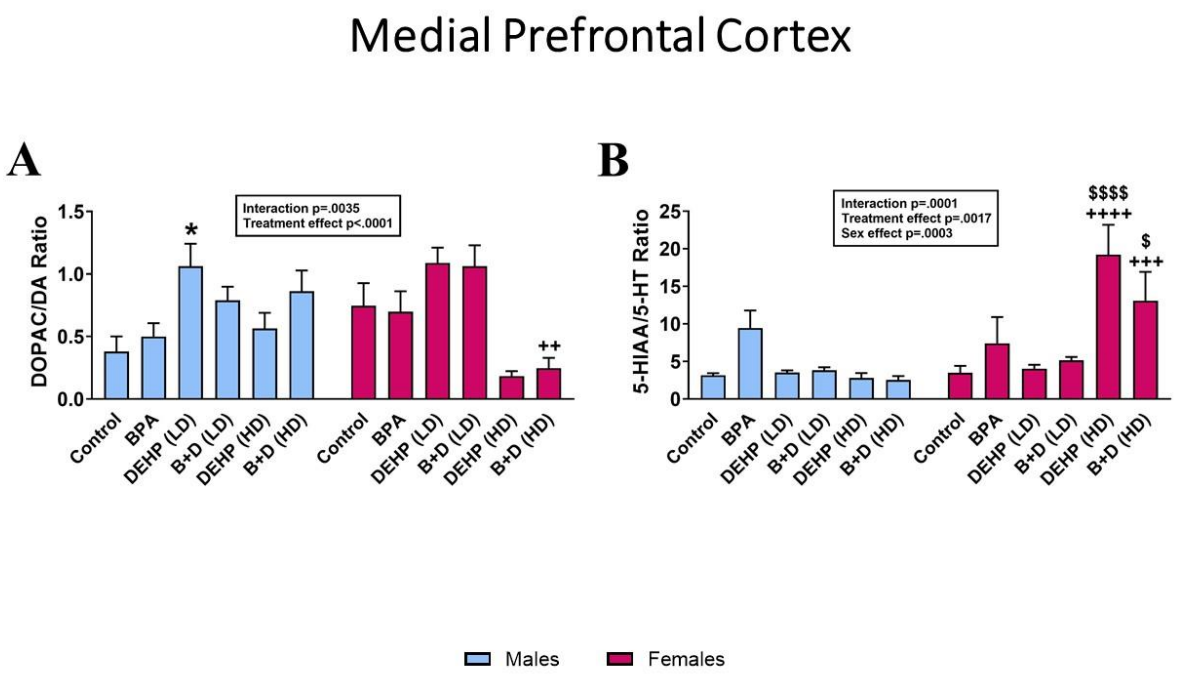

Figure 3.3. Effects of prenatal exposure to vehicle or EDCs on monoamine turnover ratios in the medial prefrontal cortex (mPFC) of male and female rat offspring. (*A*) DOPAC/DA and (*B*) 5-HIAA/5-HT ratios (mean ± SEM) in the mPFC are shown in the figure. $^{*}p<0.05$, comparison between control and EDC-exposed male offspring. $^{\$}p<0.05$, $^{\$\$\$\$}p<0.0001$, comparison between control and EDC-exposed female offspring. $^{++}p<0.01$, $^{+++}p<0.001$, $^{++++}p<0.0001$, comparison between males and females of the same treatment group. Error bars represent the standard error of the mean (SEM).

Minor changes were discovered in DOPAC levels within the mPFC, which are displayed in Table 3.2. Sex differences were observed in the BPA and DEHP (HD) groups, with the two

groups showing opposite differences between males and females. In 5-HIAA levels, DEHP (HD) female offspring once again had higher levels than control female offspring ($p < 0.0001$). Finally, females in both HD groups showed significant elevations in 5-HIAA metabolite levels compared to their male counterparts.

Paraventricular nucleus of the hypothalamus

Our data on DA levels (mean ± SEM; pg/µg protein) was especially remarkable (Figure 3.4A). One-way ANOVA results in particular revealed that male offspring in the BPA (3.6 ± 0.3; $p = 0.0093$), DEHP (LD) (4.4 ± 0.6; $p = 0.0323$), DEHP (HD) (4.0 ± 1.6; $p = 0.0202$), and B+D (HD) (4.3 ± 0.9; $p = 0.0205$) groups had significantly lower PVN DA levels than controls (12.1 ± 3.2). Furthermore, a simple linear regression determined a significant moderate positive correlation ($R = 0.5008$, $p = 0.0019$) between PVN DA levels and amount of time spent burying in the SPDB (Kaimal et al. (2022) – manuscript in review) in male offspring (Figure 3.4B), correlating the behaviors of these offspring with their neurotransmitter levels. On the contrary, 2-way ANOVA results revealed that high-dose DEHP ($p = 0.0528$) or B+D ($p = 0.0033$) treatments increased PVN DA levels in female offspring compared to their control counterparts (Table 3.2).

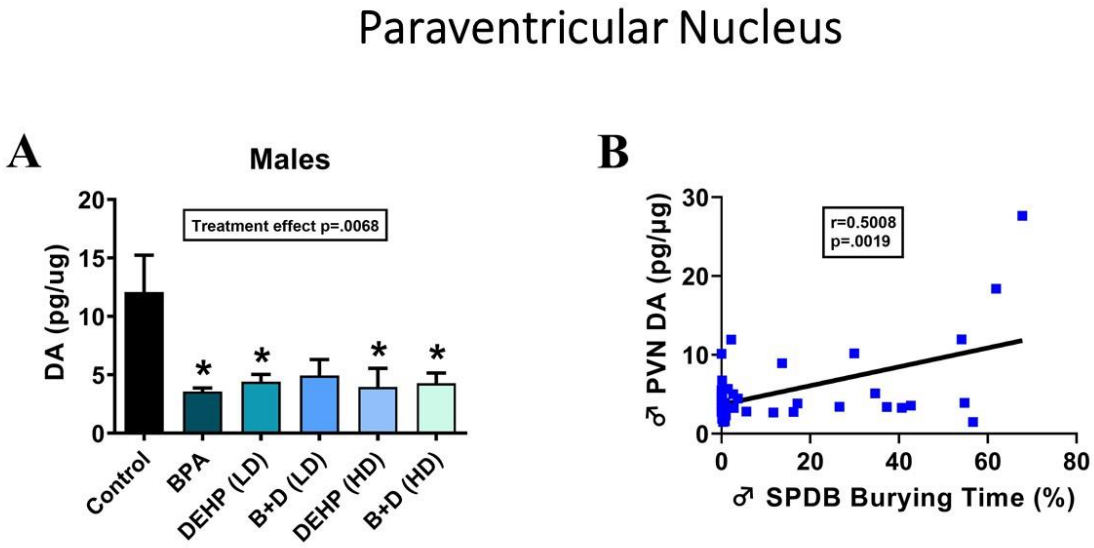

Figure 3.4. Effects of prenatal exposure to vehicle or EDCs on dopamine levels in the paraventricular nucleus (PVN) and defensive behavior of male rat offspring. (*A*) DA concentrations (mean ± SEM; pg/µg protein) in the PVN and (*B*) correlation between PVN DA levels and time spent burying in the Shock Probe Defensive Burying (SPDB) in male offspring are shown in the figure. $^{*}p<0.05$, comparison between control and EDC-exposed male offspring. Error bars represent the standard error of the mean (SEM).

Various EDC- and/or sex-dependent effects were observed in the other monoamines, metabolites, and turnover ratios in the PVN (Figure 3.5 and Table 3.2). DEHP (HD) female offspring, in particular, exhibited a robust hyperactivity in levels of NE (74.1 ± 22.7 vs. 15.4 ± 2.5; $p = 0.0001$) (Figure 3.5A), 5-HT (6.9 ± 2.5 vs. 2.2 ± 0.4; $p = 0.0490$) (Figure 3.5B), DOPAC (6.0 ± 2.2 vs. 1.4 ± 0.4; $p = 0.0009$), 5-HIAA (103.6 ± 42.4 vs. 11.0 ± 1.8; $p = 0.0003$), as well as 5-HT turnover (17.3 ± 3.7 vs. 5.7 ± 0.9; $p < 0.0001$) compared to their control counterparts. B+D (HD) females also appeared to mimic this response to a greater extent, but in 5-HT (8.0 ± 2.8 vs.

2.2 ± 0.4; p = 0.0072) and DA (4.3 ± 0.9 vs. 12.1 ± 3.2; p = 0.0033) levels only. Finally, BPA male offspring (p = 0.0504) demonstrated increased 5-HIAA/5-HT ratios than their control counterparts (Figure 3.5C).

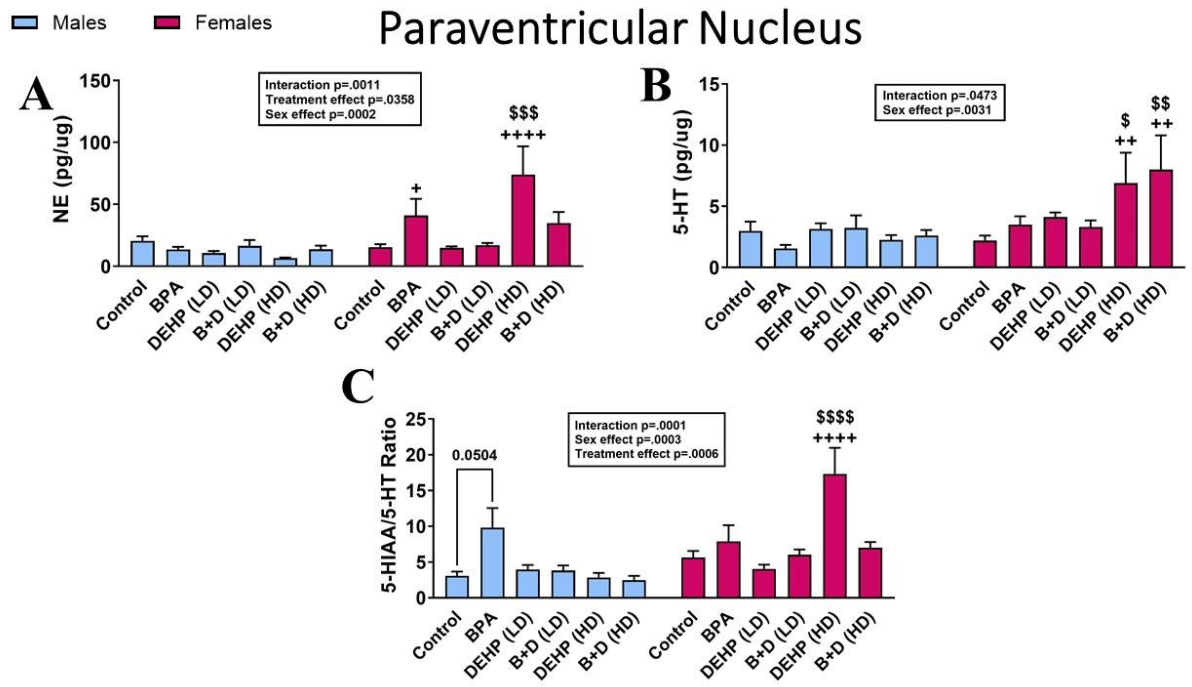

Figure 3.5. Effects of prenatal exposure to vehicle or EDCs on monoamine levels and monoamine turnover ratios in the paraventricular nucleus (PVN) of female and male rat offspring. (*A*) Norepinephrine (NE) levels (mean ± SEM; pg/µg protein), (*B*) serotonin (5-HT) levels (mean ± SEM; pg/µg protein), and (*C*) 5-HIAA/5-HT ratios (mean ± SEM) in the PVN are shown in the figure. $^{\$}p<0.05$, $^{\$\$}p<0.01$, $^{\$\$\$}p<0.001$, $^{\$\$\$\$}p<0.0001$, comparison between control and EDC-exposed female offspring. $^{+}p<0.05$, $^{++}p<0.01$, $^{++++}p<0.0001$, comparison between males and females of the same treatment group. Error bars represent the standard error of the mean (SEM).

Basolateral amygdala

Interestingly, no effects of EDC exposure were observed in monoamines within the BLA (Table 3.2). However, strong sex differences were uncovered in this region, particularly in NE and DA levels. A majority of the EDC groups displayed sex differences in BLA DA concentrations. DEHP (LD) male and female offspring were an exception, with very similar DA levels to one another, suggestive of an abolishment of sex differences. EDC exposure appeared to impact monoamine turnover rates (mean ± SEM) in the BLA instead (Figure 3.6). In particular, male offspring with low-dose B+D treatment (1.0 ± 0.2 vs. 0.4 ± 0.0; $p = 0.0137$) whereas female offspring with the corresponding high-dose treatment (1.0 ± 0.1 vs. 0.5 ± 0.0; $p = 0.0323$) had significantly higher DOPAC/DA ratios than their control counterparts, implying a sex- and dose-dependent effect. Sex differences were also generated in these 2 groups, with both groups showing a reversal of sex differences compared to each other. Finally, metabolite levels revealed that once again, HD DEHP and B+D female offspring had elevated levels of both DOPAC and 5-HIAA compared to their male counterparts and control females (Table 3.2).

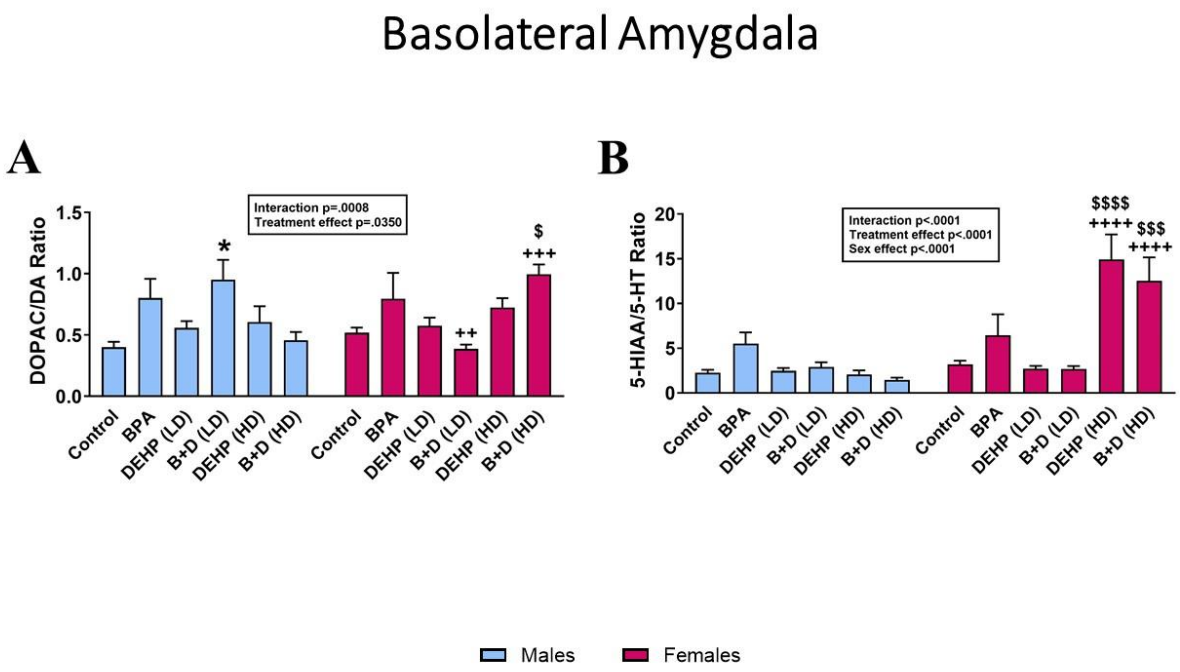

Figure 3.6. Effects of prenatal exposure to vehicle or EDCs on monoamine turnover ratios in the basolateral amygdala (BLA) of male and female rat offspring. (*A*) DOPAC/DA and (*B*) 5-HIAA/5-HT ratios (mean ± SEM) in the BLA are shown in the figure. $^{*}p<0.05$, comparison between control and EDC-exposed male offspring. $^{\$}p<0.05$, $^{\$\$\$}p<0.001$, $^{\$\$\$\$}p<0.0001$, comparison between control and EDC-exposed female offspring. $^{++}p<0.01$, $^{+++}p<0.001$, $^{++++}p<0.0001$, comparison between males and females of the same treatment group. Error bars represent the standard error of the mean (SEM).

Hippocampus

We found a pronounced increase in hippocampal NE levels (mean ± SEM; pg/µg protein) (Figure 3.7A) in B+D (LD) female offspring in comparison with control females (20.5 ± 3.6 vs. 8.8 ± 1.3; $p < 0.0001$). Furthermore, sex differences were observed in NE levels in all groups, but these were abolished in DEHP (LD) offspring again. DA levels in the HC were, not

surprisingly, substantially increased in DEHP (HD) female offspring compared to their control counterparts (13.3 ± 0.9 vs. 3.1 ± 0.9; $p < 0.0001$). Apart from that, some minor sex differences were observed in 5-HT, metabolites, and DOPAC/DA ratio, which are detailed in Table 3.2.

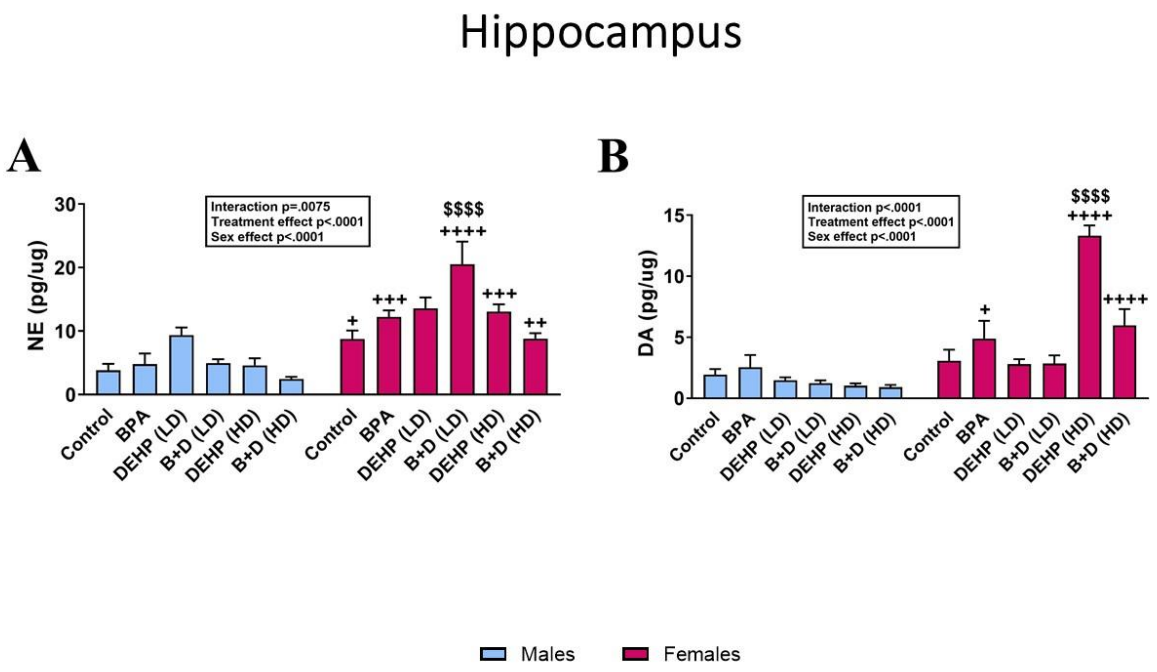

Figure 3.7. Effects of prenatal exposure to vehicle or EDCs on monoamine levels in the hippocampus (HC) of female and male rat offspring. (*A*) Norepinephrine (NE) and (*B*) dopamine (DA) concentrations (mean ± SEM; pg/µg protein) in the HC are shown in the figure. $^{\$\$\$\$}p<0.0001$, comparison between control and EDC-exposed female offspring. $^{+}p<0.05$, $^{++}p<0.01$, $^{+++}p<0.001$, $^{++++}p<0.0001$, comparison between males and females of the same treatment group. Error bars represent the standard error of the mean (SEM).

3.5. DISCUSSION

The objective of the present study was to determine the effects on brain NE, DA, 5-HT, DOPAC, and 5-HIAA levels following prenatal treatment with varying doses of EDCs either

individually or in combination. One of the major findings of the present study was the reduction in DA concentrations in the PVN within male offspring from all EDC groups, except for B+D (LD). Especially fascinating was the fact that this appeared to parallel the reduced burying time we previously noted in the shock probe defensive burying test (SPDB) in male offspring from the very same EDC groups (Kaimal et al. (2022) – manuscript in review). When we investigated this further, a significant moderate positive correlation was identified between SPDB burying time and DA levels in the PVN of male offspring, suggesting that reduced burying is moderately correlated with lower levels of PVN DA.

Reduced burying in the SPDB is often interpreted as a maladaptive decrease in active coping abilities [189]. Our findings imply that diminished PVN DA may underlie the reduced active coping defense strategies we found in males prenatally exposed to BPA, DEHP (LD), DEHP (HD), and B+D (HD). This is partially in line with a previous study that discovered reduced SPDB burying in rats treated with an atypical antipsychotic known as perospirone [220]. This particular drug serves as a dopamine D_2 and 5-HT_2 receptor antagonist, thereby reducing species-specific defensive burying in this test via modulations in dopaminergic and serotonergic activity. Although we did not observe any changes in male 5-HT levels, the lower DA levels we found in the PVN accompanied by deficits in active coping appear to be a sex-specific EDC mechanism of action that has not been identified before.

Alterations in brain DA concentrations are commonly linked with a variety of stress-related psychopathologies, including major depressive disorder [221], posttraumatic stress disorder [222], and substance use disorder [223]. Furthermore, studies have established that an increase in brain dopaminergic activity occurs in response to acute physiological and psychological stressors [224]. In contrast, our study uncovered reduced DA concentrations in the

male PVN, indicative of aberrant responses within the brain as a result of prenatal EDC treatment. Our findings imply potential dysfunctions within the mesocortical and/or mesolimbic dopaminergic pathways, and may represent deficits in motivation or cognition instead. This particular outcome requires further investigation.

Within the control group, sex differences were observed in the BLA and HC. NE concentrations were higher in female offspring in the BLA, regardless of prenatal treatment, compared to their male counterparts. We can conclude that this is a sex-specific effect unrelated to EDC exposure. Control and EDC-treated female offspring also showed higher NE than male offspring in the HC. The only exception to this was DEHP (LD) offspring, implying that DEHP (LD) in particular may eliminate typical sex differences in hippocampal NE. Additionally, hippocampal 5-HT, 5-HIAA, and DOPAC levels were also higher in control females than the males, without affecting their respective turnover ratios. More significantly, sex differences that were not observed in control offspring were induced in most EDC groups, leading to concerns about exposure to these chemicals during the gestational period.

In addition, male offspring with prenatal BPA treatment also demonstrated a trend for greater serotonin turnover in the PVN, as represented by an increase in 5-HIAA/5-HT ratio. A prior study identified reduced 5-HT and 5-HIAA levels, but no changes in 5-HT turnover, in the HC of male mice with perinatal BPA exposure at 10 mg/kg. This was coupled with a depressive behavioral phenotype at this dose of BPA as well as a lower dose of 10 µg/kg [214]. Our study is the first to our knowledge to demonstrate that treatment with an even lower dose of BPA (5 µg/kg) impacts 5-HT turnover rates in the PVN instead. Hence, one potential mechanism of action of BPA involves the specific targeting of serotonergic and dopaminergic activity in the male PVN. Elevated 5-HT turnover in the brain is associated with major depressive disorder

[225] and panic disorder [226] in humans. Moreover, a lower preference for active coping strategies is additionally observed in patients with mood disorders [227-229]. Our neurotransmitter results from this study reveal potential therapeutic targets for treating mood and anxiety disorders, in addition to broadening the knowledge on prenatal EDC exposures.

5 µg/kg of DEHP exposure in our study led to increased dopamine turnover (DOPAC/DA ratio) in the mPFC of male offspring, along with lower PVN DA. Our prior experiments found that these DEHP (LD) male offspring exhibited heightened immobility in the SPDB, and they also showed a trend for elevated serum corticosterone (CORT) levels (Kaimal et al. (2022) – manuscript in review). Consistent with our study, acute stress is associated with increases in DOPAC/DA ratio in the mPFC as well as stress-induced immobility responses in male rats [230, 231]. Furthermore, infusions of corticotropin-releasing factor (CRF) can also increase PFC ratios of DOPAC/DA in male mice [232]. The results from our experiments support and expand these findings by showing that prenatal DEHP exposure at a low dose can exacerbate these effects in male offspring.

DOPAC is a major metabolite of DA, in addition to 3-methoxytyramine (3-MT) and homovanillic acid (HVA) [233]. In the DA life cycle, once DA is synthesized and released into the synaptic cleft to bind to and activate DA receptors, it undergoes reuptake mechanisms back into the presynaptic cell. It can then be repackaged into vesicles for future release or broken down by enzymes into metabolites [233, 234]. Since DOPAC is formed from the catalysis of DA via monoamine oxidase (MAO) [233], it is reasonable to speculate that changes in MAO activity may have led to the elevated DA turnover we discovered in DEHP (LD) males. A very recent study discovered no changes in MAO activity in male offspring with perinatal high-dose DEHP treatment (30 mg/kg) [217], but a reduction in dopamine transporter levels instead. Future

studies should explore the effects of low-dose DEHP on DA reuptake and metabolism mechanisms.

Other potential mechanisms of action of DEHP to consider are mesocortical dopaminergic effects and epigenetic modifications. The mesocortical system is one of several dopaminergic systems present throughout the brain and consists of dopaminergic neurons projecting from the ventral tegmental area (VTA) to the PFC [234]. Partial loss of mPFC DA during early postnatal development via electrolytic lesions of the VTA or intraventricular infusions of the neurotoxic 6-hydroxydopamine is associated with increased DA turnover in the mPFC of adult rats [235-237]. However, male offspring in the F3 generation of mice with prenatal DEHP exposure show upregulated dopamine receptor 1 gene expression levels in the amygdala instead [161]. Prenatal exposure to DEHP can produce epigenetic alterations [202, 203, 238], which can lead to changes in gene expression that may persist throughout life. Therefore, low-dose DEHP exposure during the prenatal period may directly or indirectly lead to the loss of DA neurons in the brain or even induce epigenetic and transgenerational effects, resulting in behavioral and dopaminergic alterations in adult offspring.

Male and female offspring with high-dose DEHP treatment showed vastly different effects. The males only showed dopaminergic reductions in the PVN, whereas the females had substantially greater concentrations of monoamines, metabolites, or turnover rates in all brain regions examined. The results pertaining to monoaminergic activity in DEHP (HD) female offspring are especially fascinating because these offspring did not previously demonstrate any robust alterations in behavior (Kaimal et al. (2022) – manuscript in review). Therefore, enhanced brain monoamine signaling and a lack of behavioral changes may suggest a neuroprotective effect of this dose of DEHP in female offspring. Consistent with this theory, studies have

demonstrated that females with postnatal DEHP treatment at 1-20 mg/kg show enhanced brain functioning, specifically in the HC, in response to DEHP exposure, while their male counterparts display the opposite or no effects [239, 240]. Hence, females may exhibit resilience following DEHP exposure, and our study further indicates that this effect is observable with prenatal exposure as well.

Furthermore, our DEHP (HD) female offspring had significantly reduced circulating levels of CORT, potentially indicating an antianxiety effect [193, 241]. Studies have confirmed lower post-stress CORT levels in female rats with lactational DEHP treatment (10 mg/kg) [242]. Lower CORT levels have also been observed transgenerationally in female mice offspring three generations after F0 mice were prenatally treated with DEHP (150 mg/kg) [162]. The results from our studies illustrate that prenatal treatment with 7 mg/kg of DEHP leads to alterations in brain monoamine signaling and HPA axis activity.

Treatment with BPA and DEHP in combination increased DA turnover in the BLA in males treated with the low dose. The literature reveals that alterations in DA turnover in the amygdala are implicated in stress reactivity. Decreased DA turnover in the amygdala is found in rats displaying a depressive behavioral phenotype [243]. Conversely, elevated amygdaloid DA turnover has been discovered in rats exposed to early life stress [244], as well as in rats with unilateral left brain lesions of the mesocortical dopaminergic system, which is correlated with increased stress vulnerability [245]. Furthermore, perinatal BPA exposure at 10 or 100 µg/kg can reduce whole brain DOPAC/DA ratio in adult female mice offspring [215], whereas the effects of DEHP on the dopaminergic system are understudied. Our results reveal that prenatal treatment with a combination of low-dose BPA and DEHP in male rats does not alter behavior, but does affect DA neurotransmission specifically in the BLA.

B+D (LD) female offspring, on the other hand, had increased hippocampal NE, whereas females with individual BPA or DEHP (LD) treatment showed no changes in monoaminergic activity. In terms of behavior, B+D (LD) females engaged in increased immobility in the SPDB (Kaimal et al. (2022) – manuscript in review), indicating a strong preference for passive coping. Immobilization stress has been demonstrated to enhance NE levels in the HC, corroborating our findings [246]. Moreover, NE is also released in the hippocampus following novelty exposure and arousal, when the locus coeruleus-noradrenergic system is activated [247]. In addition to increased passive coping, our B+D (LD) females displayed intriguing behavioral effects because they also showed enhanced center zone exploration in the OFT. It is possible that the HC NE levels were elevated in response to novelty, which mediated the diverse behavioral effects observed.

In the B+D (HD) group, male offspring only demonstrated a reduction in PVN DA, while female offspring showed higher concentrations of both DA and 5-HT in the PVN, but elevated turnover of these monoamines in the BLA and of 5-HT in the mPFC. Interestingly, these female offspring did not show any robust changes in behavior based on our previous experiments (Kaimal et al. (2022) – manuscript in review). Overall, B+D (HD) females displayed vast similarities with DEHP (HD) females in their neurobehavioral effects. This suggests that 7 mg/kg of DEHP individually and in combination with 5 µg/kg of BPA may have common mechanisms of action in female offspring. More precisely, when the two chemicals are combined at these respective doses, the effects of DEHP surpass those of BPA in female offspring, possibly as a result of the higher dose of DEHP canceling out the effects of the lower BPA dose.

To summarize, the findings of this study uncovered dose- and sex-dependent alterations in stress-regulating brain regions including the mPFC, PVN, BLA, and HC. Male offspring from

all treatment groups, with the exception of B+D (LD), had reduced DA in the PVN. In contrast, female offspring exposed to 7.5 mg/kg of DEHP alone and in combination with BPA had substantially enhanced monoaminergic activity, possibly validating a neuroprotective role of DEHP treatment in females. 5 µg/kg of BPA and/or DEHP increased monoamine turnover in a brain region-specific manner in male offspring only. Finally, B+D exposure impacted offspring in a sex- and dose-dependent manner, with female offspring exposed to this mixture at the high dose being affected more and demonstrating similarities with DEHP (HD) females. Altogether, these findings fill in some of the gaps in EDC research, specifically in terms of EDC mixture effects, neurobiological mechanisms, and sex differences.

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Table 3.1. Sample sizes of control or EDC-treated dams, and their male and female offspring.

	Control	BPA	DEHP (5 µg)	DEHP (7.5 mg)	BPA+DEHP (5 µg)	BPA+DEHP (7.5 mg)
Dams	7	7	6	6	6	7
Offspring						
Males	7	7	6	6	5	7
Females	7	7	6	6	6	6

Note: BPA, bisphenol A; DEHP; diethylhexyl phthalate; EDC, endocrine disrupting chemical. Measures were obtained from dams and offspring treated with vehicle (Control), BPA (5 µg/Kg BW), low-dose DEHP (5 µg/Kg BW), high-dose DEHP (7.5 mg/Kg BW), BPA + low-dose DEHP (5 µg/Kg BW BPA + 5 µg/Kg BW DEHP), or BPA + high-dose DEHP (5 µg/Kg BW BPA + 7.5 mg/Kg BW DEHP).

Table 3.2. Neurotransmitter data of male and female offspring following low-dose (5 µg) and/or high-dose (7.5 mg) prenatal EDC exposure.

Neurotransmitter	Control		BPA (5 µg)		DEHP (5 µg)		BPA + 5 µg DEHP		DEHP (7.5 mg)		BPA + 7.5 mg DEHP		Sex Effect ANOVA *p-value*
	Male	**Female**	**Male**	**Female**	**Male**	**Female**	**Male**	**Female**	**Male**	**Female**	**Male**	**Female**	
Medial Prefrontal Cortex													
DOPAC (pg/µg)	2.0 ± 0.5	1.9 ± 0.2	4.7 ± 1.7 $^{+}$	2.3 ± 0.3 $^{+}$	1.6 ± 0.1	2.0 ± 0.2	1.7 ± 0.3	2.1 ± 0.7	1.2 ± 0.4 $^{++}$	4.5 ± 0.6 $^{++}$	2.4 ± 0.7	2.9 ± 0.5	NS
5-HIAA (pg/µg)	7.1 ± 1.8	8.0 ± 1.9	10.5 ± 2.2	32.0 ± 17.1	7.5 ± 0.6	11.3 ± 0.7	7.1 ± 0.9	13.8 ± 1.8	6.7 ± 3.0^{++++}	109.2 ± 28.0$^{\$\$\$\$,++++}$	4.1 ± 0.3^{+}	37.1 ± 13.2^{+}	p<.0001
Paraventricular Nucleus													
DA (pg/µg)	12.1 ± 3.2	5.8 ± 0.9	3.6 ± 0.3	12.7 ± 4.2	4.4 ± 0.6	7.2 ± 0.7	4.9 ± 1.4	6.2 ± 0.8	4.0 ± 1.6^{++}	25.8 ± 5.9^{++}	4.3 ± 0.9^{++++}	31.2 ± 13.9$^{\$\$,++++}$	p=.0008
DOPAC (pg/µg)	1.9 ± 0.2	1.4 ± 0.4	1.7 ± 0.5	2.9 ± 1.2	1.1 ± 0.1	1.3 ± 0.4	1.3 ± 0.2	1.1 ± 0.3	0.9 ± 0.1^{++++}	6.0 ± 2.2$^{\$\$\$,++++}$	1.5 ± 0.2	1.7 ± 0.5	p=.0310
5-HIAA (pg/µg)	8.7 ± 1.5	11.0 ± 1.8	11.8 ± 1.8	24.4 ± 6.2	11.3 ± 0.8	15.9 ± 1.4	10.4 ± 1.6	18.2 ± 1.0	4.1 ± 0.3^{++++}	103.6 ± 42.4$^{\$\$\$,++++}$	5.1 ± 0.4^{++}	63.0 ± 30.2^{++}	p=.0006
DOPAC/DA ratio	0.2 ± 0.1	0.2 ± 0.0	0.5 ± 0.1^{+}	0.3 ± 0.1^{+}	0.3 ± 0.1	0.2 ± 0.0	0.3 ± 0.0	0.2 ± 0.1	0.3 ± 0.1	0.2 ± 0.0	0.4 ± 0.1^{++}	0.1 ± 0.0^{++}	p=.0002
Basolateral Amygdala													
NE (pg/µg)	7.7 ± 2.2^{+}	13.7 ± 1.8^{+}	7.8 ± 1.8^{++++}	22.0 ± 2.2^{++++}	9.9 ± 0.5^{+}	15.3 ± 1.2^{+}	7.2 ± 0.7^{+++}	17.6 ± 2.3^{+++}	6.7 ± 1.3^{++++}	20.6 ± 2.3^{++++}	5.8 ± 1.0^{+++}	14.7 ± 2.7^{+++}	p<.0001

DA (pg/µg)	8.7 ± 1.7	12.4 ± 1.6	6.2 ± 1.3^{+++}	16.2 ± 2.5^{+++}	15.2 ± 2.5	15.1 ± 1.6	7.3 ± 1.0^{++}	15.5 ± 1.6^{++}	7.2 ± 1.9^{+++}	18.5 ± 0.8^{+++}	5.5 ± 1.4^{++}	13.6 ± 3.3^{++}	*p*<.0001
5-HT (pg/µg)	2.7 ± 0.4	3.9 ± 0.8	2.1 ± 0.4^{++}	4.9 ± 1.0^{++}	3.8 ± 0.4	5.0 ± 0.4	2.9 ± 0.6^{+}	5.6 ± 0.9^{+}	2.9 ± 0.7	3.5 ± 0.8	3.6 ± 0.6	3.0 ± 0.9	*p*=.0015
DOPAC (pg/µg)	3.5 ± 0.8	6.3 ± 0.7	4.2 ± 0.8^{++}	10.9 ± 1.9^{++}	8.2 ± 1.1	9.0 ± 1.7	7.0 ± 1.8	6.0 ± 0.8	3.8 ± 1.0^{++++}	13.6 ± 1.7$^{\$,++++}$	2.2 ± 0.4^{++++}	14.6 ± 4.1$^{\$\$,++++}$	*p*<.0001
5-HIAA (pg/µg)	5.9 ± 1.2	10.8 ± 1.3	12.4 ± 3.7	24.7 ± 7.1	8.7 ± 0.5	13.0 ± 0.9	7.4 ± 0.6	14.0 ± 1.5	8.2 ± 3.2^{++++}	43.8 ± 5.1$^{\$\$\$,++++}$	4.6 ± 0.6^{++++}	39.3 ± 14.4$^{\$\$,++++}$	*p*<.0001
Hippocampus													
5-HT (pg/µg)	0.9 ± 0.2^{+}	2.0 ± 0.6^{+}	1.8 ± 0.5	1.8 ± 0.3	1.6 ± 0.2	2.4 ± 0.3	1.0 ± 0.2^{++}	2.7 ± 0.6^{++}	0.9 ± 0.2	1.4 ± 0.2	0.9 ± 0.3	1.3 ± 0.2	*p*=.0009
DOPAC (pg/µg)	0.4 ± 0.1^{+}	1.3 ± 0.4^{+}	1.0 ± 0.5	0.9 ± 0.1	0.8 ± 0.2	1.1 ± 0.4	0.7 ± 0.1	0.7 ± 0.1	0.4 ± 0.1^{++}	1.5 ± 0.5^{++}	0.5 ± 0.2	1.1 ± 0.2	*p*=.0063
5-HIAA (pg/µg)	3.8 ± 0.8^{+}	8.1 ± 2.6^{+}	6.5 ± 1.5	9.5 ± 1.3	7.3 ± 0.5	10.0 ± 0.6	5.4 ± 0.8^{++}	11.8 ± 2.1^{++}	4.1 ± 1.1^{+++}	13.0 ± 1.3^{+++}	2.3 ± 0.2^{+++}	10.7 ± 2.4^{+++}	*p*<.0001
DOPAC/DA ratio	0.3 ± 0.1	0.5 ± 0.2	0.2 ± 0.1	0.3 ± 0.1	0.6 ± 0.1	0.4 ± 0.1	0.6 ± 0.1^{+}	0.3 ± 0.1^{+}	0.3 ± 0.1	0.1 ± 0.0	0.5 ± 0.1	0.3 ± 0.1	*p*=.0283
5-HIAA/5-HT ratio	4.2 ± 0.8	4.0 ± 1.0	8.0 ± 2.5	7.4 ± 2.3	4.9 ± 0.4	4.5 ± 0.6	5.4 ± 0.6	4.9 ± 0.8	4.9 ± 0.9	10.5 ± 2.6	3.4 ± 0.8	9.1 ± 1.5	NS

Note: EDC, endocrine disrupting chemicals; BPA, bisphenol A; DEHP, di-(2-ethylhexyl) phthalate; NE, norepinephrine; DA, dopamine; 5-HT, serotonin; DOPAC, 3,4-dihydroxyphenylacetic acid; 5-HIAA, 5-hydroxyindoleacetic acid; NS, non-significant. Data are presented as mean ± SEM. $^{\$}$ $p < 0.05$, $^{\$\$}$ $p < 0.01$, $^{\$\$\$}$ $p < 0.001$, $^{\$\$\$\$}$ $p < 0.0001$, difference between control and EDC females, two-way ANOVA followed by Tukey's post hoc analyses. $^{+}$ $p < 0.05$, $^{++}$ $p < 0.01$, $^{+++}$ $p < 0.001$, $^{++++}$ $p < 0.0001$, difference between males and females of the same treatment group, two-way ANOVA followed by uncorrected Fisher's LSD post hoc analyses.

Table S3.1. Data from Chapter 3 figures. Data are presented as mean ± SEM.

	Control		BPA (5 µg)		DEHP (5 µg)		BPA + 5 µg DEHP		DEHP (7.5 mg)		BPA + 7.5 mg DEHP	
	Males	**Females**	**Males**	**Females**	**Males**	**Females**	**Males**	**Females**	**Males**	**Females**	**Males**	**Females**
Figure 3.2 - mPFC monoamines												
NE	5.2 ± 1.0	12.0 ± 1.7	13.7 ± 4.9	15.3 ± 4.9	7.1 ± 1.0	11.8 ± 0.8	5.1 ± 0.3	14.8 ± 2.1	3.6 ± 1.1	27.9 ± 8.7	3.6 ± 0.4	12.7 ± 2.7
5-HT	2.3 ± 0.6	2.8 ± 0.5	4.0 ± 1.1	2.7 ± 0.5	2.4 ± 0.3	3.4 ± 0.4	2.1 ± 0.2	3.0 ± 0.5	1.4 ± 0.2	6.6 ± 2.0	2.2 ± 0.5	3.0 ± 0.6
DA	10.1 ± 3.5	7.45 ± 3.50	14.7 ± 6.7	9.66 ± 5.72	1.8 ± 0.3	1.95 ± 0.26	2.1 ± 0.3	1.96 ± 0.30	2.5 ± 1.0	30.43 ± 7.58	4.0 ± 1.5	19.27 ± 4.47
Figure 3.3 - mPFC monoamine turnover												
DOPAC/DA	0.4 ± 0.1	0.75 ± 0.18	0.5 ± 0.1	0.70 ± 0.16	1.1 ± 0.2	1.09 ± 0.12	0.8 ± 0.1	1.06 ± 0.17	0.6 ± 0.1	0.18 ± 0.04	0.9 ± 0.2	0.25 ± 0.08
5-HIAA/5-HT	3.2 ± 0.3	3.5 ± 0.9	9.5 ± 2.3	7.4 ± 3.5	3.5 ± 0.3	4.0 ± 0.5	3.8 ± 0.4	5.1 ± 0.4	2.8 ± 0.6	19.2 ± 4.0	2.5 ± 0.5	13.1 ± 3.8
Figure 3.4 - PVN DA & SPDB												
PVN DA	12.1 ± 3.2	5.8 ± 0.9	3.6 ± 0.3	12.7 ± 4.2	4.4 ± 0.6	7.2 ± 0.7	4.9 ± 1.4	6.2 ± 0.8	4.0 ± 1.6	25.8 ± 5.9	4.3 ± 0.9	31.2 ± 13.9
Figure 3.5 - PVN monoamines & turnover												
NE	20.6 ± 3.6	15.4 ± 2.5	13.6 ± 2.1	41.1 ± 13.3	10.7 ± 1.6	14.8 ± 1.2	16.5 ± 4.7	17.1 ± 1.7	6.5 ± 0.5	74.1 ± 22.7	13.7 ± 3.0	34.9 ± 8.9
5-HT	3.0 ± 0.8	2.2 ± 0.4	1.6 ± 0.3	3.5 ± 0.7	3.2 ± 0.5	4.1 ± 0.4	3.2 ± 1.0	3.3 ± 0.5	2.3 ± 0.4	6.9 ± 2.5	2.6 ± 0.5	8.0 ± 2.8

5-HIAA/5-HT	3.1 ± 0.6	5.7 ± 0.9	9.8 ± 2.7	7.9 ± 2.3	4.0 ± 0.6	4.1 ± 0.6	3.8 ± 0.7	6.0 ± 0.7	2.9 ± 0.6	17.3 ± 3.7	2.5 ± 0.6	7.0 ± 0.8
Figure 3.6 - BLA												
DOPAC/DA	0.40 ± 0.04	0.52 ± 0.04	0.80 ± 0.16	0.80 ± 0.21	0.56 ± 0.06	0.58 ± 0.07	0.95 ± 0.16	0.39 ± 0.03	0.61 ± 0.13	0.72 ± 0.08	0.46 ± 0.07	1.00 ± 0.08
5-HIAA/5-HT	2.3 ± 0.3	3.2 ± 0.4	5.5 ± 1.3	6.4 ± 2.3	2.5 ± 0.3	2.7 ± 0.3	2.9 ± 0.5	2.7 ± 0.3	2.1 ± 0.5	14.9 ± 2.8	1.5 ± 0.3	12.5 ± 2.6
Figure 3.7 - HC												
NE	3.8 ± 1.0	8.8 ± 1.3	4.8 ± 1.7	12.2 ± 1.0	9.4 ± 1.2	13.6 ± 1.7	5.0 ± 0.6	20.5 ± 3.6	4.6 ± 1.1	13.1 ± 1.1	2.5 ± 0.3	8.8 ± 0.9
DA	1.9 ± 0.5	3.1 ± 0.9	2.5 ± 1.0	4.9 ± 1.4	1.5 ± 0.2	2.8 ± 0.4	1.2 ± 0.2	2.8 ± 0.7	1.0 ± 0.2	13.3 ± 0.9	0.9 ± 0.2	6.0 ± 1.3

CHAPTER 4

PRENATAL BISPHENOL A AND/OR DIETHYLHEXYL PHTHALATE EXPOSURE FOLLOWED BY ADULT ESTRADIOL TREATMENT AFFECTS BEHAVIOR AND BRAIN MONOAMINES IN FEMALE RAT OFFSPRING [3]

[3] Kaimal et al. To be submitted to *Environmental Health Perspectives*.

4.1. ABSTRACT

Prenatal exposures to bisphenol A (B) and/or diethylhexyl phthalate (D) *in utero* affect neurobehavior, whereas exogenous estrogens can also have detrimental effects in females. The aim of this study was to determine the cumulative effects of prenatal exposure to B and/or D followed by chronic 17β-estradiol (E2) treatment in adulthood on neurobehavior. Pregnant Sprague-Dawley rats were orally dosed with vehicle, B (5 µg/kg body weight (BW)/day), low-dose (LD) D (5 µg/kg BW/day), high-dose (HD) D (7.5 mg/kg BW/day), a combination of B and LD-D (B+D (LD)), or a combination of B and HD-D (B+D (HD)) on gestational days 6-21. After sham or E2 treatment for 90 days, the adult female offspring underwent behavioral testing. Brains and blood were collected at sacrifice. Various serum hormones were measured, and brain monoamine levels were analyzed. Chronic treatment with E2 in control offspring increased unconditioned anxiety in the Elevated Plus Maze and reduced active coping abilities. In several of the EDC-exposed offspring, E2 treatment led to a reversal of these responses and elevated anxiety-like behavior in the Open Field Test instead. A variety of E2- and EDC dose-dependent alterations were observed in hormone levels and monoamine concentrations in the paraventricular hypothalamic nucleus (PVN) and hippocampus. Most notably, there appeared to be a disconnect between PVN noradrenergic activity and circulating corticosterone levels. Prenatal EDC exposures alter behavior, hormones, and/or brain monoamines, and adult E2 treatment may further exacerbate these effects in female offspring.

4.2. INTRODUCTION

Women are constantly exposed to exogeneous forms of estrogen, particularly at low doses, throughout their lives. Early life exposure may occur via endocrine disrupting chemicals (EDCs), including the plasticizers bisphenol A (BPA) and diethylhexyl phthalate (DEHP), present during developmental stages [206, 248]. EDCs existing in pregnant women can cross the placental barrier and adversely impact the growing fetus [90, 91], resulting in long-lasting neurodevelopmental effects and behavioral manifestations [86]. Even more concerning is the potential for exposure to these EDCs in combination with one another, the effects of which may be more harmful than individual EDC exposure [12, 13]. In addition, adult women may also be exposed to exogenous estrogen through the use of oral contraceptive pills (OCs) containing ethinyl estradiol or via hormone replacement therapy (HRT). Furthermore, the risk for anxiety and depression is approximately twice as high in women as it is in men [101], suggesting the involvement of estrogens in the etiology of these disorders. Therefore, the increased likelihood for the development of mood disorders in females could arise from the combined exposures of EDCs during early development and estrogen in adulthood.

Exposure to BPA or DEHP during the perinatal stage of development leads to detrimental effects on stress-related behaviors and hormones in female rodents. Low-dose BPA exposure (2-200 µg/kg/day) increases anxiety-like behavior in the open field test (OFT) and the elevated plus maze (EPM), but decreases OFT locomotor activity in female mice offspring [136, 143, 155]. This is exacerbated with increasing doses in females, implying a dose-dependent and sex-specific response. Furthermore, elevated basal levels of corticosterone (CORT) have been reported in female rat offspring with perinatal BPA exposure (40 µg/kg/day) [117, 154], which persist following acute stress [117].

On the other hand, low DEHP doses (5, 40, and 400 µg/kg/day) are associated with increased home cage exploration [160], although higher doses (10, 50, and 200 mg/kg/day) may lead to decreased rearing (a form of exploration) and locomotor activity in the OFT in female mice offspring [145]. This suggests non-monotonic dose responses on female exploratory and locomotor activity. Anxiogenic behavioral responses in the OFT and EPM are additionally observed in female mice with low-dose (5 and 40 µg/kg) perinatal DEHP exposure [160], and these effects are persistent even at high doses (50 and 200 mg/kg) [144, 145]. Moreover, prenatal high-dose DEHP exposure (150 mg/kg) leads to transgenerational reductions in CORT concentrations in stressed female mice offspring [162]. This implies that BPA and DEHP may produce differential, dose-dependent effects on CORT in female offspring.

Monoamines in the brain including norepinephrine (NE), dopamine (DA), and serotonin (5-HT) are strongly implicated in the etiology of anxiety and mood disorders [209, 210], and EDC exposure is also linked with these conditions. Within the adult female rodent brain, perinatal exposure to BPA dose-dependently impacts NE [164], 5-HT and its major metabolite 5-hydroxyindoleacetic acid (5-HIAA) [211, 212], as well as DA and its metabolite 3,4-dihydroxyphenylacetic acid (DOPAC) [212] in areas including the cortex and hypothalamus. Moreover, mature female offspring show decreased dopamine turnover in the brain, measured by comparing the ratios of DOPAC to DA, following perinatal treatment with doses as low as 10 µg/kg of BPA [215]. DEHP is also capable of affecting brain neurotransmitter systems. Gestational exposure to DEHP across a range of doses (20 µg/kg – 750 mg/kg) can affect DA receptor expression levels in the amygdala across generations in female mice offspring [161]. Yet, the effects on brain noradrenergic and serotonergic systems in female offspring with prenatal low-dose DEHP exposure are vastly understudied.

Estrogens have been suspected to contribute to the development of mood disorders, since hormonal fluctuations occurring during the menstrual cycle are associated with the manifestation of anxiety symptoms [249]. Additionally, the use of OCs containing ethinyl estradiol in adult women is linked with increased anxiety- and depression-related symptoms [250], whereas long-term estradiol exposure in postmenopausal women undergoing HRT may induce adverse cognitive effects [251]. Our lab has previously demonstrated that in intact female rats, chronic estradiol treatment for 12 weeks at very low doses (20 ng/day) produces anxiogenic responses coupled with reduced DA levels in the amygdala [252]. On the contrary, acute estradiol treatment at a low dose (10 µg/day) for 3 weeks results in anxiolytic behaviors but impaired object recognition performance [175]; however, estradiol at a higher dose (30 µg/day) leads to increased anxiety-like behavior and improved object recognition. These studies reveal the array of dose- and duration-dependent effects of estradiol on anxiety and cognition.

The goals of the present study were to assess: 1) the behavioral, cognitive, and hormonal effects following a dual exposure paradigm – BPA, DEHP, or BPA+DEHP exposure *in utero* followed by chronic exposure to estradiol in adulthood, and 2) the effects of EDC and/or estradiol treatment on monoaminergic activity in stress-regulating brain regions. Our hypothesis was that prenatal EDC exposure, particularly in combination, would induce alterations in brain monoaminergic activity leading to changes in circulating stress hormones and a variety of behavioral and cognitive parameters. Adult estradiol treatment would exacerbate these effects.

4.3. Materials and Methods

Animals

Adult female Sprague-Dawley rats purchased from Envigo (Indianapolis, IN) were housed in light-controlled (12:12 light-dark cycle) and temperature-controlled (23.2 ± 2 °C, 50 ± 20% relative humidity) rooms at the University of Georgia, with food and water provided *ad libitum*. The rats were fed Pico Lab Rodent Diet 20 (LabDiet) and housed in polycarbonate cages with corn cob bedding. The female breeders underwent vaginal cytology for 10 consecutive days prior to mating to track their individual estrous cycles. Females in proestrus were randomly assigned a male and the two were co-housed for one day. The presence of a vaginal plug was used to confirm the occurrence of mating. Gestational day (GD) 0 represented the day of copulation. Experimental protocols followed the National Institutes of Health's *Guide for the Care and Use of Laboratory Animals* approved by the Institutional Animal Care and Use Committee (IACUC) at the University of Georgia.

Chemicals

BPA (Catalog No. 239658; Lot MKBH2096V) and DEHP (Catalog No. 36735; Lot BCBR8079V) were obtained from Sigma Aldrich (St. Louis, MO). Stock solutions were made in dimethylsulfoxide (DMSO) to obtain complete dissolution. Doses were calculated daily based on BW and mixed with 20µl PBS for oral dosing, which occurred daily from GD 6-21. Dams were administered the vehicle or EDC treatment into the oral cavity using a pipette. The BPA dose was selected because it is significantly lower than the Environmental Protection Agency (EPA) recommended no-observed-adverse-effect-level (NOAEL) dose of 5 mg/kg/day [115] and it is also 10-fold below the tolerable daily intake (TDI) dose of 50 µg/kg/day [114]. The high dose of DEHP was selected since it is higher than the established NOAEL dose of 4.8 mg/kg/day [130],

whereas the low DEHP dose is significantly lower than this. The low dose of DEHP used in this study also lies within the range of the typical daily intake of this chemical in humans (0.5-25 µg/kg/day) [127], but is well below the EPA reference dose of 20 µg/kg/day [128].

EDC and E2 exposure paradigms

The experimental design is demonstrated in Figure 4.1. The dam was considered the experimental unit. Daily oral dosing of dams with EDCs or vehicle began on GD 6. Each dam was randomly assigned to one of 6 different treatment groups: control (10 µl Phosphate Buffered Saline or PBS; n=7), BPA (5 µg/kg BW/day; n=9), low-dose (LD) DEHP (5 µg/kg BW/day; n=6), high-dose (HD) DEHP (7.5 mg/kg BW/day; n=6), a combination of BPA and LD DEHP (5 µg/kg/day of BPA + 5 µg/kg/day of DEHP; n=6), and a combination of BPA and HD DEHP (5 µg/kg/day of BPA + 7.5 mg/kg/day of DEHP; n=7). These are displayed in Table 4.1.

Once the female offspring of these dams reached 3-5 months of age, vaginal smears were collected from them to determine estrous cyclicity, and those with regular estrous cycles were included. Two female rats from each dam were used; one was sham-implanted (control) (n=6-7/group) and the other was implanted with a slow release 17β-Estradiol (E2) pellet (n=6-9/group) (1.8 µg, Innovative Research America, Sarasota, FL). The E2 pellets release 20 ng per day over 90 days, which leads to constant estrus in rats after 60 days of exposure as a result of accelerated reproductive aging [253, 254]. Therefore, control offspring underwent behavioral testing only when they were in estrus.

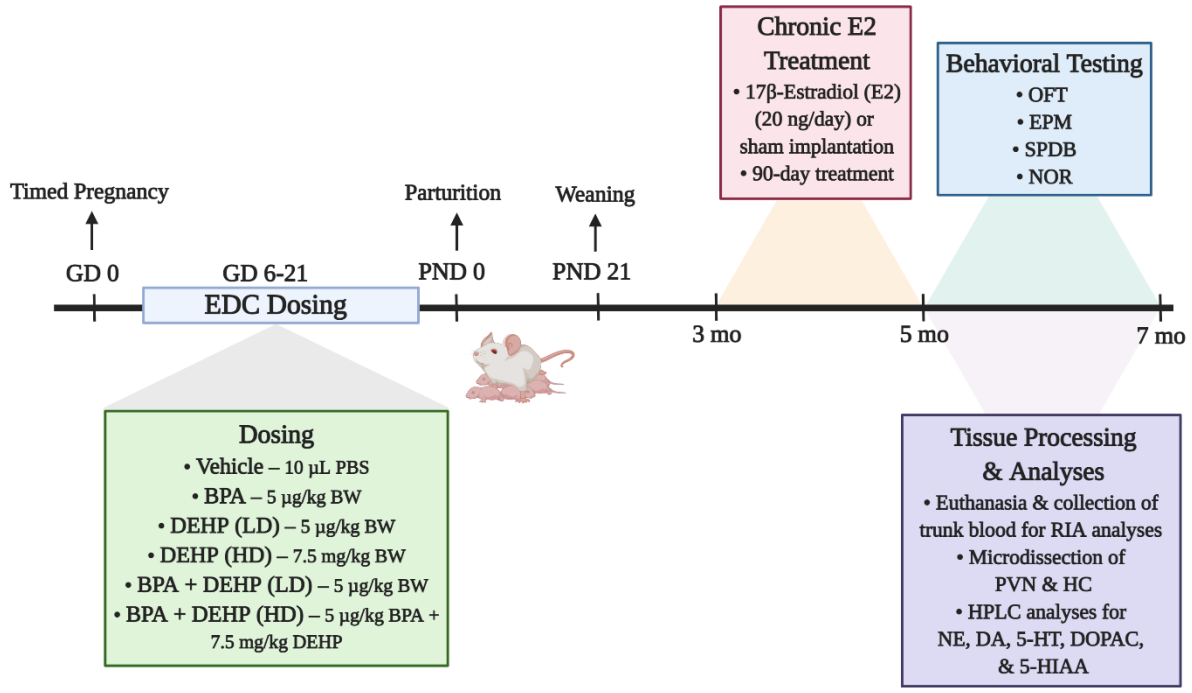

Figure 4.1. Summary of the experimental design of the study. Pregnant Sprague-Dawley dams were orally dosed daily from gestational days (GD) 6-21 with vehicle (Control) (10 µL PBS; *n*=7), BPA (5 µg/kg/day; *n*=9), low-dose (LD) DEHP (5 µg/kg/day; *n*=6), high-dose (HD) DEHP (7.5 mg/kg/day; *n*=6), a mixture of BPA + LD DEHP (5 µg/kg/day of BPA + 5 µg/kg/day of DEHP; n=6), or a mixture of BPA + HD DEHP (5 µg/kg/day of BPA + 7.5 mg/kg/day of DEHP; n=7). Adult female offspring were either sham-implanted or implanted with a slow release 90-day E2 pellet, following which all offspring underwent behavioral testing and were euthanized immediately after. Trunk blood was collected for the measurement of serum E2, corticosterone, and oxytocin using RIA. Brains were micro-dissected for PVN and HC tissues, which were then analyzed for monoamines and major metabolites using HPLC. Experimental design schematic was created using Biorender.com.

Note: EDC, endocrine disrupting chemical; PBS, Phosphate Buffered Saline; BPA, Bisphenol A; DEHP, diethylhexyl phthalate; LD, low-dose; HD, high-dose; OFT, Open Field Test; EPM, Elevated Plus Maze; SPDB, Shock Probe Defensive Burying; NOR, Novel Object Recognition Test; RIA, radioimmunoassay; PVN, paraventricular nucleus; HC, hippocampus; HPLC, high performance liquid chromatography; NE, norepinephrine; DA, dopamine; 5-HT, serotonin; DOPAC, 3,4-dihydroxyphenylacetic acid; 5-HIAA, 5-hydroxyindoleacetic acid.

Behavioral testing

The adult sham- and E2-treated female offspring (5-7 months old) were administered a battery of behavioral tests including the Open Field Test (OFT), Elevated Plus Maze (EPM), and Shock Probe Defensive Burying (SPDB). The Novel Object Recognition test (NOR) was also administered, but only to the animals in the LD group since the laboratory equipment was not available at the time of the HD animal experimentation. The order for the tests was OFT, EPM, SPDB, followed by NOR. 4 rats were tested per day on average and the rats were administered each test once. Vaginal smears were obtained from all rats, for 2-10 days prior to behavioral testing to ensure that animals were all tested in estrus. A total of 79 offspring were used for this study (see Table 4.1). The behavioral tests were conducted exactly as described in Chapter 2.

Hormone measurement

Immediately following behavioral testing, the offspring were euthanized by rapid decapitation. Trunk blood was collected, centrifuged, and serum was separated and stored at -80°C. Serum estradiol (E2), corticosterone (CORT), and oxytocin (OXT) levels were measured in duplicate using a double antibody radioimmunoassay (E2 & CORT – MP Biomedicals, Santa Ana, CA; E2 SKU: 0713810-CF; CORT SKU: 07120121; OXT – Phoenix Pharmaceuticals,

Burlingame, CA; Catalog No. RK-051-01), according to the manufacturer's protocol. CORT values were expressed as ng/ml. E2 and OXT values were expressed as pg/ml.

Brain sectioning and microdissection

Following euthanasia of offspring, brains were also dissected and stored at -80°C. A cryostat (Slee, London, UK) maintained at -10°C was used to section brains at 300 µm thickness. Following this, the paraventricular nucleus of the hypothalamus (PVN) and ventral subdivision of the HC were micro-dissected on a cold stage using the Palkovits' microdissection technique and a stereotaxis brain atlas as a reference [218]. All brain punches were stored at -80°C until further analyses.

Neurotransmitter analysis by HPLC-EC

HPLC-EC was used to analyze brain punches for NE, DA, DOPAC, 5-HT, and 5-HIAA as previously described in [219] and in Chapter 3. Brain punches were briefly homogenized in 0.05 M perchloric acid on ice and an aliquot was used for protein estimation (MicroBCA assay, Pierce, Rockford, IL). The remaining homogenate was centrifuged at 18,000 × g for 8 min at 4°C. The supernatant was injected with an internal standard (dihydroxybenzylamine, 0.05 M) into the autoinjector for HPLC analysis. Chromatograms were analyzed for neurotransmitter concentrations using the Class VP software v 7.2 (Shimadzu, Columbia, MD). Neurotransmitter concentrations in tissue samples were expressed as pg/µg of protein. Protein levels in tissue punches were measured using the micro bicinchoninic acid assay (Pierce, Rockford, IL). Samples were assayed in duplicate according to the manufacturer's protocol. Besides actual neurotransmitter values, turnover rates for DA and 5-HT were obtained by dividing the concentrations of the metabolites by the concentration of the parent neurotransmitter.

Statistical analysis

Prism 9.0.0 (GraphPad, Inc.) software was used to perform statistical analyses. All data were analyzed by two-way ANOVA (EDC exposure × sex) with behavioral parameters, hormones, or neurotransmitters as the dependent variable. Interaction effects between EDC exposure and sex were also assessed. Differences in behavioral parameters between control and EDC groups, as well as between sham and E2 groups, were analyzed using uncorrected Fisher's LSD post-hoc test. EDC effects in hormonal and neurotransmitter data were analyzed using Tukey's multiple comparisons post-hoc test. Sham and E2 differences within EDC groups in hormones and neurotransmitters were measured using uncorrected Fisher's LSD post-hoc test. P-value < 0.05 was considered to indicate a statistically significant difference. Data is expressed as mean ± standard error of mean (SEM).

4.4. RESULTS

Behavioral tests

The results from the OFT, EPM, SPDB, and NOR are shown in Figures 4.2-4.5 and Table 4.2.

Open field test

A significant EDC exposure effect ($p = 0.046$) was observed in ambulatory distance (cm, mean ± SEM) (Figure 4.2A), wherein DEHP (HD)-sham offspring (941.3 ± 76.0; $p = 0.049$) traveled less within the chamber relative to their control counterparts (1316.8 ± 167.5). No significant differences were observed in the amount of time spent ambulating or rearing within the chamber in any of the groups (Table 4.2).

Significant main effects of EDC ($p = 0.001$) and E2 ($p = 0.010$), as well as an interaction effect ($p = 0.033$), were found in time spent within the center zone (%, mean ± SEM) (Figure 4.2B). Sham-treated BPA (3.4 ± 0.7; $p = 0.044$) and B+D (LD) (3.5 ± 0.7; $p = 0.035$) offspring demonstrated anxiolytic effects with robust increases in center time compared to their control counterparts (1.8 ± 0.4). On the contrary, E2-treated offspring exposed to BPA (1.1 ± 0.3; $p = 0.002$), DEHP (LD) (1.5 ± 0.3; $p = 0.046$), or DEHP (HD) (0.4 ± 0.1; $p = 0.028$) displayed anxiogenic effects by having drastic reductions in center time than their sham counterparts. This decrease in center time was present in DEHP (HD)-E2 offspring when compared to control-E2 offspring (2.2 ± 0.6; $p = 0.025$) as well, displaying an interaction between EDC and E2 in DEHP (HD)-E2 females in particular.

In terms of center zone entries (mean ± SEM) (Figure 4.2C), main effects of EDC ($p = 0.023$) and E2 ($p = 0.010$) were observed. E2-treated females exposed to both DEHP doses showed increased anxiety-like behavior in this measure as well. A very near-significant effect was also discovered in B+D (HD)-E2 offspring (14.9 ± 1.5; $p = 0.053$), who also showed anxiogenic effects compared to their control counterparts (28.7 ± 5.8). No significant differences were observed in time spent or entries into the perimeter zone (Table 4.2).

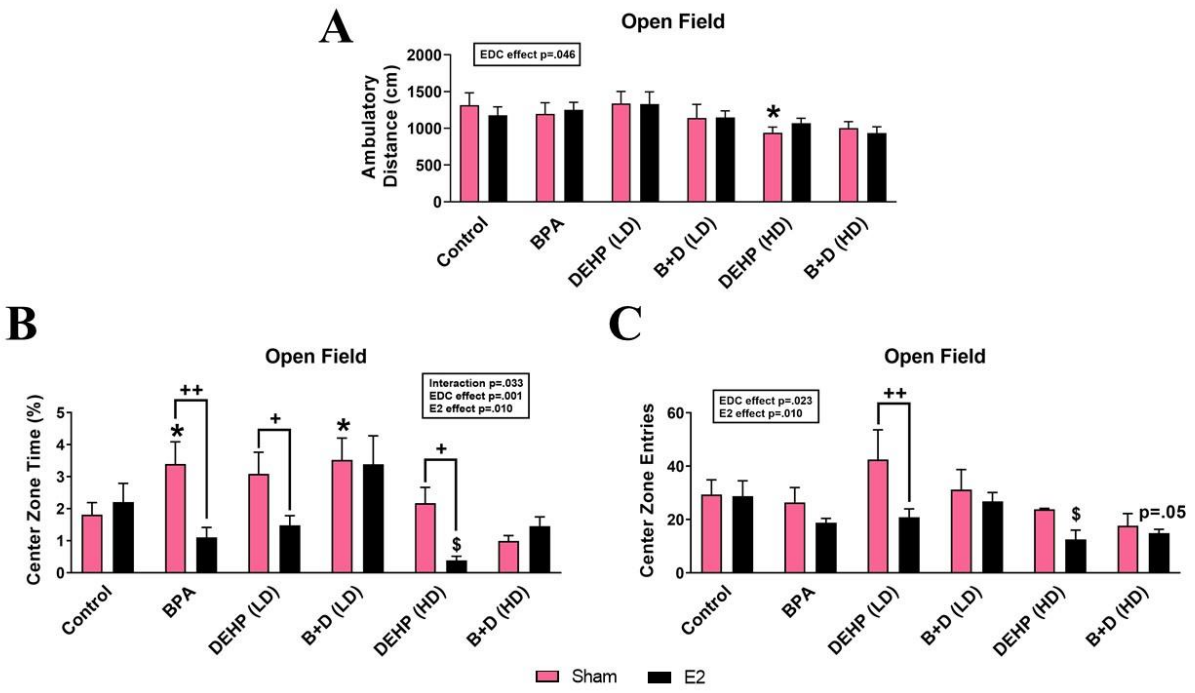

Figure 4.2. Behavioral effects of prenatal EDC exposure and/or adult E2 treatment in female rat offspring in the open field test (OFT). (*A*) Locomotor activity, (*B*) center zone time, and (*C*) center zone entries. Behavioral data were collected from adult female offspring. Data were analyzed by two-way ANOVA, followed by Fisher's LSD post hoc test. $^{*}p<0.05$, comparison between sham-implanted control and EDC-exposed female offspring. $^{\$}p<0.05$, comparison between E2-implanted control and EDC-exposed female offspring. $^{+}p<0.05$, $^{++}p<0.01$, comparison between sham- and E2-implanted female offspring of the same treatment group. Error bars represent the standard error of the mean (SEM).

Elevated plus maze

No significant differences were identified in total exploration of the EPM (Figure 4.3A). An interaction effect ($p = 0.018$) was observed in the number of entries into the open arms (mean ± SEM) (Figure 4.3B). Control-E2 females (5.0 ± 0.3) showed a robust decrease in open arm entries than their sham counterparts (11.6 ± 0.5; $p = 0.002$), representing an increase in anxiety-

like behavior. Interestingly, all of the E2-treated females prenatally exposed to EDCs, with the exception of DEHP (HD)-E2 females, demonstrated anxiolytic effects with significant increases in open arm entries compared to control-E2 offspring. DEHP (LD)-E2 (p = 0.0003) and B+D (HD)-E2 (p = 0.002) offspring showed the highest increases in open arm entries relative to their control counterparts at 150% and 120%, respectively.

Additionally, an EDC exposure effect (p = 0.007) was also determined in the amount of time spent in the central platform of the maze (Table 4.2). Sham-implanted low-dose DEHP (p = 0.004) and B+D (p = 0.009) females spent significantly less time in the center than control-sham animals, whereas B+D (HD)-E2 females (p = 0.022) spent a greater amount of time in the center than control-E2 females. No significant differences were found in open arm time, closed arm entries, or closed arm time (Table 4.2).

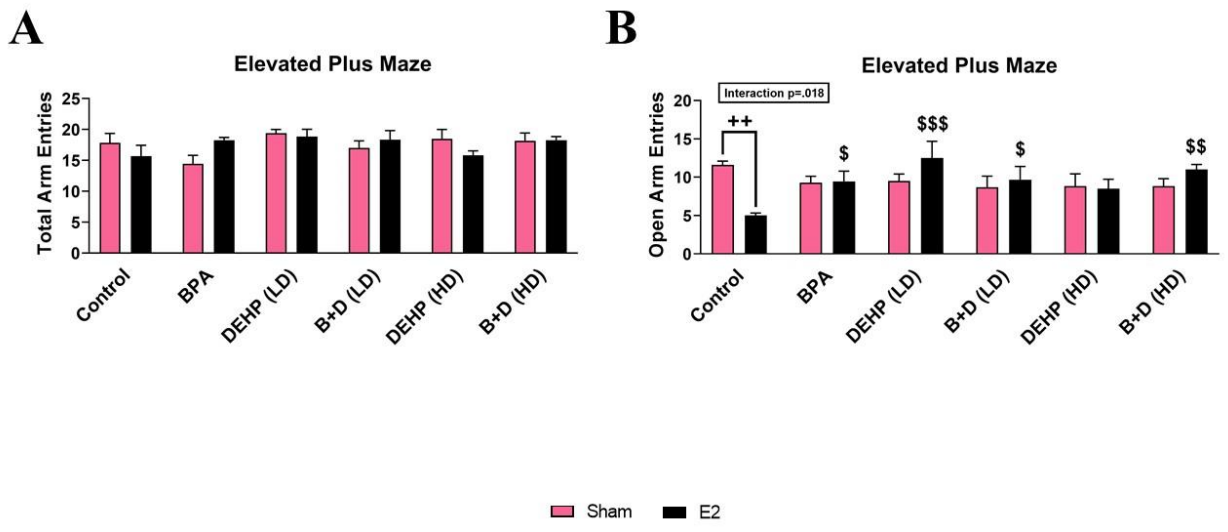

Figure 4.3. Behavioral effects of prenatal EDC exposure and/or adult E2 treatment in female rat offspring in the elevated plus maze (EPM). (*A*) Total exploration of the maze and

(*B*) number of entries into the open arms. Behavioral data were collected from adult female offspring. Data were analyzed by two-way ANOVA, followed by Fisher's LSD post hoc test. $^{\$}p<0.05$, $^{\$\$}p<0.01$, $^{\$\$\$}p<0.001$, comparison between E2-implanted control and EDC-exposed female offspring. $^{++}p<0.01$, comparison between sham- and E2-implanted female offspring of the same treatment group. Error bars represent the standard error of the mean (SEM).

Shock probe defensive burying

There was a significant interaction effect ($p = 0.010$) observed in the amount of time spent burying (%, mean ± SEM) (Figure 4.4A). EDC effects were prevalent in sham-treated BPA and DEHP (LD) offspring, whereas E2 effects were apparent in control and B+D (LD) offspring. Drastic reductions in burying time were observed in control-E2 (91.5% decrease), BPA-sham (79.7%), and DEHP (LD)-sham (85.2%) offspring compared to control-sham offspring. This suggests a decrease in active coping strategies in these offspring. This was coupled with significantly decreased bedding height (Table 4.2) in DEHP (LD)-sham females only (5.9 ± 0.4; $p = 0.026$) relative to their control counterparts (7.8 ± 0.7). Surprisingly, B+D (LD)-E2 (36.6 ± 13.1) spent considerably more time burying compared to their control (2.3 ± 1.0; $p = 0.002$) and B+D (LD)-sham counterparts (13.6 ± 6.5; $p = 0.027$), implying aberrant defensive behavioral effects. Very similar effects were also observed in burying frequency (Table 4.2).

In immobility time (%, mean ± SEM) (Figure 4.4B), we found a significant EDC effect ($p = 0.001$). In comparison with their control counterparts (2.2 ± 1.8), sham-implanted DEHP (LD) (17.8 ± 13.3; $p = 0.001$) and B+D (LD) (17.1 ± 13.0; $p = 0.002$) females showed substantial increases in immobility time, representing a shift to passive coping mechanisms. An EDC effect ($p = 0.002$) was additionally observed in immobility frequency, in which DEHP (LD)-sham females (24.7 ± 5.1; $p = 0.031$) exhibited a greater frequency of immobility as well (Table 4.2).

Significant main effects of EDC (p = 0.018) and E2 (p = 0.037) were identified in the amount of time spent exploring the probe (%, mean ± SEM) (Figure 4.4C). E2-exposed control offspring (1.0 ± 0.6; p = 0.038) demonstrated a substantial decrease in probe exploration time compared to their sham counterparts (7.7 ± 3.3). On the other hand, BPA-E2 (8.6 ± 2.1; p = 0.011) offspring spent significantly more time exploring the probe than E2-treated controls, exhibiting aberrant defensive behaviors. No significant differences were observed in probe exploration frequency (Table 4.2), rearing time (Figure 4D) or frequency (Table 4.2), grooming frequency or time (Table 4.2), or shock reactivity (Table 4.2).

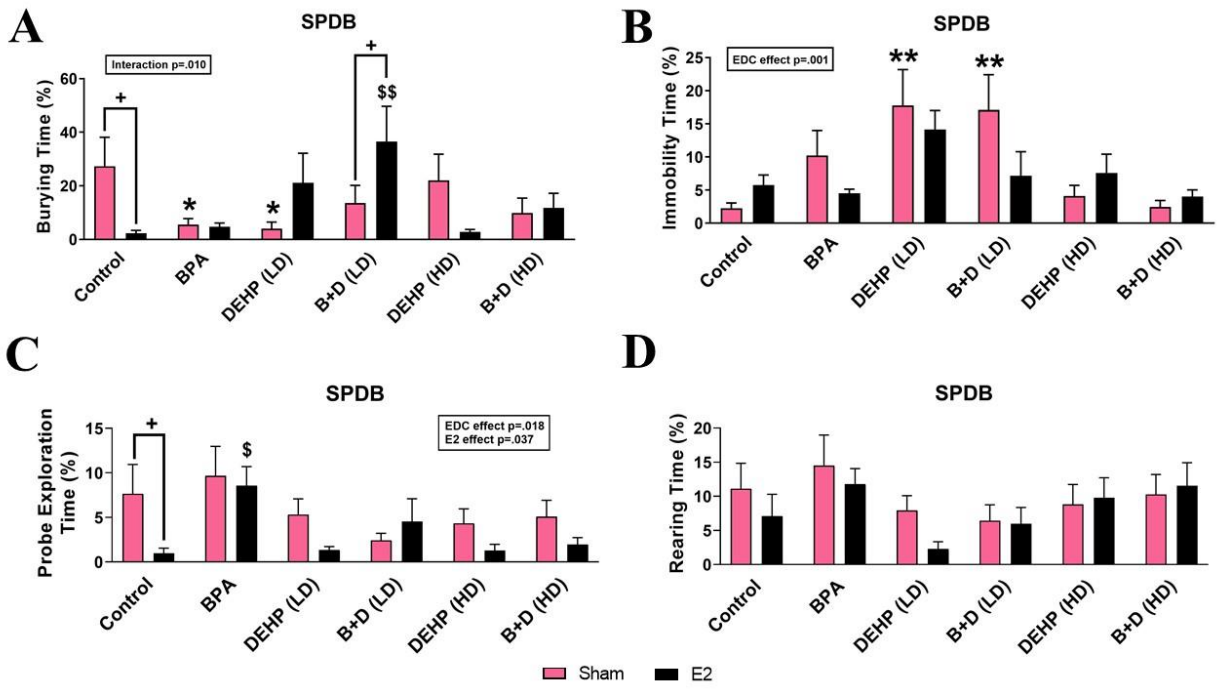

Figure 4.4. Behavioral effects of prenatal EDC exposure and/or adult E2 treatment in female rat offspring in the shock probe defensive burying (SPDB). (*A*) Amount of time spent burying, (*B*) amount of time spent immobile, (*C*) amount of time spent exploring the probe, and (*D*) amount of time spent rearing. Behavioral data were collected from adult female offspring. Data were analyzed by two-way ANOVA, followed by Fisher's LSD post hoc test. $^{*}p<0.05$,

$^{**}p<0.01$, comparison between sham-implanted control and EDC-exposed female offspring. $^{\$}p<0.05$, $^{\$\$}p<0.01$, comparison between E2-implanted control and EDC-exposed female offspring. $^{+}p<0.05$, comparison between sham- and E2-implanted female offspring of the same treatment group. Error bars represent the standard error of the mean (SEM).

Novel object recognition test

The NOR was only administered to animals in the low-dose groups due to availability of laboratory equipment that was unavailable when high-dose EDC-treated animals were tested. An interaction effect ($p = 0.023$) was observed in the training trial (T1) discrimination index (DI) (mean ± SEM) (Figure 4.5A). B+D (LD)-E2 (-0.2 ± 0.1) females spent significantly more time with the left object compared to control-E2 offspring (0.1 ± 0.1; $p = 0.004$), as well as B+D-sham offspring (0.1 ± 0.0; $p = 0.009$). This is indicative of atypical exploratory behavior in B+D (LD) females because their pattern of object exploration was reversed compared to that of sham and E2-treated control offspring. No further differences were observed in recognition index (Figure 4.5B), T1 average exploration time, or T2 DI (Table 4.2).

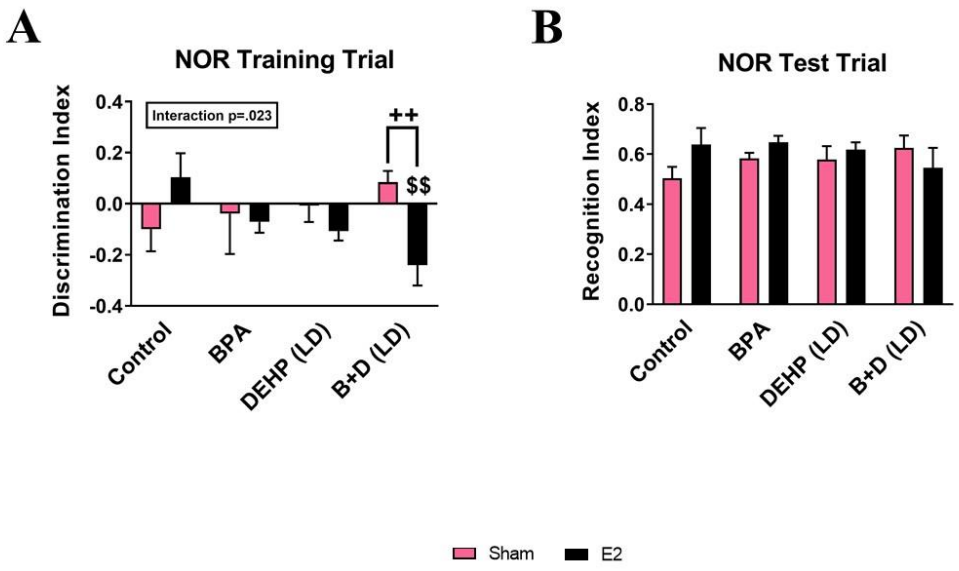

Figure 4.5. Behavioral effects of prenatal EDC exposure and/or adult E2 treatment in female rat offspring in the novel object recognition test (NOR). (*A*) Discrimination index during the training trial and (*B*) recognition index during the test trial. Behavioral data were collected from adult female offspring. Data were analyzed by two-way ANOVA, followed by Fisher's LSD post hoc test. $^{\$\$}p<0.01$, comparison between E2-implanted control and EDC-exposed female offspring. $^{++}p<0.01$, comparison between sham- and E2-implanted female offspring of the same treatment group. Error bars represent the standard error of the mean (SEM).

Hormonal levels

Expected increases in E2 levels (Figure 4.6A) were significantly different only in control ($p = 0.034$), B+D (LD) ($p = 0.033$), and DEHP (HD) ($p = 0.011$) offspring, indicating that E2 effects were abolished in the rest of the EDC groups. On the other hand, in terms of circulating CORT levels (Figure 4.6B), only the HD groups were affected. Prenatal B+D (HD) exposure

elevated CORT in sham offspring (p = 0.001), but E2-treated offspring in both B+D (HD) (p = 0. 001) and DEHP (HD) (p = 0. 042) groups showed increased CORT, demonstrating hyperactive HPA axis activity in offspring with HD EDC exposures. Finally, DEHP offspring were also impacted in OXT levels (Figure 4.6C). Interestingly, only E2-treated DEHP offspring were affected. DEHP (LD)-E2 females had significantly higher OXT levels than their DEHP (HD) exposed counterparts (p = 0.027).

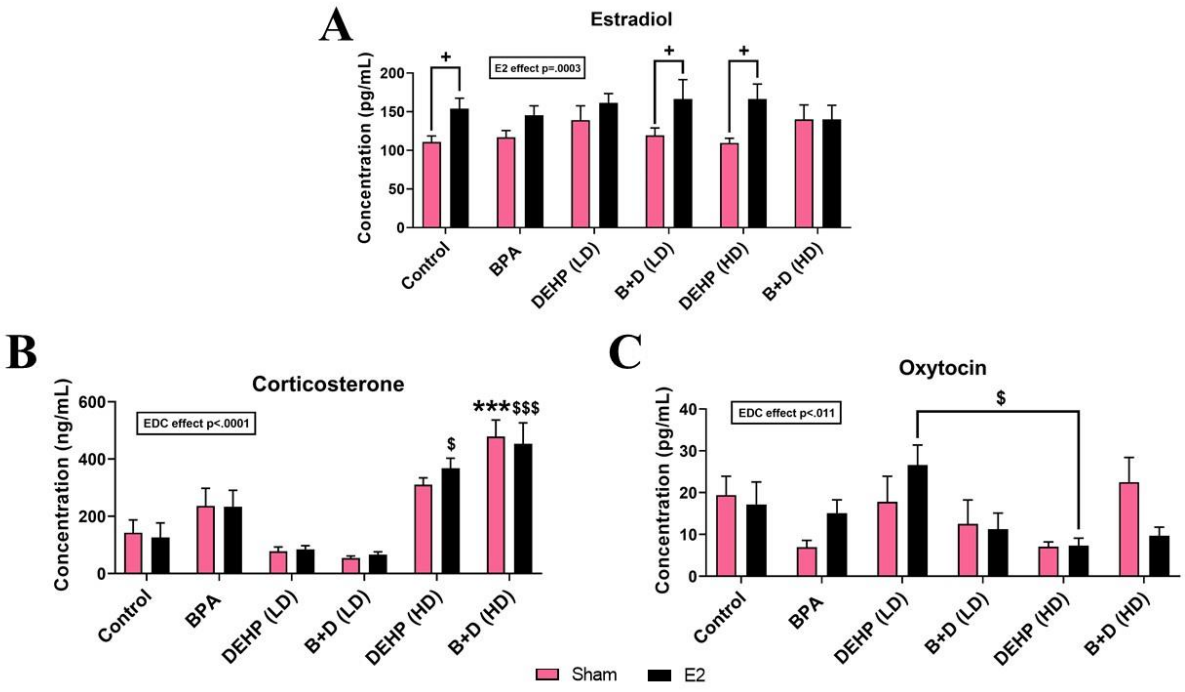

Figure 4.6. Circulating hormone levels in female rat offspring with prenatal EDC exposure and/or adult E2 treatment. (*A*) Serum estradiol (E2) levels (pg/mL), (*B*) serum corticosterone (CORT) levels (ng/mL), and (*C*) serum oxytocin (OXT) levels (pg/mL). Hormonal data were collected from adult female offspring. $^{***}p<0.001$, comparison between sham-implanted control and EDC-exposed female offspring. $^{\$}p<0.05$, $^{\$\$\$}p<0.001$, comparison between E2-implanted control and EDC-exposed female offspring. $^{+}p<0.05$, comparison between sham- and E2-

implanted female offspring of the same treatment group. Error bars represent the standard error of the mean (SEM).

Neurotransmitter levels in the paraventricular hypothalamic nucleus

Monoamine levels and DA turnover rates within the PVN are displayed in Figure 4.7. In PVN NE levels (Figure 4.7A), E2-treated control ($p = 0.036$) and BPA animals ($p = 0.025$) showed increased levels compared to their sham counterparts, and this increase was even more drastic in low-dose DEHP- ($p < 0.0001$) and B+D-E2 animals ($p < 0.0001$). Furthermore, DEHP (LD)-E2 females had higher NE levels than control-E2 females ($p = 0.038$), whereas DEHP (HD)-sham offspring had significantly increased NE levels than their control counterparts ($p = 0.050$). In 5-HT levels (Figure 4.7C), only control animals showed a significant difference, with E2-treated animals having substantially higher 5-HT levels than their sham counterparts ($p = 0.001$). This difference between sham and E2 animals was abolished in EDC-exposed offspring.

In addition, sham-implanted BPA, DEHP (HD), and B+D (HD) females had increased PVN DA levels compared to their E2-treated counterparts (Figure 4.7B). On the contrary, E2-treated offspring in all of the groups, with the exception of BPA, had significantly higher DA turnover compared to their sham counterparts (Figure 4.7D). Changes in DOPAC and 5-HIAA metabolites, as well as 5-HIAA/5-HT ratios, can be found in Table 4.3.

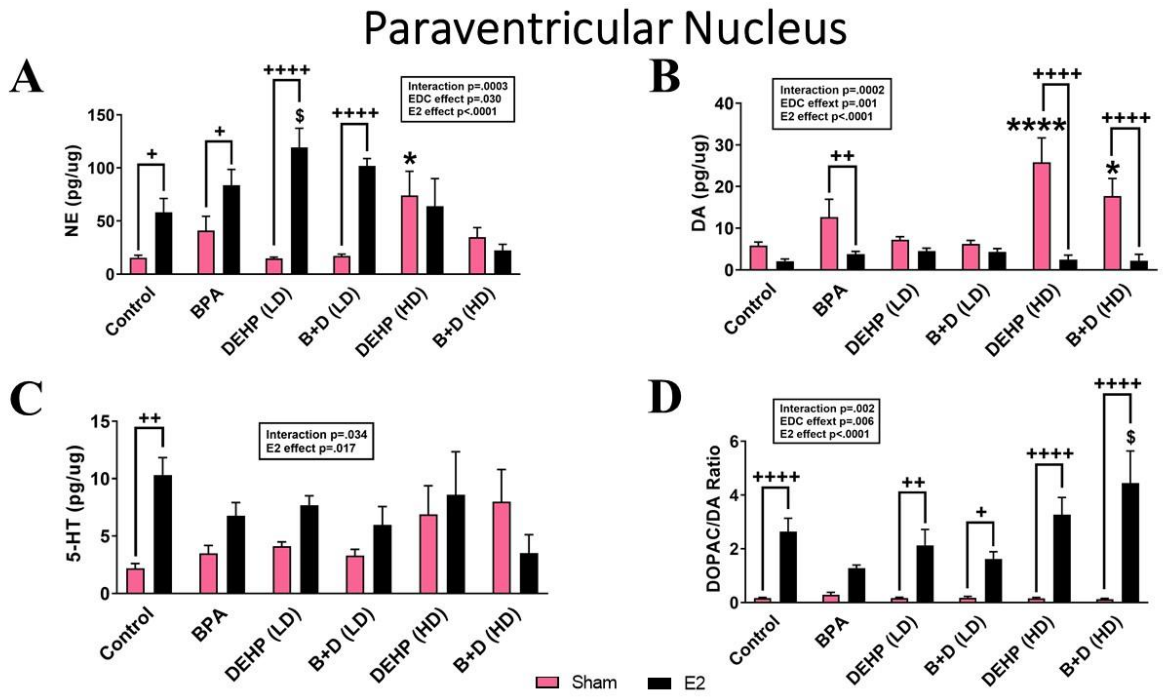

Figure 4.7. Effects of prenatal EDC exposure and/or adult E2 treatment on monoamine levels and monoamine turnover ratios in the paraventricular nucleus (PVN) of female rat offspring. (*A*) Norepinephrine (NE), (*B*) dopamine (DA), (*C*) serotonin (5-HT) concentrations (mean ± SEM; pg/µg protein), and (*D*) DOPAC/DA ratios (mean ± SEM) in the PVN are shown in the figure. $^{*}p<0.05$, $^{****}p<0.0001$, comparison between sham-implanted control and EDC-exposed female offspring. $^{\$}p<0.05$, comparison between E2-implanted control and EDC-exposed female offspring. $^{+}p<0.05$, $^{++}p<0.01$, $^{++++}$p<0.0001, comparison between sham- and E2-implanted female offspring of the same treatment group. Error bars represent the standard error of the mean (SEM).

Neurotransmitter levels in the hippocampus

Monoamine and metabolite levels within the HC are demonstrated in Figure 4.8. In terms of HC NE levels (Figure 4.8A), EDC exposure in B+D (LD) females with sham implantation produced higher concentrations of NE than their control counterparts ($p = 0.001$). Meanwhile,

control and BPA animals treated with E2 showed near-significant trends for increased NE levels than the corresponding sham offspring. DEHP (HD)-sham offspring, on the other hand, showed an EDC-induced increase in DA concentrations compared to controls ($p < 0.0001$) (Figure 4.8B). Interestingly, all sham-implanted offspring in vehicle and EDC-exposed groups had higher DA levels than their E2-treated counterparts. However, this pattern was reversed in terms of DA turnover, or DOPAC/DA ratio (Figure 4.8D). Finally, DEHP (HD)-E2 offspring displayed greater DOPAC levels (Figure 4.8C) relative to their control counterparts ($p = 0.001$). Serotonergic activity was entirely unaffected in the HC (Table 4.3).

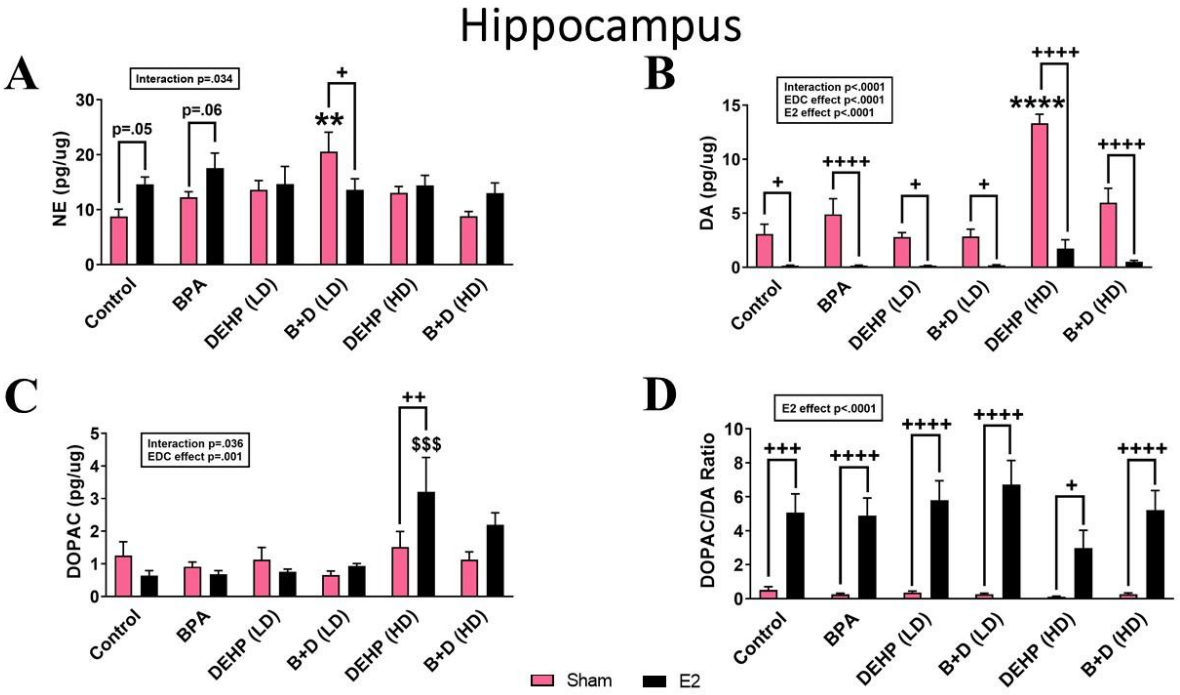

Figure 4.8. Effects of prenatal EDC exposure and/or adult E2 treatment on monoamine and metabolite levels and monoamine turnover ratios in the hippocampus (HC) of female rat offspring. (*A*) Norepinephrine (NE), (*B*) dopamine (DA), (*C*) 3,4-dihydroxyphenylacetic acid (DOPAC) concentrations (mean ± SEM; pg/µg protein), and (*D*) DOPAC/DA ratios (mean ± SEM) in the HC are shown in the figure. $^{**}p<0.01$, $^{****}p<0.0001$, comparison between sham-

implanted control and EDC-exposed female offspring. $^{\$\$\$}p<0.001$, comparison between E2-implanted control and EDC-exposed female offspring. $^{+}p<0.05$, $^{++}p<0.01$, $^{+++}p<0.001$, $^{++++}p<0.0001$, comparison between sham- and E2-implanted female offspring of the same treatment group. Error bars represent the standard error of the mean (SEM).

4.5. DISCUSSION

In this study, we aimed to determine the mechanisms underlying adverse neurobehavioral effects in females as a result of prenatal EDC exposure. We also wanted to understand how estradiol treatment in adult females interacts with prenatal EDC effects to further alter behavior, hormones, and brain neurotransmitters. Treatment with E2 in healthy control offspring did not affect locomotor activity or exploration, although it did increase anxiety-like behavior, but only in the EPM. This result is partially in accordance with a prior study from our lab [252], as well as other studies that have demonstrated elevated anxiety-like behavior in response to E2 in rodents [175, 255, 256]. In the previous study from our lab, adult female rats were treated with E2 at the same dose and duration. E2-treated rats were found to exhibit increased anxiety-like behavior in that study as well, but in the OFT rather than the EPM [252]. Nevertheless, our results contrast with a number of studies that have found diminished anxiety-related behaviors following E2 treatment [175, 257, 258].

Inconsistencies in anxiety-like behaviors across various behavioral paradigms have been reported before [259, 260]. The OFT and EPM independently evaluate different aspects of stress-related behaviors, including exploration and novelty exposure (as represented by the OFT) or unconditioned anxiety (as represented by the EPM) [183]. In addition, the effects of E2 on behavior vary depending on several factors, including the dose and duration of E2 exposure and

the age of the animal at the time of exposure [175, 261, 262]. In terms of defensive behavior, E2 treatment appeared to reduce active coping mechanisms in the form of decreased preference for burying and probe exploration. This finding contradicts other studies that have found a positive correlation between E2 administration and active coping behaviors [263, 264], or no correlation [265]. Nevertheless, several factors differ between our study and the aforementioned studies, including presence or absence of ovaries, dose of E2, and route of E2 administration. Furthermore, even though there were no significant differences between sham and E2-treated controls in immobility, control-E2 offspring spent slightly more time immobile than sham offspring in our study, suggesting a preference for passive coping [189].

These females also had significantly higher circulating E2 levels compared to their sham counterparts, as expected; however, no changes were observed in CORT or OXT levels. Considering that estradiol can influence hypothalamic-pituitary-adrenal (HPA) axis activity in an estrogen receptor subtype-dependent manner [266], as well as increase plasma OXT levels [267], it was surprising that neither CORT nor OXT were affected in control-E2 offspring. We can conclude that chronic treatment for 90 days with 20 ng/day of E2 increases unconditioned anxiety and reduces active defensive behaviors in healthy adult female rats.

Even though we did not observe any significant differences in cognition in the NOR in control animals, a near-significant trend in the recognition index ($p = 0.067$) was in fact identified in E2 offspring. In other words, E2-treated controls showed a trend for enhanced object recognition. Therefore, E2 appears to have a positive correlation with cognition and may boost cognitive abilities in adult females, which is consistent with previous studies [175, 258, 268]. An increase in sample size in our study may have produced statistically significant results.

Additionally, we also wanted to understand how chronic low-dose E2 affects monoaminergic activity in various brain regions involved in stress regulation. In the PVN, E2 increased NE levels, which was surprising because PVN NE concentrations and CORT levels represent HPA axis activation and are directly correlated, as NE infusions directly into the PVN have been shown to increase circulating CORT [269]. 5-HT infusions into the PVN are also capable of elevating CORT but to a lesser extent, and DA infusions have no impact. Yet our results revealed a disconnect between PVN noradrenergic activity and CORT. While E2 increased PVN NE levels in the control, BPA, and low-dose EDC groups, none of these groups showed changes in CORT. On the contrary, only the high-dose groups showed significant increases in CORT.

Furthermore, PVN 5-HT concentrations were only enhanced in E2-treated control offspring; this effect was eliminated in EDC-exposed offspring. Estrogens have been known to modulate serotonergic activity [270]. Moreover, the serotonergic system is involved in mood regulation and has long been implicated in mood disorders [271]. Within the PVN, specifically, E2 administration contributes to the desensitization of 5-HT1A receptors in female rats, which underlies selective serotonin reuptake inhibitor (SSRI) efficacy to treat mood disorders [272]. Our study demonstrates that E2 treatment enhances 5-HT signaling in the PVN to mediate behavior.

In addition, E2 treatment in our control females impacted dopaminergic activity in both the PVN and HC. This is partially consistent with a previous study from our lab, which also discovered anxiogenic effects coupled with reduced DA levels after E2 treatment [252]. Interestingly, in that study DA levels were only reduced in the central amygdala and not in the HC. Nevertheless, the hippocampus belongs to the limbic system along with the amygdala [273],

and chronic estradiol treatment is capable of reducing DA concentrations in limbic regions of the brain [274]. Our findings demonstrate that enhanced dopaminergic functioning in limbic regions may be necessary to reduce anxiety-like behaviors and promote active defensive behaviors.

In BPA offspring, while sham implantation led to anxiolytic effects in the OFT, E2 treatment increased anxiety-like behavior in this test. Contrastingly, E2 reduced anxiety-like behavior in the EPM in BPA-exposed offspring, which is arguably more stressful and riskier [275]. This may suggest an increase in risk-taking behaviors in females exposed to a combination of prenatal BPA and adult E2. In support of this theory, E2-treated BPA offspring also spent more time exploring the shock-probe in the SPDB, in contrast with control-E2 females, indicative of risky and inappropriate responses to the aversive stimuli. Research is lacking into the effects of BPA on risk-taking, but estradiol has been linked with increased risk-taking behaviors [276, 277]. Overall, we can conclude that BPA exposure alone and in combination with E2 in adulthood alters stress-related behaviors and produces inappropriate responses to stress.

Within the brain, E2 treatment in BPA offspring led to modest changes in the PVN and HC in comparison with sham BPA offspring, but these effects generally paralleled those of control offspring. One feature that must be highlighted is that BPA offspring appeared to be desensitized to the effects of E2 on DA turnover in the PVN, as increased DA turnover in response to E2 was abolished only in BPA offspring. BPA is known to induce estrogenic effects [278], although it can also serve as an antiestrogen by competing with endogenous E2 to block estrogenic responses [279, 280]. Correspondingly, BPA is also capable of inhibiting or antagonizing estrogenic activity within the brain when co-administered with E2 [281, 282]. Moreover, BPA could be interacting with E2 to produce epigenetic modifications. It has been

discovered that BPA can alter DNA methylation in the ERα gene in the hypothalamus and PFC of offspring, potentially underlying changes in anxiety-like behavior [143]. Hence, future studies should examine changes in gene expression and epigenetic mechanisms in BPA-exposed female offspring, especially those with E2 treatment in adulthood.

In offspring with prenatal exposure to DEHP, adult E2 treatment induced anxiogenic effects at both doses of DEHP in the OFT, although this effect was more pronounced in the HD offspring. In contrast, E2 offspring in the LD group had significantly decreased anxiety-like behavior in the EPM, while HD offspring were unaffected. DEHP (HD) exposure also reduced ambulation in sham offspring in the OFT; however, these offspring did not display changes in activity levels in the EPM. Therefore, there is no clear effect on locomotor activity in these females. While a plethora of studies exist in the literature illustrating alterations in anxiety-like behavior in females with developmental DEHP exposure [144, 145, 160, 161], ours is the first study to incorporate a dual exposure paradigm evaluating the cumulative behavioral effects of prenatal DEHP followed by adult E2.

In terms of hormones, DEHP offspring demonstrated dose-dependent effects on E2, CORT, and OXT levels. E2-treated DEHP (HD) females had significantly higher circulating E2 levels, which were coupled with increased CORT. Our results pertaining to CORT contrast with other studies that have found blunted CORT levels in DEHP-exposed females at baseline and after stress exposure [162, 242]. Yet, our findings do align with studies confirming that although DEHP suppresses E2 production [283], addition of exogenous E2 rescues this effect [284]. In addition, studies have shown an increase in CORT following E2 administration [285, 286]. We can speculate that the chronic E2 treatment may have elevated CORT levels in these offspring, thereby increasing their susceptibility to anxiety-like behavior in response to novelty.

More interestingly, LD and HD DEHP offspring with E2 treatment also exhibited significant differences in OXT levels relative to one another. DEHP (LD)-E2 females had increased circulating concentrations of serum OXT, whereas the inverse was observed in DEHP (HD)-E2 females. In rodents, OXT administration is associated with anxiolytic effects and suppressed HPA axis activation [287, 288]; similar effects are also observed in humans [289]. The results from our OXT analyses appear to support this and correspond with the behavioral effects observed in E2-treated DEHP females. This indicates that E2 treatment may interact with OXT in the body to mediate anxiety-like behavior, particularly in DEHP-exposed females.

In addition, our neurotransmitter data revealed dose- and E2-dependent effects on NE and DA. DA levels in both the PVN and HC were elevated only in HD sham offspring. This finding contrasts with previous studies that have found downregulated expression levels of dopamine receptor 2 in the brains of females with both low- and high-dose DEHP exposure [161, 290], indicative of lower dopaminergic activity. This effect has been demonstrated to persist transgenerationally [161]. E2-treated DEHP (HD) females, on the other hand, had significantly higher DOPAC levels. Since DOPAC is a major metabolite of DA and is formed as a result of DA catalysis [233], elevated hippocampal DA metabolism and turnover may be an underlying mediator of the increased anxiety-like behavior found in DEHP (HD) female offspring with E2 treatment. Overall, it is important to highlight that exposure to different doses of DEHP can lead to drastically distinct outcomes on behavior, hormones, and brain monoamines.

Similar to offspring treated with DEHP independently, exposure to a combination of BPA and DEHP yielded dose-dependent effects on behavior. In the OFT, B+D (HD) offspring engaged less in the center zone in general – indicative of anxiogenic effects – and E2-treated offspring in particular had a near-significant reduction in center zone entries. Moreover, LD

offspring instead showed altered behavior in the SPDB. Sham offspring preferred passive coping strategies, whereas their E2-treated counterparts engaged in active coping. This is significant because E2 treatment in B+D (LD) offspring completely reversed the effects observed in control offspring. Interestingly, E2-treated B+D offspring exposed to both doses exhibited an antianxiety effect in the EPM. E2 treatment also led to deficits in object exploration in LD offspring, without impacting object recognition. Overall, adult E2 treatment can interact with prenatal B+D exposure, particularly at a low dose, to alter behaviors and cognition.

In addition to behavior, dose-dependent alterations were also observed in hormones in B+D-exposed offspring. Expected increases in serum E2 levels were only discovered in LD offspring. The reasons pertaining to a lack of changes in E2 levels in the HD group are unclear. It is possible that treatment with a combination of BPA and DEHP (HD) desensitizes offspring to the effects of E2. Specifically, BPA may counteract the effects of DEHP (HD) on circulating E2, since only DEHP (HD)-E2 offspring showed increased serum E2. In contrast, B+D (HD) offspring, regardless of E2 treatment, had drastically elevated CORT levels. This finding is consistent with our OFT results, in which B+D (HD)-E2 females demonstrated increased anxiety-like behavior. Increased CORT may be an underpinning source of the anxiogenic effect. Moreover, although sham-implanted B+D (HD) offspring possessed greater levels of circulating CORT, they did not demonstrate any behavioral manifestations, implying that they may only be affected physiologically and not behaviorally. It is also possible that these offspring may instead show alterations in other facets of behavior and cognition not examined in the present study, including social behavior or spatial memory.

We also determined modest changes in brain neurotransmitter levels in B+D offspring. In general, B+D offspring seemed to mirror the effects observed in their LD or HD DEHP

counterparts, especially in the PVN. For example, B+D (HD)-sham offspring had significantly higher PVN DA levels, similar to DEHP (HD)-sham offspring. One study examined the effects of perinatal BPA and DEHP mixtures on activity of tyrosine hydroxylase, the rate-limiting enzyme of dopamine, in midbrain dopaminergic nuclei [205]. B+D mixtures counteracted the effects of the chemicals individually on DA activation, in contrast with our study. In the HC, an interesting effect noted was enhanced NE levels in sham-implanted B+D (LD) females. Hippocampal NE is released following novelty exposure and arousal, activating the locus coeruleus-noradrenergic system [247]. B+D (LD)-sham females showed enhanced center zone exploration in the OFT. It is possible that NE levels were elevated in the HC in response to novelty, which mediated the behavioral effects observed in these offspring.

In summary, healthy control females treated with chronic E2 in adulthood had increased unconditioned anxiety and engaged less in active coping strategies, but showed a trend for marginally enhanced object recognition. E2 treatment increased anxiety-like behavior in a majority of the EDC-exposed offspring in response to novelty in the OFT, but reduced unconditioned anxiety in the EPM. In the SPDB, sham-implanted EDC offspring were more affected, whereas E2 treatment only impacted BPA and B+D (LD) offspring. Low-dose E2-treated B+D offspring also showed alterations in object exploration in the NOR. These findings are indicative of aberrant behaviors following E2 treatment in offspring with prenatal EDC exposures. Finally, a variety of changes were apparent in response to EDC and/or E2 treatment in circulating hormones and monoamines in the PVN and HC. In conclusion, this study provides evidence that prenatal EDC exposures and adult exogenous E2 treatment cumulatively alter behavior, hormones, and brain monoaminergic activity in a dose-dependent manner. This calls

for additional review and modifications of current regulatory practices regarding harmful EDC exposures, and broadens the knowledge on chronic estradiol exposures in adult females.

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Table 4.1. Sample sizes of control or EDC-treated dams, and sham- or E2-treated female offspring.

	Control	BPA	DEHP (5 µg)	DEHP (7.5 mg)	BPA+DEHP (5 µg)	BPA+DEHP (7.5 mg)
Dams	7	9	6	6	6	7
Offspring						
Sham	7	7	6	6	6	6
E2	7	9	6	6	6	7

Note: BPA, bisphenol A; DEHP; diethylhexyl phthalate; EDC, endocrine disrupting chemical; E2, estradiol. Measures were obtained from dams and offspring treated with vehicle (Control), BPA (5 µg/Kg BW), low-dose DEHP (5 µg/Kg BW), high-dose DEHP (7.5 mg/Kg BW), BPA + low-dose DEHP (5 µg/Kg BW BPA + 5 µg/Kg BW DEHP), or BPA + high-dose DEHP (5 µg/Kg BW BPA + 7.5 mg/Kg BW DEHP).

Table 4.2. Behavioral data of sham and E2-treated female offspring following low-dose (5 µg) and high-dose (7.5 mg) prenatal EDC exposure.

Measure	Control		BPA (5 µg)		DEHP (5 µg)		BPA + 5 µg DEHP		DEHP (7.5 mg)		BPA + 7.5 mg DEHP	
	Sham	**E2**	**Sham**	**E2**	**Sham**	**E2**	**Sham**	**E2**	**Sham**	**E2**	**Sham**	**E2**
OFT												
Ambulation (% time)	21.6 ± 2.8	17.8 ± 1.8	17.6 ± 2.4	18.5 ± 1.9	22.5 ± 2.7	18.0 ± 1.2	18.2 ± 3.4	17.3 ± 1.1	15.0 ± 1.3	16.4 ± 1.2	16.4 ± 1.6	15.7 ± 1.6
Rearing (frequency)	82.7 ± 14.9	86.0 ± 10.0	69.2 ± 1.4	72.5 ± 4.0	80.2 ± 4.7	87.8 ± 10.7	91.7 ± 10.7	66.4 ± 3.9	114.0 ± 29.7	61.2 ± 2.5	83.2 ± 6.7	70.3 ± 9.2
Rearing (% time)	21.6 ± 4.4	27.4 ± 4.8	25.6 ± 4.7	18.8 ± 2.0	24.0 ± 2.7	25.2 ± 3.4	24.7 ± 2.5	23.5 ± 2.4	23.1 ± 2.2	19.6 ± 2.1	16.4 ± 1.4	17.5 ± 2.1
Perimeter zone (entries)	92.5 ± 12.9	78.6 ± 9.9	86.3 ± 7.5	92.1 ± 11.8	61.3 ± 4.1	87.7 ± 2.5	71.3 ± 12.6	72.3 ± 5.9	52.3 ± 8.1	76.8 ± 9.4	62.0 ± 6.2	75.6 ± 11.7
Perimeter zone (% time)	39.6 ± 6.5	33.4 ± 5.5	36.9 ± 4.8	41.1 ± 4.6	26.9 ± 6.2	39.7 ± 6.2	36.6 ± 2.5	38.4 ± 1.0	32.2 ± 8.8	37.8 ± 5.7	42.8 ± 6.2	49.4 ± 4.9
EPM												
Central platform (% time)	22.9 ± 4.6	11.9 ± 1.9	21.2 ± 3.6	17.5 ± 1.9	10.3 ± 1.5**	10.5 ± 1.2	10.8 ± 2.2**	13.4 ± 2.2	16.7 ± 4.7	17.2 ± 3.6	18.8 ± 2.4	21.2 ± 3.0$^{\$}$
Open arms (% time)	42.6 ± 5.4	27.5 ± 5.3	53.6 ± 7.8	45.3 ± 6.9	48.5 ± 8.6	57.2 ± 10.2	10.8 ± 2.3	39.3 ± 7.7	33.6 ± 5.1	44.4 ± 7.1	39.7 ± 6.9	46.8 ± 3.2
Closed arms (# of entries)	8.8 ± 1.0	10.8 ± 0.5	7.4 ± 1.6	10.0 ± 1.6	11.5 ± 1.8	9.3 ± 2.2	10.8 ± 2.4	10.5 ± 0.8	12.0 ± 0.8	9.7 ± 0.9	12.0 ± 2.1	9.0 ± 1.0
Closed arms (% time)	34.0 ± 8.0	60.0 ± 6.3	24.8 ± 5.7	36.9 ± 6.6	41.0 ± 7.4	32.1 ± 10.9	10.8 ± 2.5	46.7 ± 8.6	41.4 ± 7.5	38.1 ± 5.8	40.7 ± 7.3	31.6 ± 3.4
SPDB												
Burying (frequency)	25.2 ± 6.8^{+}	7.3 ± 3.1^{+}	9.7 ± 3.2	6.8 ± 1.7	7.0 ± 3.5*	18.2 ± 8.7	14.2 ± 7.2^{a}	30.7 ± 10.1$^{\$\$,a}$	21.2 ± 7.5	6.2 ± 2.0	10.3 ± 5.4	12.9 ± 5.0
Immobility (frequency)	12.7 ± 5.8	16.7 ± 2.2	17.7 ± 4.8	11.1 ± 1.4	24.7 ± 5.1*	24.8 ± 3.3	19.5 ± 4.8	12.8 ± 3.2	10.8 ± 4.5	9.2 ± 2.7	9.8 ± 4.0	8.0 ± 1.6
Probe exploration (frequency)	14.3 ± 4.4	10.1 ± 4.3	13.4 ± 3.1	12.1 ± 2.5	13.3 ± 4.9	4.5 ± 1.1	9.8 ± 3.0	9.2 ± 4.1	8.2 ± 2.3	5.3 ± 2.4	9.2 ± 2.1	9.1 ± 3.0
Rearing (frequency)	19.3 ± 4.8	12.1 ± 5.4	20.6 ± 4.3	17.3 ± 3.5	14.8 ± 3.9	8.0 ± 2.7	13.5 ± 5.5	12.7 ± 4.0	15.8 ± 4.0	8.8 ± 2.0	17.7 ± 4.0	15.2 ± 3.3

Grooming (frequency)	0.0 ± 0.0	0.0 ± 0.0	0.4 ± 0.3	0.6 ± 0.4	0.5 ± 0.3	0.2 ± 0.2	0.5 ± 0.3	0.0 ± 0.0	0.0 ± 0.0	0.7 ± 0.4	0.2 ± 0.2	0.6 ± 0.4
Grooming (% time)	0.0 ± 0.0	0.0 ± 0.0	0.5 ± 0.3	1.4 ± 0.9	0.7 ± 0.6	0.9 ± 0.9	0.5 ± 0.3	0.0 ± 0.0	0.0 ± 0.0	1.2 ± 0.9	0.1 ± 0.1	0.5 ± 0.4
Bedding height (cm)	7.8 ± 0.7	7.4 ± 0.6	6.3 ± 0.6	6.1 ± 0.3	5.9 ± 0.4	7.1 ± 0.8	6.8 ± 0.6	8.4 ± 0.6	6.4 ± 0.6	6.1 ± 0.4	6.3 ± 0.5	6.4 ± 0.6
Shock reactivity	1.7 ± 0.2	1.3 ± 0.2	1.1 ± 0.1	1.1 ± 0.1	1.0 ± 0.0	1.3 ± 0.2	1.0 ± 0.0	1.2 ± 0.2	1.3 ± 0.2	1.0 ± 0.0	1.0 ± 0.0	1.4 ± 0.2
NOR												
T1 Average Exploration (% time)	19.6 ± 4.6	21.3 ± 4.3	20.9 ± 1.2	19.4 ± 1.5	22.0 ± 1.6	20.0 ± 2.6	24.6 ± 2.5	15.1 ± 2.3	N/A	N/A	N/A	N/A
T2 Discrimination Index	0.01 ± 0.1	0.3 ± 0.1	0.2 ± 0.1	0.3 ± 0.1	0.2 ± 0.1	0.2 ± 0.1	0.2 ± 0.1	0.1 ± 0.2	N/A	N/A	N/A	N/A

Note: EDC, endocrine disrupting chemicals; E2, estradiol; BPA, bisphenol A; DEHP, di-(2-ethylhexyl) phthalate; OFT, open field test; EPM, elevated plus maze; SPDB, shock probe defensive burying; NOR, novel object recognition test. Data are presented as mean ± SEM. Data were analyzed using two-way ANOVA, followed by uncorrected Fisher's LSD post hoc analyses. * $p < 0.05$, ** $p < 0.01$, significantly different from Control-Sham group. $^{\$}$ $p < 0.05$; $^{\$\$}$ $p < 0.01$, significantly different from Control-E2 group. $^{+}$ $p < 0.05$, significant difference between Sham and E2-treated Control offspring. ^{a} $p = 0.05$, difference between Sham and E2-treated BPA + 5µg DEHP offspring.

Table 4.3. Neurotransmitter data of sham and E2-treated female offspring following low-dose (5 µg) and high-dose (7.5 mg) prenatal EDC exposure.

Neurotransmitter	Control		BPA (5 µg)		DEHP (5 µg)		BPA + 5 µg DEHP		DEHP (7.5 mg)		BPA + 7.5 mg DEHP	
	Sham	**E2**	**Sham**	**E2**	**Sham**	**E2**	**Sham**	**E2**	**Sham**	**E2**	**Sham**	**E2**
Paraventricular Nucleus												
DOPAC (pg/µg)	1.4 ± 0.4	4.6 ± 0.7	2.9 ± 1.2	4.6 ± 0.8	1.3 ± 0.4^{+++}	8.6 ± 1.8^{+++}	1.1 ± 0.3^{+}	6.1 ± 0.8^{+}	6.0 ± 2.2	6.0 ± 2.9	1.7 ± 0.5	3.7 ± 1.0
5-HIAA (pg/µg)	11.0 ± 1.8	36.9 ± 6.5	24.4 ± 6.2	20.3 ± 4.4	15.9 ± 1.4	26.2 ± 4.2	18.2 ± 1.0	17.7 ± 2.4	103.6 ± 42.4$^{***,++}$	28.3 ± 11.6^{++}	63.0 ± 30.2^{+}	15.7 ± 4.2^{+}
5-HIAA/5-HT ratio	5.7 ± 0.9	3.7 ± 0.4	7.9 ± 2.3^{+}	3.0 ± 0.3^{+}	4.1 ± 0.6	3.6 ± 0.7	6.0 ± 0.7	3.4 ± 0.4	17.3 ± 3.7$^{****,++++}$	4.1 ± 0.8^{++++}	7.0 ± 0.8	6.4 ± 1.2
Hippocampus												
5-HT (pg/µg)	2.0 ± 0.6	2.3 ± 0.3	1.8 ± 0.3	1.8 ± 0.3	2.4 ± 0.3	1.5 ± 0.3	2.7 ± 0.6	1.8 ± 0.4	1.4 ± 0.2	2.7 ± 0.6	1.3 ± 0.2	2.1 ± 0.5
5-HIAA (pg/µg)	8.1 ± 2.6	12.3 ± 1.0	9.5 ± 1.3	11.2 ± 1.8	10.0 ± 0.6	9.3 ± 1.3	11.8 ± 2.1	10.6 ± 1.1	13 ± 1.3	15.7 ± 1.6	10.7 ± 2.4	14.4 ± 2.4
5-HIAA/5-HT ratio	4.0 ± 1.0	5.8 ± 0.6	7.4 ± 2.3	6.6 ± 0.9	4.5 ± 0.6	7.1 ± 1.4	4.9 ± 0.8	7.1 ± 1.4	10.5 ± 2.6	6.8 ± 1.1	9.1 ± 1.5	8.2 ± 1.9

Note: EDC, endocrine disrupting chemicals; E2, estradiol; BPA, bisphenol A; DEHP, di-(2-ethylhexyl) phthalate. Data are presented as mean ± SEM. *** $p < 0.001$, **** $p < 0.0001$, significant difference between sham-implanted Control and EDC females, two-way ANOVA followed by Tukey's post hoc analyses. $^{+}$ $p < 0.05$, $^{++}$ $p < 0.01$, $^{+++}$ $p < 0.001$, $^{++++}$ $p < 0.0001$, significant difference between sham and E2-treated females of the same treatment group, two-way ANOVA followed by uncorrected Fisher's LSD post hoc analyses.

Table S4.1. Data from Chapter 4 figures. Data are presented as mean ± SEM.

	Control		BPA (5 µg)		DEHP (5 µg)		BPA + 5 µg DEHP		DEHP (7.5 mg)		BPA + 7.5 mg DEHP	
	Sham	**E2**	**Sham**	**E2**	**Sham**	**E2**	**Sham**	**E2**	**Sham**	**E2**	**Sham**	**E2**
Figure 4.2 - OFT												
Ambulatory distance	1316.8 ± 167.5	1176.8 ± 117.4	1196.8 ± 153.1	1250.9 ± 103.7	1337.3 ± 165.0	1329.9 ± 167.8	1140.9 ± 185.9	1148.7 ± 90.0	941.3 ± 76.0	1070.8 ± 67.2	1004.5 ± 87.1	938.3 ± 83.8
Center zone time	1.8 ± 0.4	2.2 ± 0.6	3.4 ± 0.7	1.1 ± 0.3	3.1 ± 0.7	1.5 ± 0.3	3.5 ± 0.7	3.4 ± 0.9	2.2 ± 0.5	0.4 ± 0.1	1.0 ± 0.2	1.5 ± 0.3
Center zone entries	29.3 ± 5.6	28.7 ± 5.8	26.4 ± 5.5	18.8 ± 1.7	42.5 ± 11.1	20.8 ± 3.2	31.17 ± 7.6	26.8 ± 3.3	23.8 ± 0.4	12.5 ± 3.5	17.7 ± 4.6	14.9 ± 1.5
Figure 4.3 - EPM												
Total arm entries	17.8 ± 1.5	15.7 ± 1.7	14.4 ± 1.4	18.3 ± 0.4	19.4 ± 0.6	18.8 ± 1.2	17.0 ± 1.2	18.3 ± 1.5	18.5 ± 1.5	15.8 ± 0.7	18.2 ± 1.3	18.3 ± 0.6
Open arm entries	11.6 ± 0.5	5.0 ± 0.3	9.3 ± 0.8	9.4 ± 1.3	9.5 ± 0.9	12.5 ± 2.2	8.7 ± 1.5	9.7 ± 1.7	8.8 ± 1.6	8.5 ± 1.2	8.8 ± 1.0	11.0 ± 0.7
Figure 4.4 - SPDB												
Burying time	27.3 ± 10.8	2.3 ± 1.0	5.5 ± 2.2	4.7 ± 1.4	4.0 ± 2.4	21.1 ± 11.1	13.6 ± 6.5	36.6 ± 13.1	22.0 ± 9.8	2.8 ± 0.9	9.8 ± 5.6	11.8 ± 5.4
Immobility time	2.2 ± 0.8	5.7 ± 1.5	10.2 ± 3.8	4.5 ± 0.6	17.8 ± 5.4	14.1 ± 2.9	17.1 ± 5.3	7.2 ± 3.6	4.1 ± 1.6	7.6 ± 2.8	2.4 ± 1.0	4.0 ± 1.0
Probe exploration time	7.7 ± 3.3	1.0 ± 0.6	9.7 ± 3.3	8.6 ± 2.1	5.3 ± 1.8	1.3 ± 0.4	2.4 ± 0.8	4.5 ± 2.6	4.3 ± 1.6	1.3 ± 0.7	5.1 ± 1.8	2.0 ± 0.8
Rearing time	11.1 ± 3.7	7.1 ± 3.2	14.5 ± 4.5	11.8 ± 2.3	7.9 ± 2.2	2.3 ± 1.1	6.4 ± 2.3	6.0 ± 2.4	8.8 ± 2.9	9.8 ± 2.9	10.3 ± 2.9	11.6 ± 3.4
Figure 4.5 - NOR												

T1 Discrimination index	-0.10 ± 0.09	0.10 ± 0.09	-0.04 ± 0.16	-0.07 ± 0.04	-0.01 ± 0.06	-0.11 ± 0.04	0.08 ± 0.04	-0.24 ± 0.08	-	-	-	-
T2 Recognition index	0.50 ± 0.05	0.64 ± 0.07	0.58 ± 0.02	0.65 ± 0.03	0.58 ± 0.05	0.62 ± 0.03	0.62 ± 0.05	0.55 ± 0.08	-	-	-	-
Figure 4.6 - Hormones												
E2	110.7 ± 8.0	153.9 ± 13.5	116.8 ± 8.8	145.5 ± 12.2	139.1 ± 18.4	161.7 ± 11.7	119.5 ± 9.5	166.3 ± 25.1	109.7 ± 5.9	166.3 ± 19.4	140.0 ± 18.7	140.0 ± 18.4
CORT	142.6 ± 44.7	125.6 ± 51.0	236.3 ± 61.6	233.7 ± 57.1	77.8 ± 14.8	84.7 ± 12.3	54.2 ± 7.2	65.5 ± 10.1	310.3 ± 24.2	367.9 ± 34.8	478.6 ± 57.6	453.5 ± 72.6
OXT	19.4 ± 4.5	17.2 ± 5.4	6.9 ± 1.6	15.1 ± 3.2	17.8 ± 6.1	26.6 ± 4.8	12.5 ± 5.7	11.3 ± 3.8	7.1 ± 1.1	7.3 ± 1.8	22.5 ± 5.9	9.7 ± 2.1
Figure 4.7 - PVN NTs												
NE	15.4 ± 2.5	58.3 ± 12.9	41.1 ± 13.3	83.7 ± 14.9	14.8 ± 1.2	119.1 ± 18.2	17.1 ± 1.7	101.9 ± 7.0	74.1 ± 22.7	63.8 ± 26.2	34.9 ± 8.9	22.3 ± 5.8
DA	5.8 ± 0.9	2.1 ± 0.5	12.7 ± 4.2	3.8 ± 0.7	7.2 ± 0.7	4.5 ± 0.7	6.2 ± 0.8	4.3 ± 0.8	25.8 ± 5.9	2.5 ± 1.1	17.7 ± 4.3	2.2 ± 1.5
5-HT	2.2 ± 0.4	10.3 ± 1.5	3.5 ± 0.7	6.8 ± 1.2	4.1 ± 0.4	7.7 ± 0.8	3.3 ± 0.5	6.0 ± 1.6	6.9 ± 2.5	8.6 ± 3.8	8.0 ± 2.8	3.5 ± 1.6
DOPAC/DA	0.17 ± 0.02	2.6 ± 0.5	0.29 ± 0.10	1.3 ± 0.1	0.17 ± 0.03	2.1 ± 0.6	0.18 ± 0.05	1.6 ± 0.3	0.15 ± 0.04	3.3 ± 0.6	0.13 ± 0.03	4.4 ± 1.2
Figure 4.8 - HC NTs												
NE	8.8 ± 1.3	14.6 ± 1.3	12.2 ± 1.0	17.6 ± 2.7	13.6 ± 1.7	14.7 ± 3.2	20.5 ± 3.6	13.6 ± 2.0	13.1 ± 1.1	14.4 ± 1.9	8.8 ± 0.9	13.0 ± 1.9
DA	3.1 ± 0.9	0.15 ± 0.05	4.9 ± 1.4	0.16 ± 0.03	2.8 ± 0.4	0.15 ± 0.02	2.8 ± 0.7	0.19 ± 0.05	13.3 ± 0.9	1.73 ± 0.81	6.0 ± 1.3	0.50 ± 0.13
DOPAC	1.3 ± 0.4	0.6 ± 0.2	0.9 ± 0.1	0.7 ± 0.1	1.1 ± 0.4	0.8 ± 0.1	0.7 ± 0.1	0.9 ± 0.1	1.5 ± 0.5	3.2 ± 1.1	1.1 ± 0.2	2.2 ± 0.4
DOPAC/DA	0.51 ± 0.19	5.1 ± 1.1	0.26 ± 0.06	4.9 ± 1.0	0.37 ± 0.08	5.8 ± 1.1	0.27 ± 0.05	6.7 ± 1.4	0.11 ± 0.03	3.0 ± 1.0	0.26 ± 0.07	5.2 ± 1.2

CHAPTER 5

PRENATAL EXPOSURE TO BISPHENOLS AFFECTS PREGNANCY OUTCOMES AND OFFSPRING DEVELOPMENT IN RATS [4]

[4] Kaimal et al. 2021. *Chemosphere*. 276:130118.

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5.1. ABSTRACT

The objective of this study was to evaluate the effects of gestational exposure to low doses of bisphenol A (BPA), bisphenol S (BPS), and bisphenol F (BPF) on pregnancy outcomes and offspring development. Pregnant Sprague-Dawley rats were orally dosed with vehicle, 5 µg/kg body weight (BW)/day of BPA, BPS and BPF, or 1 µg/kg BW/day of BPF on gestational days 6-21. Pregnancy and gestational outcomes, including number of abortions and stillbirths, were monitored. Male and female offspring were subjected to morphometry at birth, followed by pre- and post-weaning body weights, post-weaning food and water intakes, and adult organ weights. Ovarian follicular counts were also obtained from adult female offspring. We observed spontaneous abortions in over 80% of dams exposed to 5 µg/kg of BPF. BPA exposure increased Graafian follicles in female offspring, while BPS and BPF exposure decreased the number of corpora lutea, suggesting reduced ovulation rates. Moreover, BPA exposure increased male kidney and prostate gland weights, BPF decreased epididymal adipose tissue weights, and BPS had modest effects on male abdominal adipose tissue weights. Prenatal BPS exposure reduced anogenital distance (AGD) in male offspring, suggesting possible feminization, whereas both BPS and BPA induced oxidative stress in the testes. These results indicate that prenatal exposure to BPF affects pregnancy outcomes, BPS alters male AGD, and all three bisphenols alter certain organ weights in male offspring and ovarian function in female offspring. Altogether, it appears that prenatal exposure to BPA or its analogs can induce reproductive toxicity even at low doses.

5.2. INTRODUCTION

The adverse health effects of BPA, a ubiquitous endocrine disrupting chemical (EDC) in the environment, have been extensively investigated. There is abundant evidence associating BPA exposure with repercussions on development, reproduction, metabolism, and neurobehavior [136, 291-293]. Therefore, in an effort to phase out the use of BPA in consumer products, manufacturers have turned to the use of chemicals that are structurally similar to BPA in BPA-free products. Among the most commonly used substitutes of BPA are its structural analogues BPS and BPF [11].

BPS is used in industrial applications including certain agents found in cleaning products, and in thermal paper products such as cashier's receipts [17]. BPF is found in epoxy resins [18] and is a contaminant in a variety of fresh and canned foods including vegetables, meats, and dairy products [19, 20]. Both BPF and BPS are also used in a variety of applications such as structural adhesives, dental materials, electrical varnishes, industrial applications such as grouts, coatings, flooring, tank and pipe linings, and road and bridge deck sealants [11, 17]. They have additionally been detected in a variety of consumer products including food packaging and plastics, and in personal care items such as hair care products, lotions, and toothpaste [21]. In the environment, BPS and BPF are particularly prevalent in indoor dust, water, sediment, and sewage [39-42]. Exposure to these chemicals occurs through the dermal, oral, and inhalation routes. Consequently, these chemicals have been found in human urine samples in concentrations comparable to BPA [45]. Research into the health consequences of BPS and BPF is expanding, with increasing evidence identifying similarities in the adverse effects of these analogues with those of BPA itself [11].

Rodent studies examining the effects of perinatal exposure to BPS report dose-dependent changes in offspring body weight (BW) and organ weights. Male mice offspring with low-dose BPS treatment (100 ng/g BW) show increases in BW and specific organ weights [294]. Interestingly, male and female rats with perinatal exposure to very low doses of BPS (10 and 50 µg/kg BW) have lower food intake [295]. BPS can cross the placental barrier [296], but its ability to do so is ten times less than that of BPA [297]. The current Environmental Protection Agency (EPA) recommended no-observed-adverse-effect-level (NOAEL) for BPS is 10 mg/kg/day [298], which is relatively higher than the doses used in the aforementioned studies.

Studies investigating the effects of perinatal BPF exposure in rodent models to date have primarily focused on neuroendocrine, metabolic, oxidative stress, and behavioral endpoints in the offspring [36, 299, 300], but none have studied the gestational effects or offspring development. Nevertheless, studies examining direct exposure to high-dose BPF (20-750 mg/kg BW) report lower BWs in mature male and female rats coupled with increased organ weights [301, 302], and studies in male mice have used BPF at doses of 0.044 or 4.4 mg/kg/day and report less weight gain [303]. Although a NOAEL for BPF has not been published yet, the EPA has unofficially classified it as a strong developmental hazard [304] and a moderate reproductive hazard [305]. Moreover, BPF is known to cross the placental barrier and reach the fetus [306].

The prenatal period is a critical window of development [84] during which exposure to exogenous compounds – including EDCs – can impact fetal development. The fetus is particularly vulnerable during this sensitive period with limited capacity to metabolize and process these chemicals [93]. This may result in long-lasting tissue level changes that contribute to adverse health outcomes in adulthood [85, 307, 308]. In this study, we aimed to uncover pregnancy and developmental outcomes that result from prenatal exposures to BPA, BPS, and

BPF at environmentally relevant doses that are significantly lower than the established NOAEL doses and those used in prior studies.

5.3. MATERIALS AND METHODS

Animal Husbandry

Adult female and male Sprague-Dawley (SD) rats (3 months old) were obtained from Envigo (Indianapolis, IN). They were housed in light- (12:12 light-dark cycle) and temperature-controlled rooms (23 ± 2 °C, 50 ± 20% relative humidity) within accredited animal facilities. Animals were housed in polycarbonate cages and food (LabDiet 5053) and water were provided *ad libitum*. We did not control for bisphenol exposures from the environment (cages, water bottles etc.) since all animals were maintained in the same environment. All animal procedures were compliant with the National Institutes of Health's *Guide for the Care and Use of Laboratory Animals* and were approved by the Institutional Animal Care and Use Committees (IACUC) at the University of Georgia.

EDC Exposure Paradigm

The experimental design is depicted in Figure 5.1. Prior to mating, vaginal cytology was performed on the female breeders for 10 consecutive days to document the regularity of estrous cycles. Following this, a female in proestrus and a randomly assigned male were co-housed for 1 day. Mating was confirmed by the presence of a vaginal plug. The day of copulation was marked as GD 0. Each dam was randomly assigned to one of 4 different treatment groups: control (10µl Phosphate Buffered Saline or PBS; n=9), BPA (5µg/kg BW/day; n=6), BPF (5 or 1µg/kg BW/day; n=10), and BPS (5µg/kg BW/day; n=13). The dam was considered the experimental unit. At GD 6, females were orally exposed to environmentally relevant doses of EDC or vehicle.

The dams remained group-housed with others of the same treatment group for the duration of the exposure and were separated into individual cages on GD 22. The dams remained with their litters until weaning. The samples sizes varied between different treatment groups because the experiment was repeated twice, and animals from both studies are reported here. Furthermore, one BPS dam had only 4 pups. These offspring were not used in this study but were instead used in related experiments that are not reported here.

Fig. 1

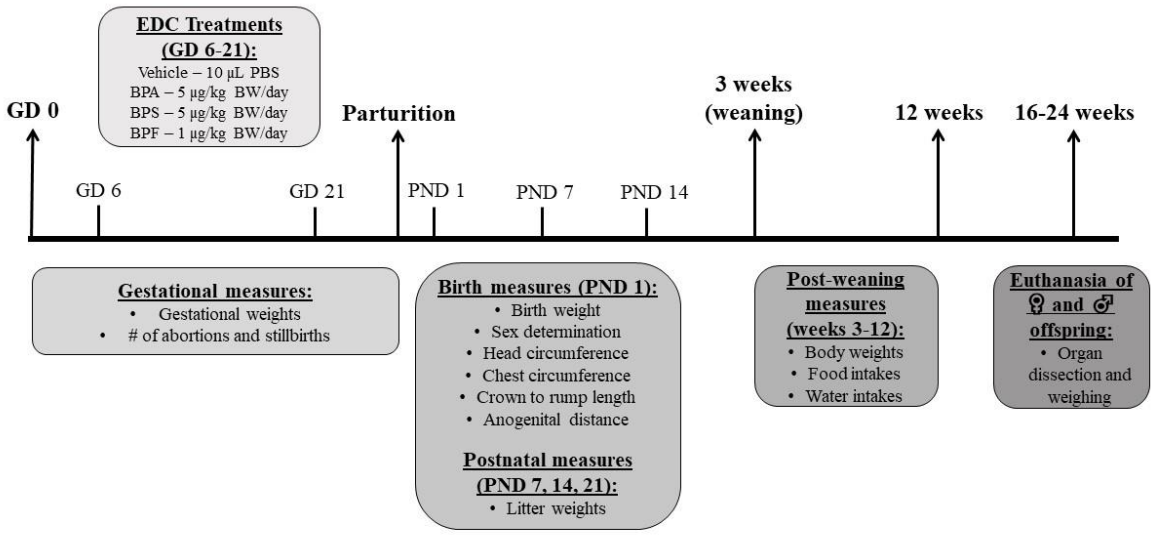

Figure 5.1. Schematic depicting the experimental design. Pregnant Sprague-Dawley dams were orally dosed daily from gestational days (GD) 6-21 with vehicle (control) (10 µL PBS; *n*=9), BPA (5 µg/kg/day; *n*=6), BPS (5 µg/kg/day; *n*=13), or BPF (5 or 1 µg/kg/day; *n*=7 and 10 respectively). Several measurements were obtained during gestation and on postnatal days (PND) 1, 7, and 14 as well as following weaning (week 3) until euthanasia (weeks 16-24). Note: EDC, endocrine disrupting chemical; PBS, Phosphate Buffered Saline; BPA, Bisphenol A; BPS, Bisphenol S; BPF, Bisphenol F; BW, body weight.

BPA (Catalog No. 239658; Lot MKBH2096V; ≥99% purity), BPF (Catalog No. 51453; Lot BCBQ5566V; ≥98% purity), and BPS (Catalog No. 43034; Lot BCBV2462; ≥98% purity) were obtained from Sigma Aldrich (St. Louis, MO). Stock solutions were made in dimethylsulfoxide (DMSO) to obtain complete dissolution. Doses were calculated daily based on BW and small aliquots were mixed with 10µl PBS for oral dosing. Daily oral dosing occurred from GD 6-21, during which the vehicle or EDC solution (~15µl) was discharged into the oral cavity with a micropipettor to avoid causing irritation to the gastrointestinal tract and potential stress to the pregnant dam. This procedure was relatively quick and induced minimal stress. All dams received vehicle or EDC treatments daily. Dams were group-housed (3 to a cage) based on treatment and it is likely that they were exposed to chemicals that were excreted in the feces and urine of their cage-mates. The BPS and BPA doses were selected because they are well below the EPA recommended NOAEL doses of 10 mg/kg/day for BPS and 5 mg/kg/day for BPA [115, 298]. The BPA dose we used is also 10-fold lower than the current daily reference dose of 50 µg/kg/day [114]. No NOAEL is currently set for BPF. However, the lowest-observed-adverse-effect level (LOAEL) for BPF is 20 mg/kg/day based on a sub-acute oral toxicity study [301], and a proposed tolerable daily intake value is 11 µg/kg/day for BPF [309]. The BPF dose we used for this study was significantly lower than both of these doses. The dams were separated into individual cages on GD 22, and they remained in separate cages with their litter until weaning.

Gestational and Offspring Measurements

The number of pregnant dams and number of abortions per dam were tracked. Body weights of dams were obtained daily from GD 6. Any sudden reduction in body weight and return to pre-breeding weight was considered an abortion. Each dam needed to gain an average

of 35 g to confirm pregnancy. Sudden weight loss below this body weight was considered an abortion. Dams that aborted typically stopped gaining weight by gestational days 15 or 16. The number of stillbirths and live births were recorded following parturition. Gestational index was defined as the ratio of the number of dams with live litters to the number of pregnant dams, and was calculated using the equation: (# of dams with live litters / # of pregnant dams) x 100. Stillbirth index was defined as the ratio of the number of stillbirths to the total number of pups on PND1, and was calculated using the equation: (# of stillborn pups / # of total pups born) x 100.

Pups were typically counted within 24 hours of initiation of the delivery process. Sex was determined on PND 1, and morphometric measurements were collected individually for the live pups. These measurements included head circumference, chest circumference, crown to rump length (measured from the midpoint on the top of the head to the base of the tail), and anogenital distance (AGD). Since it was not possible to identify the pups individually prior to weaning, weekly litter weights were collected on postnatal days (PND) 1, 7, 14, and 21 until weaning. Litter weights were divided by the litter sizes to obtain average pre-weaning BW for male and female pups, which is reported in this paper. After weaning, animals were identified by ear punches and individual BWs were obtained until they were 12 weeks old. Pups were housed by sex and litter, with three or four pups from the same litter per cage. BWs, food intakes, and water intakes were recorded at 3, 6, and 12 weeks of age. Post-weaning BWs were collected to determine if there was any catch-up growth in the event of intrauterine growth restriction.

Tissue Collection and Preparation

Adult male and female offspring in diestrus (as confirmed by vaginal cytology) were euthanized by rapid decapitation in adulthood (at 16-24 weeks of age). Blood was collected following euthanasia, and organs and tissues were dissected, weighed, and stored for further

processing. Organs collected included the pituitary gland, thymus, heart, lungs, liver, spleen, adrenal glands, kidneys, abdominal adipose tissue (AAT), epididymal adipose tissue (EAT) from males or ovarian adipose tissue (OAT) from females, perirenal adipose tissue (PAT), and reproductive organs (ovaries and uterus in females, paired testes, prostate glands, and seminal vesicles in males). All organ weights that were measured at the time of sacrifice were normalized to the body weight of the animals.

Oxidative Stress in the Testis of Weanlings

At the time of weaning, some pups from each treatment group were culled. Testes from male pups were fixed in formalin and subjected to immunohistochemistry (IHC) for 8-hydroxy deoxy guanosine (8-OHdG), a DNA oxidation product and a marker of oxidative stress [310]. Four µm sections were deparaffinized in xylene and rehydrated in graded alcohol and PBS. They were subject to permeabilization in 0.25% Triton in PBS for 10 minutes. Endogenous peroxidase activity was quenched using 3% hydrogen peroxide followed by blocking using PBS-Caesin for 1 hour. After rinsing in PBS-tween, sections were incubated with primary antibody (8-hydroxy deoxy guanosine antibody tagged with HRP; Santa Cruz biotechnology, Dallas, TX; Cat. No.SC393871 ; 1:100) overnight at room temperature. They were rinsed in PBS-tween followed by PBS before adding DAB substrate (Vector labs, Burlingame, CA). The sections were counter stained with methylene blue before dehydration and coverslipping. The slides were scanned at 40x using an Aperio AT2 digital whole slide scanner (Leica Microsystems, Buffalo Grove, IL) and the images were obtained using the Aperio eSlide viewer software.

Testosterone Measurement

Following euthanasia, trunk blood was collected from adult male rats, and the serum was separated and stored at -80°C for hormone assays. Serum testosterone levels were measured by a

double antibody radioimmunoassay (MP Biomedicals, Santa Ana, CA; SKU:0718910-CF) according to the manufacturer's protocol. 50 µl serum volume was used in duplicates. Values were expressed as ng/ml.

Morphometric Analysis of Ovaries

Four sections from the ovaries (4µm thick, 20 mm apart) were collected and stained with hematoxylin and eosin using standard protocols. The slides were scanned at 40x using an Aperio AT2 digital whole slide scanner (Leica Microsystems, Buffalo Grove, IL) and the images were analyzed using the Aperio eSlide Manager and viewer software. The follicles were characterized as primordial, primary, secondary, tertiary, Graafian, corpus luteum, or atretic, according to Myers et al. [311]. A primordial follicle was defined as an oocyte surrounded by a single layer of squamous cells. A primary follicle was defined as an oocyte surrounded by a single layer of cuboidal cells. A secondary follicle was defined as an oocyte surrounded by multiple layers of cuboidal granulosa cells with or without antral space development. A tertiary follicle was any follicle with a confluent antral space. A Graafian follicle was an oocyte located on a cumulus oophorous containing multiple layers of granulosa cells and a single, confluent, large antral space (size of the follicle is greater than 300µm). A corpus luteum is a dense body composed of luteal cells. Finally, atretic follicles were degenerating follicles with inflammatory cells and macrophages. The entire section was evaluated and the different follicles and corpora lutea were counted for statistical analysis.

Statistical Analysis

Prism 8.0 (GraphPad, Inc.) and R statistical software were used to perform statistical analyses. Chi-square tests of homogeneity were applied to the number of normal pregnancies, abortions, stillbirths and sex ratios. We used Tukey's post hoc analysis for multiple comparisons.

In addition, in exploratory data analysis, standard checks for heterogeneity of variances were performed and necessary transformations were applied, if needed. Gestational weight gain and stillborn weights were analyzed using one-way ANOVA. Gestational weight gain by day was analyzed using repeated measures two-way ANOVA, with treatment and time as variables. Differences in stillbirth index were analyzed using the Kruskal-Wallis test. Pre-weaning litter sizes and body weights were analyzed using analysis of covariance (ANCOVA), with treatment and time as covariates.

Morphometric measures, as well as post-weaning measures including BWs, food intakes, and water intakes were analyzed using a linear mixed effect model, with treatment as a fixed effect and dam as a random effect, followed by Tukey's multiple comparisons post hoc analyses to identify differences between the control and EDC groups. Serum testosterone levels, relative organ weights (organ weights normalized to body weight), and absolute organ weights were analyzed by one-way ANOVA, with treatment as a variable. Tukey's multiple comparisons post hoc test was used to identify differences between the control and EDC groups. Finally, the number of ovarian follicles per treatment group was analyzed by one-way ANOVA, followed by Tukey's multiple comparisons post hoc test. Prism software was used for ANOVA and Chi-Square tests, and R software was used to analyze linear mixed effect models and ANCOVA tests. P-value < 0.05 was considered to indicate a statistically significant difference. Data was expressed as mean ± standard error of mean (SEM).

5.4. RESULTS

Gestational and Birth Measurements

Figure 5.2 and Table 5.1 provide information regarding pregnancy outcomes. In the BPF group, dams were initially treated with a dose of 5 µg/kg/day; however, this dose produced spontaneous abortions in a majority (86%) of the dams ($X^2 = 32.3$, $p < 0.0001$) (Figure 5.2A). We were able to detect the abortions by closely monitoring the body weight of the dam throughout gestation. Dams treated with 5 µg/kg BPF stopped gaining weight within one week of treatment (around day 15 or 16 of pregnancy), suggesting that this dose of BPF was lethal to the developing fetus (Figure 5.2B). Therefore, the BPF dose was lowered to 1 µg/kg/day for a new set of dams, and the abortion rate was reduced from 86% to 9.1% (Table 5.1). As a result, the gestational index increased from 14% in the high dose BPF group to 90% in the low dose BPF group (Table 5.1). In comparison, the gestational index was 100% in the other treatment groups.

Furthermore, dams treated with BPA, BPS, and 1 µg of BPF gained weight at rates comparable to control dams throughout the gestational period (Figure 5.2C). In contrast, dams exposed to 5 µg of BPF showed a drastic decrease in gestational weight gain ($F = 6.6$; $p = 0.0003$), providing further confirmation that a majority of the pregnancies in this group resulted in abortions. Finally, BPS- and BPF (1 µg)-treated dams had stillborn pups, while the control and BPA groups had none. Stillborn pups were observed in 2 out of 13 BPS dams and 2 out of 11 low dose BPF dams (Table 5.1).

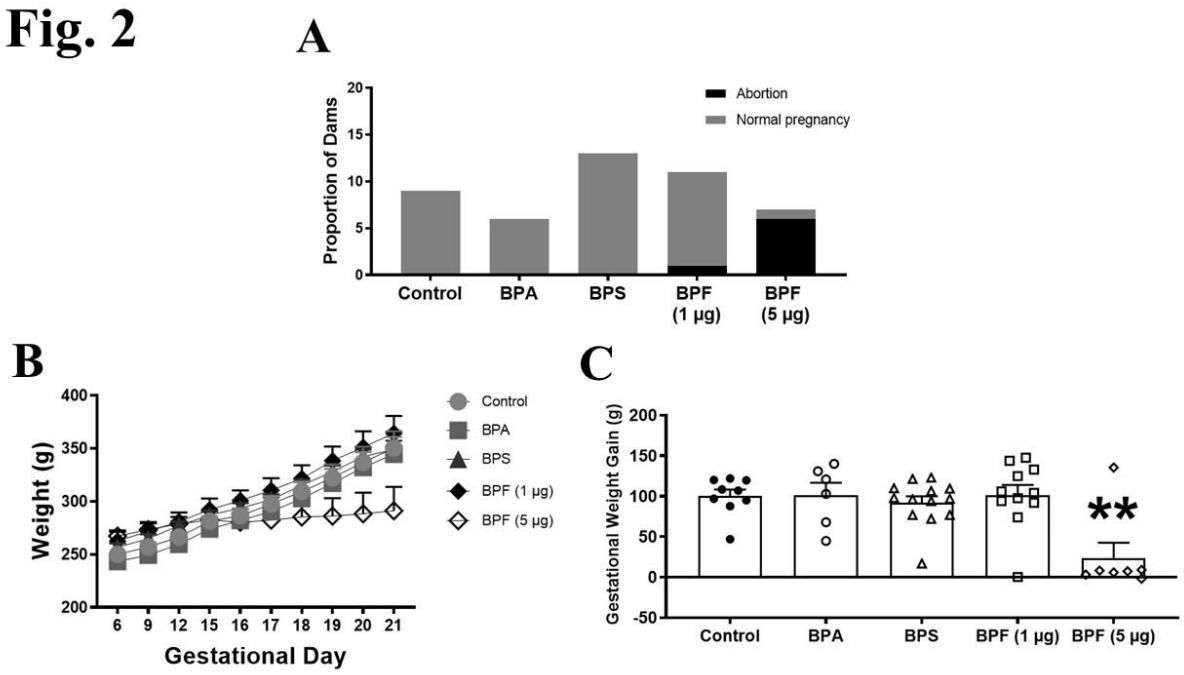

Figure 5.2. Pregnancy outcomes and weight gain during gestation in dams following exposure to vehicle or bisphenols during gestational days 6-21. (*A*) Proportion of dams with normal pregnancies or abortions (complete litter loss), (*B*) gestational weight gain per day, and (*C*) overall gestational weight gain in dams treated with vehicle (control) (*n*=9), BPA (5 µg/kg BW; *n*=6), BPS (5 µg/kg BW*;* *n*=13), high dose BPF (5 µg/kg BW; *n*=7) or low dose BPF (1 µg/kg BW; *n*=11) during pregnancy. $^{**}p<0.01$, one-way ANOVA, followed by Tukey's multiple comparisons between bisphenol groups and the control group.

In terms of pre-weaning growth parameters, there were no significant effects of treatment on litter size or body weight (Table 5.2). There were also no differences in the sex ratio between the treatment groups (Table 5.2).

Morphometric Measurements

Table 5.2 and Figure 5.3A list the measurements obtained at birth from offspring of each group including head circumference, chest circumference, crown to rump length, and anogenital

distance (AGD). A significant treatment effect was observed in male AGD (p = 0.02) (Figure 5.3A). Male offspring exposed to BPS had lower AGD (0.26 ± 0.01) than control males (0.32 ± 0.02; p = 0.02). No significant differences were observed in female AGD or any of the other morphometric measures (Table 5.2).

Oxidative Stress in the Testis of Weanlings and Adult Serum Testosterone

Since BPS male offspring showed a reduction in AGD, oxidative stress in the testes and serum testosterone levels were next examined. Figure 5.3B depicts representative IHC images of the testis from weanlings and Figure 5.3C shows serum testosterone levels from adult male rats. There was an increase in the production of 8-OHdG, a marker of oxidative stress, in BPA- and BPS-exposed testes. From their location in the germinal epithelium, it appears that the cells with marked levels of 8-OHdG are primary spermatocytes. On the contrary, control and BPF males did not show oxidative stress in the testes. No significant differences were observed in testosterone levels at postnatal weeks 16-24.

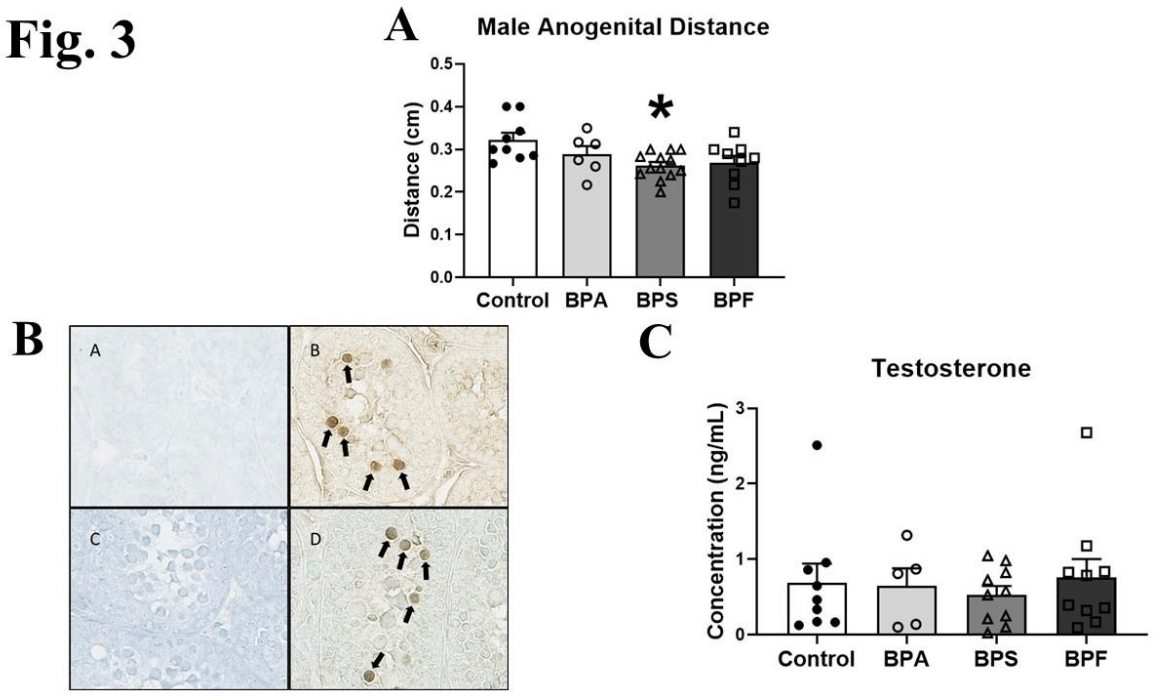

Figure 5.3. Reproductive measures from male offspring with prenatal exposure to vehicle or bisphenols. (*A*) BPS exposure reduced anogenital distance in male offspring at PND 1 (Control: *n*=9, BPA 5µg/kg BW: *n*=6, BPS 5µg/kg BW: *n*=13, BPF 1µg/kg BW: *n*=9). (*B*) Representative IHC images of testes sections show accumulation of 8-OHdG in the seminiferous tubules of male offspring with BPA (panel B) and BPS (panel D) exposure. (*C*) Serum testosterone levels were not significantly different between treatment groups in adulthood (Control: *n*=9, BPA 5µg/kg BW: *n*=5, BPS 5µg/kg BW: *n*=10, BPF 1µg/kg BW: *n*=10). $^{*}p<0.05$, linear mixed effect model, followed by Tukey's multiple comparisons between control and EDC groups. Error bars represent the standard error of the mean (SEM).

Post-Weaning Measurements

Table 5.3 displays post-weaning BWs, food and water intakes of male and female offspring at 3, 6, and 12 weeks of age. No significant differences were observed in any of the post-weaning measures.

Organ Weights

Table 5.3 provides the relative organ weights (g/kg BW, mean ± SEM) as well as absolute organ weights (g, mean ± SEM) obtained from adult male and female offspring. Modest treatment effects were observed in the relative weights of kidneys ($F = 3.8$; $p = 0.02$) (Figure 5.4A) and epididymal adipose tissue (EAT) ($F = 3.1$; $p = 0.04$) (Figure 5.4C) in male offspring. Male offspring exposed to BPA (7.15 ± 0.24) had slightly increased kidney relative weights compared to control males (6.53 ± 0.09; $p = 0.02$). Relative EAT weights were markedly reduced in male offspring exposed to BPF (6.83 ± 0.96) compared to controls (10.19 ± 0.75; $p = 0.03$).

Male offspring exposed to BPF had lower EAT absolute weights (2.88 ± 0.45; $p = 0.04$) (Table 5.3). On the other hand, there were no apparent effects of EDCs on female kidneys

(Figure 5.4B) or ovarian adipose tissue (OAT) (Figure 5.4D). While there were no effects of EDC exposure on the testes, significant treatment effects were apparent in the absolute weights (F = 3.0; p = 0.04) (Figure 5.5C) as well as relative weights (F = 3.4; p = 0.03) (Figure 5.5D) of the prostate gland in male offspring. Male offspring prenatally exposed to BPA had significantly higher prostate gland absolute weights (1.11 ± 0.11; p = 0.04) and relative weights (2.54 ± 0.29; p = 0.03). Finally, significant treatment effects were apparent in the absolute weights (F = 3.3; p = 0.04) and relative weights (F = 3.1; p = 0.04) of the seminal vesicles, but no differences between any of the treatment groups were found following post hoc analyses (Table 5.3). No significant differences were observed in the other organ weights as well (Table 5.3).

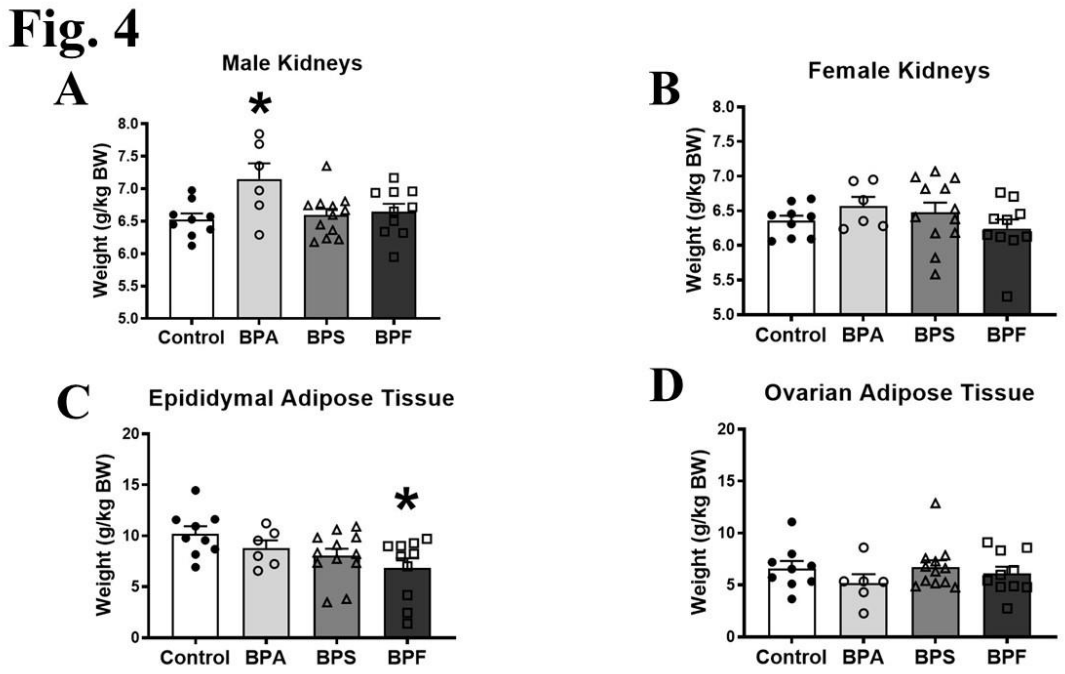

Figure 5.4. Kidney and gonadal adipose tissue weights of adult male or female offspring with prenatal exposure to vehicle or bisphenols. Offspring were exposed to vehicle (control) (n=9), BPA (5µg/kg BW; n=6), BPS (5µg/kg BW; n=12), or low dose BPF (1µg/kg BW; n=10) *in utero*. BPA exposure increased relative weights of the kidneys (*A*) and BPF exposure

decreased relative weights of the epididymal adipose tissue (*C*) in male offspring. In contrast, no changes were observed in female organ weights, including kidneys (*B*) or ovarian adipose tissue (*D*). $^{*}p<0.05$, one-way ANOVA, followed by Tukey's multiple comparisons between control and EDC groups. Error bars represent the standard error of the mean (SEM).

Fig. 5

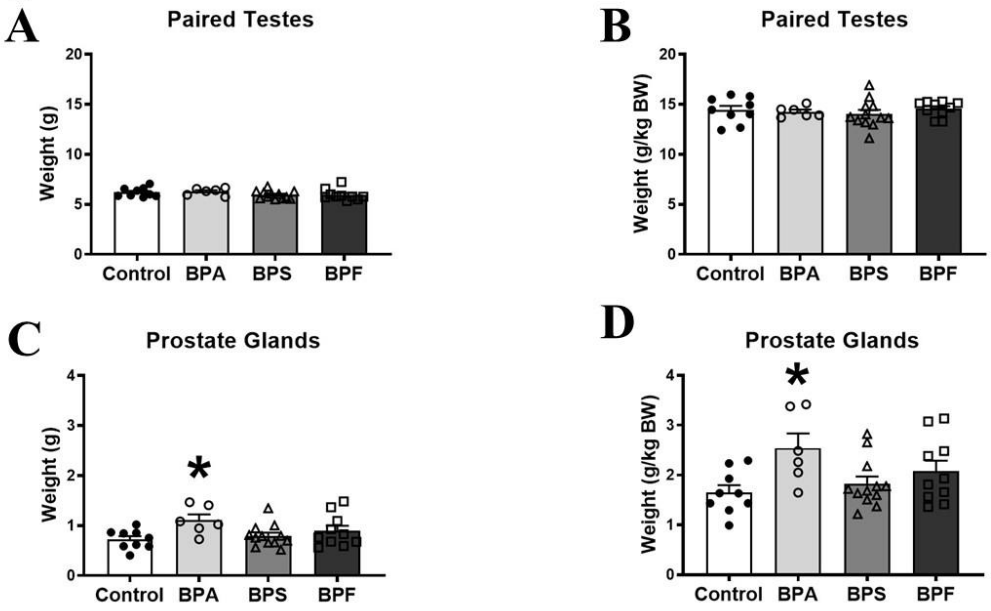

Figure 5.5. Paired testes and prostate gland weights of adult male offspring exposed to EDCs *in utero*. Prenatal EDC exposure did not alter absolute weight (*A*) or relative weight (*B*) of the testes. However, prenatal exposure to BPA increased prostate gland absolute weights (*C*) and prostate gland relative weights (*D*). Organs were harvested from male offspring prenatally exposed to vehicle (control) (*n*=9), BPA (5µg/kg BW; *n*=6), BPS (5µg/kg BW; *n*=12), or BPF (1µg/kg BW; *n*=10) when they were 16-24 weeks of age. $^{*}p<0.05$, one-way ANOVA, followed by Tukey's multiple comparisons between control and EDC groups. Error bars represent the standard error of the mean (SEM).

Ovarian Morphology

Figures 5.6 and 5.7 depict changes in ovarian structures observed in each treatment group. A non-significant trend for a treatment effect was apparent in the number of Graafian follicles ($F = 2.5$; $p = 0.08$) (Figure 5.6A). A trend for increased number of Graafian follicles was apparent in the BPA group compared to control (2.6 ± 0.43 vs. 0.7 ± 0.47; $p = 0.05$). Additionally, a significant treatment effect was found in the number of corpora lutea (CL) ($F = 6.1$; $p = 0.002$) (Figure 5.6C). Prenatal exposure to BPS (9.5 ± 2.41; $p = 0.01$) significantly reduced the number of CL when compared to control offspring (20.1 ± 2.26), and BPF (11.9 ± 1.35) had the same effect, but it did not reach statistical significance ($p = 0.07$). BPA offspring did not show any differences in CL compared to controls.

Finally, there was a significant treatment effect ($F = 3.4$; $p = 0.03$) in the number of primary follicles (Table 5.4); however, post hoc analyses revealed no significant differences between treatment groups. No significant differences were observed in the number of atretic follicles (Figure 5.6B), old CL (Figure 5.6D), primordial, secondary, or tertiary follicles (Table 5.4).

Fig. 6

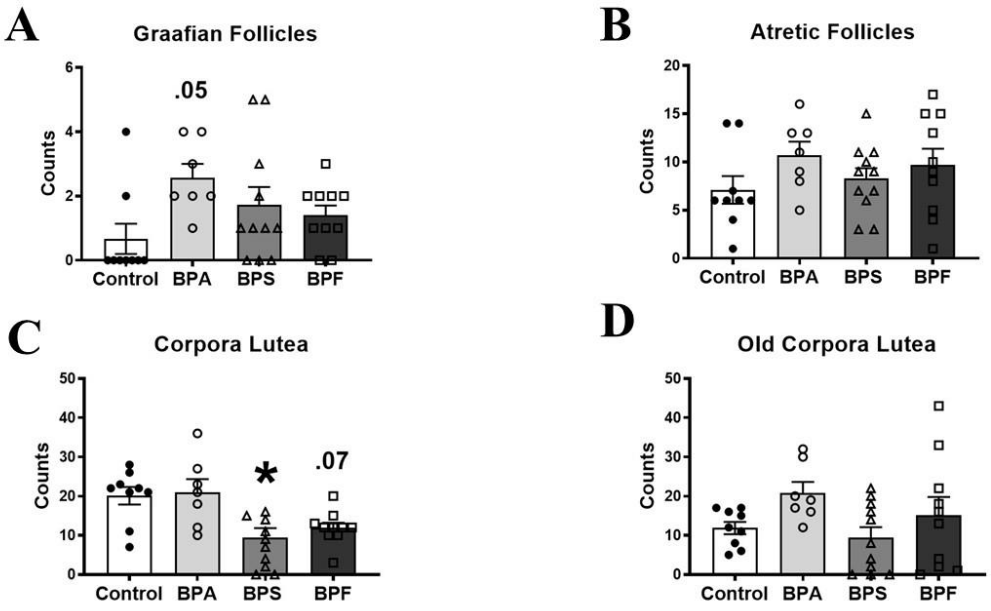

Figure 5.6. Ovarian follicle counts from adult female offspring exposed to BPA, BPS or BPF *in utero*. Numbers of Graafian follicles (*A*) atretic follicles (*B*), CL (*C*) and old CL (D) from female offspring after prenatal exposure to vehicle (control) (*n*=9), BPA (5µg/kg BW; *n*=7), BPS (5µg/kg BW; *n*=11), or low dose BPF (1µg/kg BW; *n*=10). Data were obtained from ovaries of adult female offspring sacrificed when they were in diestrus. $^{*}p<0.05$, one-way ANOVA, followed by Tukey's multiple comparisons between BPS and the control group. Error bars represent the standard error of the mean (SEM).

Fig. 7

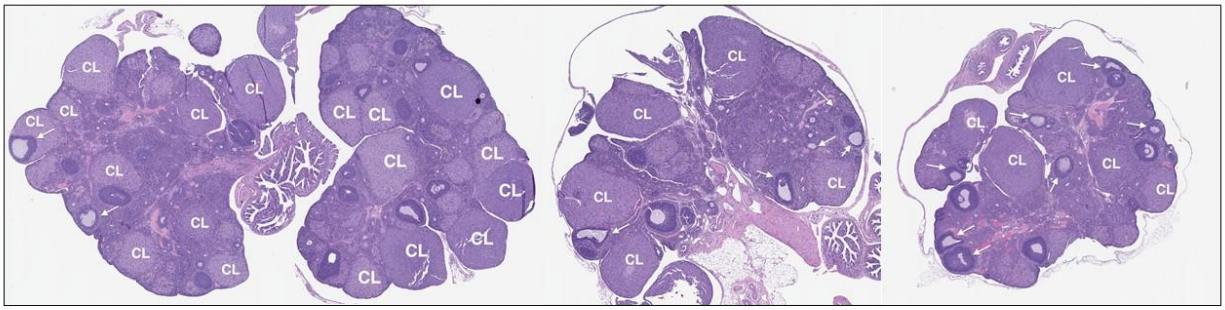

Figure 5.7. Representative sections of the ovary from the different treatment groups. CL: corpus luteum; arrows indicate atretic follicles. Ovaries were obtained from adult female offspring sacrificed when they were in the state of diestrus.

5.5. DISCUSSION

The results of this study indicate for the first time that BPS and BPF even at low doses were capable of producing significant effects on the reproductive system. While BPF at doses comparable to BPA and BPS (5µg/kg BW) induced spontaneous abortions in pregnant dams, lower doses of BPF (1µg/kg BW) still induced abortions, but at a significantly lower rate. Both BPF and BPS decreased the number of CL in the ovaries suggesting a possible reduction in ovulation. BPA on the other hand, produced a modest increase in Graafian follicles. In male offspring, BPS decreased the anogenital distance in male offspring which could indicate possible feminization or compromised testicular function while BPF reduced epididymal adipose tissue

weights. Moreover, exposure to BPA increased kidney and prostate gland weights. Taken together, it appears that prenatal exposure to low doses of BPA or its analogues is capable of inducing reproductive toxicity both in male and female offspring.

Our findings regarding the gestational outcomes are among the most insightful results of this study. We discovered that even a dose as low as 5 µg/kg/day of BPF could lead to abortions in 86% of the dams, indicating its high potential for reproductive toxicity. Lowering the dose to 1 µg/kg/day produced a dose-dependent reduction in the abortion rate, indicating that this is a very real effect. Since the dams were group housed, it is likely that they were exposed to additional BPF that was excreted in the urine and feces of their cage-mates. Also, the estrogenic potential of BPF is similar to or slightly greater than that of BPA [11], probably contributing to its ability to induce abortions. Human studies have associated BPA exposure with recurrent spontaneous abortions [312], and rodent studies further show that gestational BPA exposure at doses of 5-40 mg/kg/day are correlated with increased rates of abortion in mice [313, 314]. Recent studies investigating the mechanisms by which BPA produces abortions indicate that BPA may disrupt blastocyst formation and increase generation of reactive oxygen species that contribute to mitochondrial and DNA damage in the developing embryo [315]. In rats, the first heart beat is evident on day 11 of gestation and certain organs such as the lung and liver are detectable with ultrasound from day 16 [316]. Since the dams began to lose weight by GD15, it is safe to say that BPF produces an acute toxic effect on the developing vital organs in the embryo leading to abortions. Interestingly, BPA and BPS did not cause abortions at the same dose indicating that BPF is a more potent developmental toxicant. Further studies are needed to determine the possible underlying mechanisms.

Besides inducing spontaneous abortions, BPF along with BPS also decreased the number of corpora lutea (CL). This is the first report to indicate a reduction in CL number with prenatal BPS and BPF exposure which would suggest inhibition of ovulation [317]. Ovulation is a complex process that involves a number of hypothalamic neurotransmitters, releasing hormones and pituitary hormones. Luteinizing hormone (LH) and follicle stimulating hormone (FSH) play an important role in ovulation and it is possible that prenatal exposure to BPF and BPS inhibits their secretion. Another reason for the reduction of these hormones could be the fact that BPF and BPS have higher progestogenic activity than BPA [80]. Progesterone is known to suppress both FSH and LH secretion [318]. Since the exposure to these EDCs occurred *in utero*, it is likely that they altered the expression of hormone receptors that manifest in adulthood possibly as reduced secretion of LH and FSH and lower rates of ovulation. In contrast to BPS and BPF, prenatal exposure to BPA increases the number of Graafian follicles. It is possible that BPA could have stimulated the hypothalamic-pituitary-gonadal axis due to its weak estrogenic action [319]. It will be useful to assess the levels of gonadotrophic and gonadal hormones in these offspring.

Even though EDC-induced changes did not affect other morphometric parameters, we observed a modest reduction in the AGD of male offspring prenatally exposed to BPS. No prior studies to our knowledge have explored the relationship between prenatal BPS exposure and AGD in the offspring. Perinatal treatment with BPA at low (50 µg/kg) [320] and high doses (0.25-50 mg/kg) [321] have been shown to decrease AGD in male offspring. This effect has been observed in humans as well [322, 323], causing further concern about BPA exposure during gestation. AGD is a sensitive biomarker of fetal androgen exposure and could predict testicular development and function in later life [324, 325]. A reduction in AGD suggests feminization of

the male reproductive tract [326]. However, we did not observe any significant changes in serum testosterone levels in adult animals. Interestingly, we observed an increase in the accumulation of 8-OHdG in the BPA and BPS-exposed groups, indicative of oxidative stress possibly in primary spermatocytes within the seminiferous tubules. These changes were apparent in male offspring at the time of weaning. Further studies are needed to determine if prenatal exposure to BPA and BPS do alter the male reproductive system/function in adult animals.

Male offspring exposed to BPA had higher relative kidney weights compared to control males. This is supported by another study that used much higher doses of BPA and found increases in the weight of the liver, adrenal, spleen, pituitary and brain besides the kidney [327]. The reason for the increase in kidney weight is not clear, however, it could suggest altered tissue function as seen in diabetes or obesity [328]. Other than the kidney, prenatal exposure to BPA also increased the weight of the prostate gland. This is supported by studies in mice where gestational exposure to BPA at low doses of 2-50 µg/kg/day [329-331] increased prostate weights. This is especially concerning because prenatal BPA exposure was found to increase the risk for prostate cancer in rats [332] and pre-cancerous lesions of the prostate have been observed in male rats after prenatal exposure to BPA [333].

While prenatal exposure to BPA increased kidney and prostate gland weights, exposure to BPS significantly reduced epididymal fat weight without affecting other fat depots. This is in contrast to another study that found lower visceral adipose tissue mass in male offspring only due to reduced food intake [295]. We did not observe any change in food intake in male or female offspring after prenatal BPS exposure. Further analysis of the metabolic parameters is essential to determine why other fat depots were not affected with BPS exposure.

Finally, differences in the rodent models used, doses of EDCs, and duration of exposure (prenatal vs. perinatal) could have all contributed to the differences in observations in terms of organ weights compared to previous studies [327, 334]. In addition, a major limitation of our study was that the pregnant dams were group housed while receiving EDC treatment. We did not control for any potential contamination of the housed animals with bisphenol metabolites that may have been released through urine or feces. However, all animals used in this experiment were housed in similar cages and were provided water in glass bottles. Therefore, any bisphenol exposure from the environment would have been similar across treatments. The only way to examine this is to measure the bisphenol levels in the animals and determine any differences between EDC-exposed animals and control animals.

Regardless, results from this study provide robust associations between prenatal programming with low doses of BPF or BPS and adverse effects on gestational outcomes, offspring morphometry and changes in organ weights that are apparent in adulthood. These results are concerning because BPF and BPS appear to exert actions different from those of BPA, and require further investigation. To our knowledge, this is the first study to address the sex-specific differences in developmental parameters of male and female offspring with prenatal low-dose BPS and BPF exposures. These studies underline the need to revisit current regulatory practices on EDCs with the hope that they are appropriately modified to protect public health.

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Table 5.1. Pregnancy outcomes of control and treated dams.

Parameter	Control	BPA (5µg)	BPS (5µg)	BPF (5µg)	BPF (1µg)
Pregnant dams (n)	9	6	13	7	11
Normal Deliveries (n)	9	6	13	1	10
Total number of offspring (n)	83	85	160	17	133
Abortions (% of dams)	0	0	0	86.71	9.09
Stillbirths and live births					
Total live offspring (n)	83	85	158	17	130
Live birth index/dam (%)[a]	100 ± 0	100 ± 0	98.75 ± 0.88	100 ± 0	97.74 ± 1.29
% of dams with stillbirths	0	0	15.38	0	18.18
Total number of stillborn pups (n)	0	0	2	0	3
Stillbirth index/dam (%)[b]	0	0	1.25 ± 0.88	0	2.26 ± 1.29
Average stillborn weight (g)	-	-	0.95 ± 0.64	-	1.04 ± 0.70
Gestational index (%)[c]	100	100	100	14.29	90.91

Note: BPA, bisphenol A; BPS, bisphenol S; BPF, bisphenol F. Measures were obtained from offspring with prenatal exposure to Vehicle (control), BPA (5 µg/Kg BW), BPS (5 µg/Kg BW), high-dose BPF (5 µg/Kg BW), or low-dose BPF (1 µg/Kg BW). Data are presented as mean ± SEM. [a] Livebirth Index = (# of live pups / # of total pups born) x 100. [b] Stillbirth Index = (# of stillborn pups / # of total pups born) x 100. [c] Gestational Index = (# of dams with live litters / # of pregnant dams) x 100.

Table 5.2. Pre-weaning measures of control and prenatally-exposed offspring.

Parameter	Control		BPA		BPS		BPF (low dose)	
Litter size								
PND 1	10.38 ± 1.85		14.17 ± 1.35		12.31 ± 0.96		13.44 ± 1.22	
PND 7	9.88 ± 1.68		14.00 ± 1.39		12.08 ± 0.98		12.89 ± 1.17	
PND 14	9.75 ± 1.65		13.50 ± 0.99		12.00 ± 0.97		12.78 ± 1.13	
PND 21	9.63 ± 1.60		13.50 ± 0.99		12.00 ± 0.97		12.78 ± 1.13	
	Males	**Females**	**Males**	**Females**	**Males**	**Females**	**Males**	**Females**
Body weight (g)								
PND 1	16.58 ± 1.88	14.05 ± 1.81	13.48 ± 1.03	15.44 ± 1.44	17.55 ± 4.93	14.47 ± 1.31	13.49 ± 0.86	14.60 ± 1.33
PND 7	34.04 ± 3.66	31.11 ± 3.85	29.79 ± 2.93	32.98 ± 3.64	45.47 ± 13.42	32.89 ± 2.72	30.43 ± 1.95	33.87 ± 3.27
PND 14	68.24 ± 7.95	62.36 ± 6.42	53.76 ± 5.62	58.74 ± 5.96	80.95 ± 21.14	61.26 ± 4.85	55.96 ± 2.83	62.21 ± 6.29
PND 21	108.13 ± 13.22	99.66 ± 13.00	88.10 ± 9.19	96.09 ± 9.42	124.10 ± 37.25	88.88 ± 8.03	84.10 ± 7.58	94.54 ± 12.37
Sex ratio	40	43	45	39	82	77	62	59
Head circumference (cm)	3.72 ± 0.47	3.52 ± 0.45	4.08 ± 0.04	4.06 ± 0.05	3.85 ± 0.06	3.84 ± 0.07	3.87 ± 0.12	3.86 ± 0.10
Chest circumference (cm)	4.53 ± 0.10	4.40 ± 0.07	4.52 ± 0.13	4.44 ± 0.13	4.35 ± 0.06	4.30 ± 0.07	4.33 ± 0.15	4.26 ± 0.12
Crown to rump length (cm)	4.26 ± 0.55	4.00 ± 0.53	4.81 ± 0.10	4.68 ± 0.10	4.62 ± 0.08	4.53 ± 0.08	4.51 ± 0.11	4.47 ± 0.12
Anogenital Distance (cm)		0.13 ± 0.02		0.11 ± 0.00		0.11 ± 0.01		0.11 ± 0.00

Note: BPA, bisphenol A; BPS, bisphenol S; BPF, bisphenol F; PND, postnatal day. Measures were obtained from offspring with prenatal exposure to Vehicle (control), BPA (5 µg/Kg BW), BPS (5 µg/Kg BW), or low dose BPF (1 µg/Kg BW). Sex ratio, head and chest circumferences, and crown to rump lengths were determined on PND 1. Data are presented as mean ± SEM.

Table 5.3. Post-weaning parameters and organ weights in male and female offspring after prenatal bisphenol exposure.

Parameter	Control		BPA		BPS		BPF (low dose)	
	Males	**Females**	**Males**	**Females**	**Males**	**Females**	**Males**	**Females**
Post-Weaning								
Body weights (g)								
Week 3	51.50 ± 1.01	48.09 ± 0.78	43.70 ± 0.86	42.04 ± 0.93	45.02 ± 0.92	43.86 ± 0.99	47.05 ± 1.12	44.01 ± 1.01
Week 6	189.80 ± 1.57	148.98 ± 1.46	178.36 ± 1.98	139.51 ± 1.40	178.25 ± 2.09	139.13 ± 1.45	180.45 ± 2.24	142.61 ± 1.47
Week 12	359.74 ± 4.25	235.97 ± 4.95	347.97 ± 3.10	215.57 ± 3.67	357.85 ± 3.10	232.63 ± 2.64	345.32 ± 4.02	235.87 ± 3.49
Food intakes (g/day)								
Week 3	9.80 ± 0.22	8.93 ± 0.13	9.07 ± 0.30	8.51 ± 0.23	9.62 ± 0.16	8.74 ± 0.16	9.54 ± 0.13	8.66 ± 0.12
Week 6	22.34 ± 0.31	17.02 ± 0.45	23.18 ± 0.25	16.44 ± 0.20	21.64 ± 0.41	15.60 ± 0.19	21.53 ± 0.31	15.96 ± 0.22
Week 12	23.58 ± 0.20	18.25 ± 0.68	24.31 ± 0.24	17.37 ± 0.20	23.42 ± 0.23	16.13 ± 0.25	22.19 ± 0.29	17.02 ± 0.24
Water intakes (mL/day)								
Week 3	14.77 ± 0.75	14.03 ± 0.68	13.40 ± 0.49	14.82 ± 0.49	14.74 ± 0.30	14.04 ± 0.26	15.51 ± 0.31	13.68 ± 0.22
Week 6	35.69 ± 0.80	28.70 ± 0.48	30.80 ± 2.12	29.49 ± 0.57	33.31 ± 0.68	27.17 ± 0.42	33.96 ± 0.40	28.07 ± 0.45
Week 12	37.17 ± 0.33	32.35 ± 0.41	37.70 ± 1.12	33.03 ± 0.46	37.10 ± 0.53	32.74 ± 0.60	36.31 ± 0.53	33.23 ± 0.82
Relative Organ Weights (g/kg BW)								
Pituitary gland	0.05 ± 0.00	0.05 ± 0.00	0.04 ± 0.00	0.04 ± 0.00	0.04 ± 0.00	0.04 ± 0.00	0.04 ± 0.00	0.04 ± 0.00
Thymus	0.72 ± 0.10	0.95 ± 0.13	0.52 ± 0.03	0.58 ± 0.03	0.61 ± 0.07	0.82 ± 0.08	0.76 ± 0.10	0.94 ± 0.12
Heart	3.48 ± 0.11	3.92 ± 0.14	3.54 ± 0.12	4.33 ± 0.38	3.70 ± 0.18	4.21 ± 0.31	3.38 ± 0.11	3.80 ± 0.13
Lungs	4.41 ± 0.16	6.03 ± 0.48	4.73 ± 0.30	5.76 ± 0.32	4.46 ± 0.11	4.65 ± 0.27	4.61 ± 0.24	5.65 ± 0.28
Spleen	1.68 ± 0.06	2.18 ± 0.12	1.68 ± 0.12	1.93 ± 0.10	1.66 ± 0.04	2.16 ± 0.07	1.71 ± 0.03	2.19 ± 0.07
Liver	28.57 ± 1.46	27.09 ± 0.95	30.89 ± 1.40	28.16 ± 1.14	29.19 ± 1.52	28.26 ± 1.39	31.10 ± 1.72	26.37 ± 0.92
Adrenal glands	0.16 ± 0.01	0.28 ± 0.01	0.19 ± 0.02	0.28 ± 0.02	0.16 ± 0.01	0.27 ± 0.02	0.17 ± 0.01	0.31 ± 0.02
Testes and epididymis	14.42 ± 0.43	-	14.26 ± 0.22	-	14.04 ± 0.40	-	14.56 ± 0.24	-

Seminal vesicles	4.04 ± 0.34	-	4.15 ± 0.22	-	3.14 ± 0.23	-	3.30 ± 0.32	-
Uterus + ovaries	-	2.52 ± 0.19	-	2.64 ± 0.11	-	2.72 ± 0.19	-	2.36 ± 0.15
Abdominal adipose tissue	4.89 ± 0.54	2.37 ± 0.30	3.31 ± 0.84	2.28 ± 0.30	3.32 ± 0.3[a]	2.85 ± 0.41	3.50 ± 0.33	2.51 ± 0.29
Perirenal adipose tissue	1.81 ± 0.17	1.89 ± 0.60	1.52 ± 0.24	1.41 ± 0.28	1.39 ± 0.14	1.89 ± 0.17	1.32 ± 0.11	1.64 ± 0.31
Absolute Organ Weights								
Pituitary gland (mg)	11.74 ± 0.62	12.16 ± 0.44	11.90 ± 0.67	11.58 ± 0.50	11.37 ± 0.62	11.30 ± 0.58	11.12 ± 0.42	11.06 ± 0.74
Thymus (g)	0.30 ± 0.04	0.25 ± 0.03	0.23 ± 0.01	0.17 ± 0.01	0.26 ± 0.03	0.20 ± 0.02	0.30 ± 0.04	0.24 ± 0.03
Heart (g)	1.50 ± 0.06	1.05 ± 0.04	1.56 ± 0.07	1.09 ± 0.04	1.58 ± 0.08	1.04 ± 0.06	1.39 ± 0.07	0.99 ± 0.03
Lungs (g)	1.90 ± 0.07	1.60 ± 0.13	2.09 ± 0.16	1.62 ± 0.10	1.90 ± 0.04	1.51 ± 0.08	1.88 ± 0.08	1.49 ± 0.09
Spleen (g)	0.73 ± 0.04	0.57 ± 0.04	0.74 ± 0.06	0.54 ± 0.03	0.71 ± 0.02	0.54 ± 0.01	0.70 ± 0.02	0.57 ± 0.01
Kidneys (g)	2.83 ± 0.12	1.69 ± 0.05	3.16 ± 0.15	1.85 ± 0.05	2.83 ± 0.12	1.63 ± 0.06	2.74 ± 0.12	1.65 ± 0.06
Liver (g)	12.52 ± 1.02	7.23 ± 0.33	13.66 ± 0.86	7.95 ± 0.40	12.68 ± 1.02	7.08 ± 0.37	12.88 ± 1.01	6.35 ± 0.58
Adrenal glands (mg)	69.41 ± 4.12	74.41 ± 3.51	82.42 ± 7.68	79.92 ± 4.92	66.37 ± 4.40	67.66 ± 5.01	70.18 ± 3.98	81.51 ± 5.17
Testes and epididymis (g)	6.21 ± 0.15	-	6.28 ± 0.14	-	5.96 ± 0.11	-	5.96 ± 0.18	-
Seminal vesicles (g)	1.68 ± 0.17	-	1.81 ± 0.11	-	1.33 ± 0.11	-	1.36 ± 0.11	-
Uterus + ovaries (g)		0.66 ± 0.04		0.75 ± 0.05		0.68 ± 0.04		0.62 ± 0.04
Abdominal adipose tissue (g)	2.13 ± 0.25	0.64 ± 0.08	1.43 ± 0.36	0.65 ± 0.09	1.45 ± 0.16	0.72 ± 0.11	1.46 ± 0.17	0.67 ± 0.09
Epididymal / ovarian adipose tissue (g)	4.43 ± 0.37	1.77 ± 0.20	3.87 ± 0.30	1.47 ± 0.26	3.53 ± 0.37	1.70 ± 0.16	2.88 ± 0.45*	1.61 ± 0.16
Perirenal adipose tissue (g)	0.80 ± 0.10	0.51 ± 0.16	0.67 ± 0.10	0.40 ± 0.09	0.61 ± 0.08	0.48 ± 0.05	0.55 ± 0.06	0.43 ± 0.08

Note: BPA, bisphenol A; BPS, bisphenol S; BPF, bisphenol F; BW, body weight. Measures were obtained from offspring with prenatal exposure to Vehicle (control), BPA (5 µg/Kg BW), BPS (5 µg/Kg BW), or low dose BPF (1 µg/Kg BW). Data are presented as mean ± SEM. * $p < 0.05$, one-way ANOVA, followed by Tukey's multiple comparisons between control and BPF males. a $p = 0.07$, one-way ANOVA, followed by Tukey's multiple comparisons between control and BPS males.

Table 5.4. Ovarian follicle counts from adult female offspring with prenatal EDC exposure.

Ovarian Follicle	Control	BPA (5 µg)	BPS (5 µg)	BPF (1 µg)
Primordial follicles	23.56 ± 3.72	33.43 ± 4.27	31.00 ± 2.91	25.80 ± 5.36
Primary follicles	8.89 ± 1.95	12.00 ± 2.26	5.18 ± 1.15	6.70 ± 0.99
Secondary follicles	15.44 ± 3.75	20.29 ± 3.36	14.18 ± 2.34	12.80 ± 2.40
Tertiary follicles	4.78 ± 1.15	5.00 ± 1.09	7.91 ± 1.07	4.90 ± 0.84

Note: BPA, bisphenol A; BPS, bisphenol S; BPF, bisphenol F. Data are presented as mean ± SEM. Ovarian follicle counts were obtained from female offspring with prenatal exposure to vehicle, 5 µg/kg BPA, 5 µg/kg BPS, or 1 µg/kg BPF. Data were analyzed using one-way ANOVA, followed by Tukey's multiple comparisons post hoc analyses for differences between control and EDC groups.

Table S5.1. Data from Chapter 5 figures. Data are presented as mean ± SEM.

	Control		BPA (5 µg)		BPS (5 µg)		BPF (5 µg)		BPF (1 µg)	
Figure 5.2 - Pregnancy outcomes	Abortions	No Abortions	Abortions	No Abortions	Abortions	No Abortions	Abortions	No Abortions	Abortions	No Abortions
# of dams w/ abortions	0	9	0	6	0	13	6	1	1	10
	Males	**Females**	**Males**	**Females**	**Males**	**Females**	**Males**	**Females**	**Males**	**Females**
Avg. gestational weight gain	-	100.4 ± 7.8	-	101.3 ± 15.3	-	92.2 ± 7.7	-	24.0 ± 18.6	-	101.6 ± 12.3
Figure 5.3 - Male reproductive outcomes										
AGD	0.32 ± 0.02	-	0.29 ± 0.02	-	0.26 ± 0.01	-	-	-	0.27 ± 0.02	-
Testosterone	0.69 ± 0.25	-	0.65 ± 0.23	-	0.53 ± 0.12	-	-	-	0.76 ± 0.24	-
Figure 5.4 - Male and female organ weights										
Male kidneys	6.53 ± 0.09	-	7.15 ± 0.24	-	6.59 ± 0.10	-	-	-	6.65 ± 0.12	-
Female kidneys	-	6.36 ± 0.08	-	6.57 ± 0.13	-	6.48 ± 0.14	-	-	-	6.24 ± 0.13
EAT	10.19 ± 0.75	-	8.81 ± 0.74	-	8.05 ± 0.69	-	-	-	6.83 ± 0.96	-
OAT	-	6.61 ± 0.70	-	5.20 ± 0.84	-	6.73 ± 0.64	-	-	-	6.11 ± 0.64
Figure 5.5 - Male repro. organ weights										
Testes abs. weights	6.21 ± 0.15	-	6.28 ± 0.15	-	5.96 ± 0.11	-	-	-	5.96 ± 0.18	-
Testes rel. weights	14.42 ± 0.43	-	14.26 ± 0.22	-	14.04 ± 0.40	-	-	-	14.56 ± 0.24	-

Prostate abs. weights	0.73 ± 0.06	-	1.11 ± 0.11	-	0.80 ± 0.06	-	-	-	0.90 ± 0.10	-
Prostate rel. weights	1.65 ± 0.14	-	2.54 ± 0.29	-	1.83 ± 0.14	-	-	-	2.08 ± 0.21	-

Figure 5.6 - Female ovarian follicle counts

Graafian follicles	-	0.67 ± 0.47	-	2.57 ± 0.43	-	1.73 ± 0.56	-	-	-	1.40 ± 0.31
Atretic follicles	-	7.11 ± 1.43	-	10.71 ± 1.39	-	8.27 ± 1.08	-	-	-	9.70 ± 1.68
Corpora lutea	-	20.11 ± 2.26	-	21.00 ± 3.37	-	9.46 ± 2.41	-	-	-	11.90 ± 1.35
Old corpora lutea	-	11.89 ± 1.57	-	20.86 ± 2.80	-	9.46 ± 2.63	-	-	-	15.20 ± 4.57

CHAPTER 6

DISCUSSION

6.1. Summary of Findings

The use of EDCs is widespread in the environment, leading to public health concerns due to the plethora of health consequences associated with EDCs. The overarching goal of our study was to apply the DOHaD concept to gain a deeper understanding of it – specifically, we wanted to examine how fetal programming with EDCs may predispose rat offspring to stress-related behaviors in adulthood. We hoped to determine if there are sex differences in neurobehavioral effects, if these effects are dose-dependent, and if subsequent insults in adulthood – in this case, estradiol treatment – exacerbate the effects induced prenatally. Through a series of experiments, we exposed offspring to EDCs *in utero*, then continued to track their development into adulthood, when we subjected them to a battery of behavioral testing to assess their responses. Following this, we collected their trunk blood and tissues at sacrifice, and processed and analyzed these samples for various hormonal and neurotransmitter changes. We determined a myriad of sex-specific and dose-dependent effects on behavior, hormones, and brain monoamines. The following is a summary of our major findings, which are also summarized in Figure 6.1.

Prenatal BPA and/or DEHP, behavior, and the stress axis – sex differences

The first major aim of our experiments was to create a behavioral profile of adult offspring with gestational exposure to BPA, low-dose or high-dose DEHP, or a combination of

BPA and DEHP at both doses. Female and male rat offspring underwent behavioral paradigms that are commonly used to evaluate anxiety-like behaviors, including the Open Field Test (OFT), Elevated Plus Maze (EPM), and Shock Probe Defensive Burying (SPDB). While both male and female offspring demonstrated altered behavioral responses in a stressor-specific manner, male offspring appeared to show a more pronounced effect. Most of the EDC-treated male offspring buried the probe less in the SPDB, displaying an aberrant reduction in active coping strategies. DEHP (LD) males showed a robust shift to a passive behavioral phenotype. DEHP (HD) males, on the contrary, exhibited an alleviation of anxiety-like behavior in the EPM, which corresponded with a feminization of behavior.

Female offspring, particularly with low-dose EDC treatment, displayed aberrant behaviors in response to novelty and an aversive stimulus. The fact that low-dose EDC-exposed females displayed anxiolytic effects in the OFT does not necessarily represent a beneficial effect of EDCs because this behavior deviated from that of healthy control counterparts. This implies that a certain level of anxiety may be an appropriate and even adaptive response to a novel environment. Furthermore, EDC-exposed female offspring also showed diminished physical reactivity to the shock-probe in the SPDB compared to controls, suggesting that EDC treatment during gestation may hinder their abilities to physically react and respond appropriately to stress.

In terms of HPA axis activity, male offspring with low-dose EDC treatments appeared to be more severely impacted. DEHP (LD) males showed a trend for elevated levels of circulating CORT, yet they also had lower adrenal gland weights, along with B+D (LD) males. On the other hand, females prenatally exposed to BPA, DEHP (HD), or their combination had blunted CORT levels. These results altogether demonstrate that prenatal EDC exposure may predispose offspring to persistent, inappropriate responses to a variety of stressors. Moreover, male and

female offspring respond differently to EDC treatments in terms of behavior and HPA axis reactivity, underscoring the need for incorporating the study of sex differences in EDC studies.

Prenatal BPA and/or DEHP and brain monoaminergic activity – sex differences

Our second major aim was to develop a profile of monoamines and metabolites within brain regions involved in the regulation of stress responses. The brain regions analyzed were the mPFC, PVN, BLA, and HC. Once again, we used adult male and female rat offspring with gestational EDC exposures. These offspring were also previously subjected to behavioral testing. Following their behavioral testing and sacrifice, the brain tissue samples from these offspring were then analyzed for neurotransmitter content using HPLC.

Our most striking finding from this experiment was the fact that EDC-exposed male offspring had changes in dopaminergic activity in the PVN that corresponded with their behavior in the SPDB. Specifically, male offspring treated with BPA, low-dose and high-dose DEHP, and B+D (HD) showed deficits in PVN DA levels, and this was moderately correlated with their reduced burying levels in the SPDB. In addition, we found increased DA and 5-HT turnover in multiple brain areas in male offspring with low-dose EDC exposures. On the contrary, female offspring treated with high-dose DEHP alone or in combination with BPA demonstrated a hyperactivity of brain monoaminergic activity. This was in stark contrast with their behavioral and CORT results, which were modest and represented diminished activity. Yet, low-dose EDC treatments did not lead to any robust monoaminergic changes in female offspring. Collectively, our results pertaining to monoaminergic activity fill in some of the research gaps on prenatal exposures to EDC mixtures and their effects on the brain.

Prenatal BPA and/or DEHP followed by adult estradiol treatment in females

The third aim of our experiments was to incorporate a dual exposure paradigm to elucidate the "organizational" vs. "activational" effects of exogenous hormonal exposures in female offspring. The experimental procedure began with exposing offspring to BPA, low-dose or high-dose DEHP, or their combination during gestation. This was followed by chronic treatment with 17β-estradiol (E2) in adulthood. The rest of the procedure followed the same experimental design we used for our prior experiments, comprising of behavioral testing followed by tissue processing for hormones and brain neurotransmitters.

This experiment provided fascinating data particularly on the ways in which E2 treatment alone alters behavioral and neuroendocrine mechanisms in control females. Firstly, we discovered that E2 increases unconditioned anxiety-like behaviors in the EPM and reduces active defensive behaviors in our healthy controls, implying that E2 alone affects fear and anxiety-related responses in females with no prior exposure to EDCs. This was also associated with a trend for improved cognition in these females, which potentially mediates their anxiety-related behaviors. In terms of hormones and neurotransmitters, although E2 treatment did not affect CORT or OXT levels, it did increase noradrenergic activity in the PVN and HC and serotonergic activity in the PVN. It also appeared to suppress dopaminergic activity in both regions, especially in the HC.

E2 treatment in EDC-exposed offspring resulted in some intriguing behavioral findings. In general, adult treatment with chronic E2 either generated effects that were not apparent in control females (as observed in the OFT) or reversed the effects identified in controls (as observed in the EPM and SPDB). This is indicative of aberrant behavioral alterations. Moreover, the most remarkable results from our hormonal analyses were noted in DEHP offspring exposed

to the high dose. E2 treatment in these offspring significantly elevated CORT concentrations compared to the corresponding controls, but reduced OXT levels in comparison with their low-dose DEHP counterparts. DEHP (HD)-E2 females exhibited a robust EDC-induced increase in anxiety-like behavior in the OFT, which may have resulted from the hormonal changes.

Our neurotransmitter findings, in contrast, did not provide any clear results. Compared to control offspring, sham-implanted EDC offspring generally demonstrated a greater number of monoaminergic changes than their E2-treated counterparts. In the PVN, some highlights were that E2 treatment increased NE levels in low-dose EDC groups but lowered DA levels in high-dose groups. E2 treatment abolished 5-HT effects in all EDC groups compared to controls. E2 also enhanced DA turnover in all groups except for BPA. Similarly in the HC, E2 treatment decreased DA levels and elevated DA turnover in both control and EDC groups. Overall, this experiment provided important insights into the neurobehavioral consequences of prenatal EDC exposure and subsequent E2 treatment later in life in female offspring. We are especially hopeful that our findings encourage further research into female-specific health outcomes.

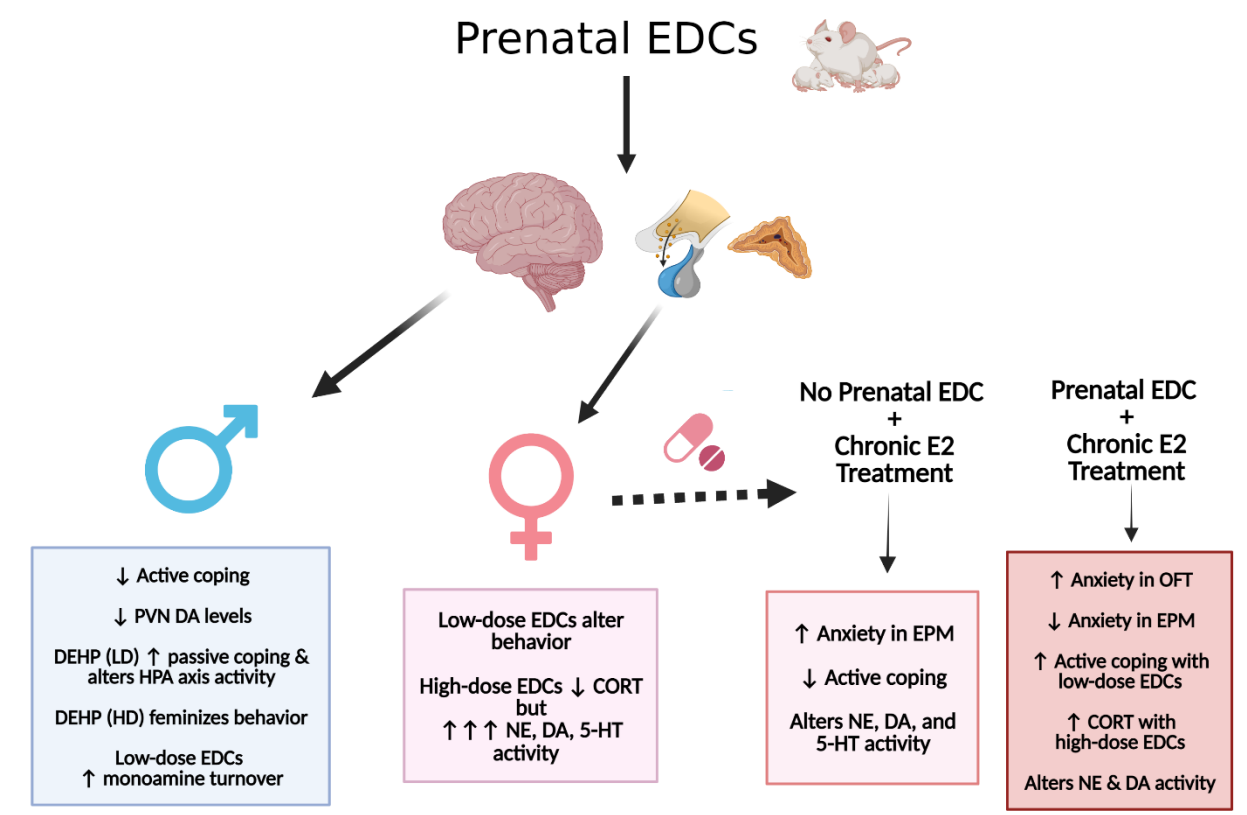

Figure 6.1. Schematic overview of the effects of exogenous hormonal compounds on behavior and the brain. Fetal programming with various EDCs impacts the brain and HPA axis in ways that result in sex- and dose-dependent effects on stress reactivity in offspring. Subsequent chronic treatment with estradiol (E2) in adult female offspring interacts with prenatal EDC exposure to further alter neurobehavior. Schematic was created using Biorender.com.

Note: EDC, endocrine disrupting chemical; DEHP, diethylhexyl phthalate; LD, low-dose; HD, high-dose; CORT, corticosterone; PVN, paraventricular nucleus; HC, hippocampus; HPLC, high performance liquid chromatography; NE, norepinephrine; DA, dopamine; 5-HT, serotonin; EPM, elevated plus maze; OFT, open field test.

Prenatal BPA analogues, pregnancy outcomes, and offspring development

The fourth and final experiment was distinct from the prior ones in that it focused on BPA analogues and their effects on gestational and developmental endpoints, rather than neurobehavior. The aim of this experiment was to assess the outcomes of fetal programming with low doses of BPA or its analogues BPS and BPF on the aforementioned endpoints. We also sought to identify any sex differences in these effects. Regarding gestational endpoints, the main finding was that BPF treatment at 5 µg/kg significantly increased spontaneous abortions in dams, indicative of reproductive toxicity at this dose. The dose of BPF used was lowered to 1 µg/kg for a different set of dams as a result. In the offspring, a variety of other reproductive consequences were observed. Male offspring demonstrated reduced anogenital distances and increased oxidative stress in the testes following BPS exposure. Changes in certain organ weights were also observed in male offspring. Female offspring were found to have a reduced quantity of corpora lutea with exposure to both BPS and BPF (1 µg/kg), implying lower rates of ovulation. This experiment contributes valuable information to the growing research on BPA analogues, highlighting the fact that they are not safer than BPA and their uses need to be limited.

6.2. Future Directions

The studies performed in this dissertation can be continued in a number of different ways. To begin, the behavioral experiments for BPA-, DEHP-, and B+D-exposed offspring have been replicated for offspring treated with BPS and BPF as well, and await analyses. It would be fascinating to probe deeper into the cognitive effects of these exposures by also examining other components of cognition, including spatial memory using the MWM. The molecular mediators underlying the adverse outcomes of BPA analogues, including brain monoaminergic activity and circulating hormone levels, would be important to examine next. Another interesting direction

this study could be taken is to further examine offspring brains for GABAergic and glutamatergic activity, since these amino acid neurotransmitters are also strongly implicated in neuropsychiatric disorders and are affected by EDC exposures.

Gene expression studies can also help strengthen our understanding of this research field, specifically in terms of genes implicated in mood and anxiety disorders. Specific biomarkers that would also be worth evaluating within the brain are brain-derived neurotrophic factor (BDNF), CRF, MAO activity, and inflammatory markers such as cytokines. Assessing changes in hormone receptor levels is also essential, including those of glucocorticoids, mineralocorticoids, ER subtypes (ERα and ERβ), oxytocin, and vasopressin. Furthermore, studies investigating epigenetic effects of low-dose EDC exposures and preconception EDC exposures would make excellent future studies. Although this is not an exhaustive list of future directions, it underscores the knowledge that needs to be expanded upon in this exciting field.

6.3. Conclusions

The original work outlined in this dissertation can help pave the way for future translational studies into human neuropsychiatric disorders. Altogether, our findings indicate that fetal programming with EDCs impact circulating hormones and neurotransmitter systems in the brain to alter stress responses in a sexually dimorphic manner. While EDC exposures during gestation more adversely impacted neurobehavior in male offspring compared to females, vehicle- and EDC-exposed females further treated with E2 in adulthood showed more deleterious effects later in life. This is consistent with other studies that have discovered that male offspring are more sensitive *in utero* [335, 336]. Our studies confirm that female offspring may be more susceptible to the "activational" effects of environmental hormonal exposures. The low-dose effects of EDCs are especially concerning, and the BPA substitutes BPS and BPF are not safe

alternatives but require further examination. Through these studies, we wanted to advance the research available on EDC mixtures and low-dose EDC effects due to the unique dose-response characteristics of EDCs. We also illustrate that consequences from environmental exposures *in utero* are not only confined to early development, but persist throughout life. Notably, exogenous estrogens in adult females may interact with prenatal EDC exposures to fundamentally alter behavior. We hope that these data inspire change so that current EDC regulations are updated to improve public health.

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