ABSTRACT

REBULI, MEGHAN ELIZABETH. Sex Specific Impact of Environmental Endocrine Disruptors on Neuroendocrine Development and Behavior. (Under the direction of Heather B. Patisaul.)

Endocrine disrupting compounds (EDCs) are exogenous chemicals that interfere with the endocrine system and hormone action. EDC action, particularly on sex hormones, indicates that they may participate in the aberrant organization or activation of sex differences within the brain. Bisphenol A (BPA) is one such EDC and has previously reported to affect sex-specific neuroendocrine physiology, estrogen signaling, and behavior. The mechanisms underlying these brain and behavior outcomes are largely unknown and defining them would allow for determination of their human relevance and potential contribution to human disease, such as neuropsychiatric disorders. This work investigates the potential for BPA to act on neuroendocrine physiology and behavior in three ways: (1) neonatal alteration of estrogen receptor expression by prenatal exposure to BPA, (2) juvenile and adult alteration of estrogen receptor expression by subchronic exposure to BPA, and (3) juvenile and adult alteration of anxiety and activity behavior by perinatal exposure to BPA. Sprague Dawley rats were gavaged on gestational days 6–21 for neonatal and perinatal exposures, and further dosed until postnatal day 90 for subchronic exposures, with vehicle, 2.5 to 2700 μg/kg bw/day BPA, or 0.5, 5, or 10 μg/kg bw/day ethinyl estradiol. Estrogen receptor expression in the hypothalamus and amygdala was investigated using in situ hybridization, while behavior was evaluated using a battery of behavioral tests including the elevated plus maze, open field and zero maze. Significant effects of BPA were observed in the neonate on estrogen receptor expression in the mediobasal hypothalamus and amygdala in both sexes. In the juvenile and adult, effects of BPA on estrogen receptor expression were

primarily seen in subregions of the preoptic area of female rats. Finally, there were no consistent effects of BPA on anxiety or activity behaviors, however study limitations limit the ability make resolute conclusions on BPA's potential to alter affective behaviors. These results indicate that estrogen receptor expression in the neonatal, juvenile and adult brain can be altered by BPA and may be one mechanism by which BPA can alter brain and behavior outcomes.

Sex Specific Impact of Environmental Endocrine Disruptors on Neuroendocrine Development and Behavior

by
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DEDICATION

To my family. Thank you for your love, support, and motivation, which has inspired me throughout my academic career and pushed me to complete my goals.

BIOGRAPHY

Meghan Elizabeth Rebuli was born December 2, 1989 in Havelock, North Carolina. She attended Havelock High School, where she was elected Student Body President, participated as captain of the girls' soccer team, was section leader in the marching band, and won Homecoming Queen before graduating in 2007.

Her undergraduate institution was North Carolina State University, where she was selected as a Park Scholar, studied abroad in Antigua, Guatemala, was a member of Alpha Epsilon Delta, and participated as an undergraduate researcher. She graduated with a Bachelor of Science in Biological Sciences, cum laude, with a concentration in Human Biology, and a minor in Spanish in 2011.

After completion of her undergraduate career she continued on at North Carolina State University to complete her doctoral studies in the Zoology graduate program researching neuroendocrine development and behavior. During her graduate studies she received two teaching awards for her efforts as a teaching assistant and presented her research at multiple national and international conferences.

After graduation she plans to continue her research as a postdoctoral trainee at the University of North Carolina, Chapel Hill.

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ABBREVIATIONS

1	first test day
2	second test day
3V	third ventricle
AXXXX	Aroclor XXXX
AALAC	Assessment and Accreditation of Laboratory Animal Care
AC	anterior commissure
ADHD	Attention Deficit Hyperactivity Disorder
Ahi	amygdalo-hippocampal area
AhR	arylhydrocarbon receptor
AMYG	amygdala
ANOVA	analysis of variance
ARC	arcuate hypothalamic nucleus
rARC	rostral arcuate nucleus
cARC	caudal arcuate nucleus
ASD	autism spectrum disorder
AVPV	anteroventral periventricular nucleus
BPA	bisphenol A
BPA-G	BPA-glucuronide
bw	body weight
CA1	Region I of hippocampus proper
CC	corpus callosum
CEBS	National Toxicology Program Chemical Effects in Biological Systems
CLARITY	Consortium Linking Academic and Regulatory Insights on BPA Toxicity
CM	central medial thalamic nucleus
CMC	carboxymethylcellulose sodium salt
CPu	Caudate Putamen
DES	diethylstilbestrol
DG	dentate gyrus
EAC	endocrine active compound
EDC	endocrine disrupting compound
EE	ethinyl estradiol
EPM	elevated plus maze
ER	estrogen receptor
ERα	estrogen receptor alpha
ERβ	estrogen receptor beta
ERK	extracellular signal-regulated kinase
ESR	estrogen receptor
ESR1/*Esr1*	estrogen receptor alpha
ESR2/*Esr2*	estrogen receptor beta
f	effect size
F	female

FAO	Food and Agricultural Organization
FDA	Food and Drug Administration
GD	gestational day
GH	growth hormone
GLP	good laboratory practice
GnRH	gonadotropin releasing hormone
HPA	hypothalamic-pituitary-adrenal axis
HPG	hypothalamic-pituitary-gonadal axis
ic	internal capsule
ISHH	*in situ* hybridization histochemistry
LH	luteinizing hormone
LHβ	luteinizing hormone beta polypeptide
LOAEL	lowest-observed-adverse-effect level
LOD	limit of detection
LSD	least significant difference
LV	lateral ventricle
M	male
MBH	mediobasal hypothalamus
MePD	medial amygdala
MPOA	medial preoptic area
NCTR	National Center for Toxicological Research
NCSU	North Carolina State University
NIEHS	National Institute of Environmental Health Sciences
NOAEL	no-observed-adverse-effect level
NTP	National Toxicology Program
OF	open field
PBDE	polybrominated diphenyl ether
PCB	polychlorinated biphenyl
PFOA	perfluorooctanoic acid
PLCo	posterolateral cortical amygdaloid nucleus
PMCo	posteromedial cortical amygdaloid nucleus
PND	postnatal day
POA	preoptic area
PRL	prolactin
r	empirical correlation
ROI	region of interest
SD	Sprague Dawley
SDN	sexually dimorphic nucleus
TCDD	2,3,7,8 – tetrachlorodibenzo-p-dioxin
VMN	ventromedial hypothalamic nucleus
VMNvl	ventrolateral division of the ventromedial hypothalamic nucleus
cVMNvl	caudal ventrolateral division of the ventromedial hypothalamic nucleus
rVMNvl	rostral ventrolateral division of the ventromedial hypothalamic nucleus

WHO	World Health Organization
ZM	zero maze

CHAPTER 1— Effects of endocrine disrupting compounds on neurodevelopmental sex differences in perinatal and adolescent rodents: a review

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Abstract

Background

Brain sex differences are found in nearly every region of the brain. They are organized during gestation and early adolescence. EDCs interfere with hormone action and are thought to interfere with the formation of sex differences. EDCs may be considered an environmental factor contributing to the increased diagnosis of neuropsychiatric disorders that emerge during adolescence (suggesting developmental etiology) and present with a sex bias.

Objective

This review focuses on the available evidence for the ability of EDCs to impact the emergence of brain sex differences, with a focus on effects detected at or before adolescence.

Methods

Studies were identified by searching PubMed using the keywords endocrine disrupting compound, EDC, endocrine active compound, EAC, brain, neuro, and development. Endpoints reviewed are brain gene, protein, hormone, and transcript expression, as well as brain morphology. Studies on behavioral endpoints were not included. All peer-reviewed papers published by January 31, 2015 were incorporated.

Results

The hypothalamus was found to be particularly affected by estrogenic EDCs in a sex, time, and exposure dependent manner. The hippocampus also appears vulnerable to endocrine disruption by BPA and PCBs although there is little evidence from the available data set to make any conclusions about sex-specific effects. Gestational EDC exposure can alter fetal neurogenesis and gene expression. PCB, dioxin, and estrogenic EDC exposures, were found to alter larger brain areas and organizational sex differences in the neonatal brain, such as the cortex and cerebellum.

Conclusion

The developmental EDC exposure literature demonstrates evidence of altered neurodevelopment as early as fetal life, with sex specific effects observed throughout the brain even before puberty.

Key Words

Endocrine disrupting compounds, neurodevelopment, sex differences, developmental exposure, rodent

Introduction

Brain sex differences are fundamental to understanding neurophysiology and behavior (Becker et al., 2007; G.J. de Vries, 1984). These differences are present as early as gestation and found in nearly every region of the brain, particularly in the hypothalamus (G. J. De Vries, 2004; Ohtani-Kaneko, 2006). Many neuropathological and neuropsychiatric disorders have a sex bias in their prevalence but it is unclear why (Paus, Keshavan, & Giedd, 2008). Some of these disorders, including attention-deficit/hyperactivity disorder (ADHD),

and Autism spectrum disorder (ASD), emerge in childhood, suggesting that sex differences in etiology and risk occur in development. Understanding how hormones and other factors shape neural sexual dimorphisms, and sex-specific vulnerability to neuropsychiatric illness is critical for understanding these disorders, yet sex differences are underreported in the literature and available neurodevelopmental research is biased towards males (Woodruff, Kibbe, Paller, Turek, & Woolley, 2014). One area of study in which brain sexual differentiation has consistently been a central focus is endocrine disruption. Because endocrine disrupting chemicals (EDCs) interfere with hormone action, it is hypothesized that they may contribute to neurobehavioral effects by altering sexual differentiation. Accounting for sex is thus an important aspect of EDC research and the outcomes are informative for the broader neuroscience community. This review examines available evidence for the ability of EDCs to impact the emergence of brain sexual dimorphisms, with a focus on effects detected at or before puberty.

ADHD and ASD disproportionally affect boys while anxiety and depressive disorders are more common in girls (CDC, 2012; Costello, Mustillo, Erkanli, Keeler, & Angold, 2003; Ramtekkar, Reiersen, Todorov, & Todd, 2010; Tang & Pinsky, 2015). Rates of pediatric psychiatric disorders, most notably ADHD and ASD, are rapidly rising, suggesting an environmental component to their etiology. This sex bias indicates that sex hormones may be involved, but potential mechanisms remain unclear. ASD, for example, has been associated with prenatal androgen excess and attenuation of sex differences in some studies (Baron-Cohen et al., 2014; Baron-Cohen, Knickmeyer, & Belmonte, 2005), but genome-wide

studies have revealed that a complex mixture of gene by environment interactions likely contribute to etiology(Merikangas, Nakamura, & Kessler, 2009).

EDCs may be one environmental factor contributing to greater prevalence of adolescent neuropsychiatric disorders (J. S. Brown, Jr., 2009; de Cock, Maas, & van de Bor, 2012; Diamanti-Kandarakis et al., 2009; Schug, Janesick, Blumberg, & Heindel, 2011). The Endocrine Society defines an EDC as an exogenous chemical substance or mixture that alters the structure or function(s) of the endocrine system (Diamanti-Kandarakis et al., 2009). This can occur through a range of mechanisms including direct agonism/antagonism of sex hormone receptors (A.C. Gore & Dickerson, 2012) and abrogation of sex differences in hormone receptor expression (Cao, Joyner, Mickens, Leyrer, & Patisaul, 2014; Cao, Mickens, McCaffrey, Leyrer, & Patisaul, 2012; Cao et al., 2013). Thus, accounting for sex and sexual dimorphisms in the developing (gestational through pubertal) brain is fundamental to EDC research.

EDC effects on behavior, including sexually dimorphic behaviors that may be related to onset of neuropsychiatric disorders, have been comprehensively reviewed (Leon-Olea et al., 2014; Lephart, Setchell, Handa, & Lund, 2004; Palanza, Gioiosa, vom Saal, & Parmigiani, 2008; Palanza, Morellini, Parmigiani, & vom Saal, 1999; Patisaul & Adewale, 2009; Schantz & Widholm, 2001), so this review focuses on molecular, cellular and neuroanatomical neural endpoints including gene expression, neurogenesis, neural plasticity and epigenetic changes. Rodent studies, with an emphasis on those reporting outcomes stratified by sex, were identified and included. Effects reported prior to puberty were the primary focus of this review because pediatric psychiatric disorders, most notably ADHD

and ASD, clearly have fetal origins and emerge in infancy and early childhood. A better understanding of how EDCs can affect sex differences in neurodevelopment can guide knowledge on their role in human health and the onset of adolescent neuropsychiatric disorders (Grandjean & Landrigan, 2006).

Methods

The review includes rodent studies (published by January 31, 2015) in which exposure was gestational and/or neonatal and assessment was made prior to early adulthood (before postnatal day (PND) 37). Studies were identified by searching PubMed using the keywords: endocrine disrupting compound, EDC, endocrine active compound, EAC, brain, neuro, and development. Effects in adults are available in numerous studies, but beyond the scope of the present review. Thus, studies with older animals were included only if animals younger than PND 37 were part of the experimental design. Studies on behavioral endpoints were not included.

Details regarding the reviewed studies including the species, strain, sex, exposure route, exposure window, dose, endpoint, and age at testing are summarized in four tables grouped by brain region of interest: hypothalamus (Table 1); hippocampus (Table 2); cortex, cerebellum, and mid-brain(Table 3); and whole brain and embryonic brain regions (Table 4). Estrogenic EDCs were hypothesized to be much more likely to influence the hypothalamus (Table 1), while thyroid hormone-like EDCs are thought to be much more influential in the hippocampus (Table 2). Tables 3 and 4 are studies that mostly concentrated on gene and protein expression at ages that did not allow for individual subregion analysis and

investigated larger regions, like cortex and cerebellum (Table 3) and whole brain or larger embryonic brain regions (Table 4).

Results

Hypothalamus (Table 1)

The hypothalamus is the apical coordinator for homeostatic functions including stress, emotion, reproduction, feeding, and the regulation of sex hormones production and circulation (Campbell, 2005). In rodents, sexual dimorphisms within hypothalamic regions are organized primarily during the perinatal period by aromatized testosterone (Lenz & McCarthy, 2010) thus disruption of estrogen signaling is hypothesized to be a primary route in which ECDs alter neuroendocrine development. Therefore it is not entirely surprising the most of the available literature obtained for this review focused on three of the most well-known estrogen-altering EDCs: Bisphenol A (BPA; exposures ranged from 2.5 μg/kg bw/day to 50 mg/kg bw/day), polychlorinated biphenyls (PCBs) (exposures ranged from 1 mg/kg to 10 mg/kg), and the phytoestrogen, genistein (exposures ranged from 250 μg to 10 mg/kg bw/day). Exposures were mostly gestational (7 studies), with several extending into the neonatal period (6 studies) and the remaining focused only on neonatal exposure.

Of the 19 studies identified (Table 1), 15 included both sexes, reported outcomes based upon sex, and reported sex-specific effects. Sex specific effects varied by region of interest and compound investigated. For example, gestational exposure to the PCB mixture Aroclor 1221 (A1221) via subcutaneous injection to pregnant rat dams (1mg/kg), altered the expression of numerous estrogen-sensitive genes in the anteroventral periventricular nucleus (AVPV) of females, but not males (Dickerson, Cunningham, & Gore, 2011; Walker, Goetz,

& Gore, 2014), while the arcuate nucleus (ARC) was found to be affected in males, but not females (Walker et al., 2014). Affected genes included steroid hormone receptors (*Ar*, *Thra*, *Gper*), neuropeptide receptors (*Gnrh1* and *Kiss1r*), methyl transferase (*Dnmt1*), and clock genes (*Arntl* and *Per2*) (Walker et al., 2014). Other studies have also identified the developing AVPV as having sex-specific vulnerability to EDCs. Neonatal genistein demasculinizes tyrosine hydroxylase immunoreactivity in the male AVPV (Patisaul, Fortino, & Polston, 2006, 2007) and defeminized (reduced) kisspeptin fiber density in the female AVPV and ARC (Losa et al., 2011). The AVPV is a highly sexually dimorphic region which coordinates the luteinizing hormone (LH) surge in females and is thus essential for ovulation (Simerly, 2002). These observed impacts on AVPV sexual differentiation are consistent with the large body of literature reporting EDC-related effects on estrous cyclicity, fecundity, and ovulation (Crain et al., 2008; Peretz et al., 2014).

Developmental EDC exposure also impacted neuropeptides and their receptors other hypothalamic regions, somatostatin receptor expression (Facciolo, Alo, Madeo, Canonaco, & Dessi-Fulgheri, 2002; Facciolo, Madeo, Alo, Canonaco, & Dessi-Fulgheri, 2005), progestin receptor expression (Hays, Carpenter, & Petersen, 2002), and kisspeptin gene expression (Dickerson et al., 2011; Navarro et al., 2009). In the periventricular and ventromedial (VMN) nuclei, increased levels of somatostatin receptor 2 were found with BPA exposure at PND 10, as well as increased levels of the same receptor at PND 23 in the periventricular nucleus (Facciolo et al., 2002). Increased expression of somatostatin receptor 3 was also found in the VMN and ARC at PND 7 after BPA exposure (Facciolo et al., 2005). In the preoptic area (POA) at PND 3, sex differences in the expression of progestin receptor were

not found to be altered by dioxin, however it was found to alter GAD 67 levels, reducing the naturally occurring sex difference in the rostral portion of the POA (Hays et al., 2002). In whole hypothalamus, BPA decreased kisspeptin mRNA expression in both females and males at PND 30 (Navarro et al., 2009). Also in the POA, A1221 reduced kisspeptin receptor expression, specifically in males, at PND 1 (Dickerson et al., 2011). These examples illustrate the variety of ways EDCs can affect hypothalamic development, either not affecting sex differences by altering both sexes, reducing sex differences, or only affecting one sex (Ceccarelli, Della Seta, Fiorenzani, Farabollini, & Aloisi, 2007; Dickerson et al., 2011; Hays et al., 2002; Navarro et al., 2009).

The developing hypothalamus has sex-specific vulnerability to EDCs with the POA and mediobasal hypothalamus (MBH) being the most intensely studied and robustly affected. Estrogen receptor and aromatase expression are highly sexually dimorphic and altered by developmental EDC exposure; thus potentially serving as a central mechanism by which other aspects of hypothalamic sexual differentiation are altered by EDCs. Exposures encompassing both the gestational and postnatal period seemed to be the most effective in altering steroid receptor expression, while exposures limited to later postnatal development seemed to result in more transient effects.

Because the hypothalamus is critically important for the regulation of mood and emotion, as well as sociality (Engel et al., 2010; Eskenazi et al., 2013; Hoffman, Webster, Weisskopf, Weinberg, & Vieira, 2010; Miodovnik et al., 2011), the animal outcomes reviewed here are consistent with epidemiological data in children associating BPA and PCBs with ASD, ADHD, and anxiety disorders (J. M. Braun et al., 2011; Cheslack-Postava

et al., 2013; Eubig, Aguiar, & Schantz, 2010; Sagiv et al., 2010). Interestingly, no immune related endpoints have been investigated to date in young animals, even though some immune cells like microglia (Wood, 2011; Wu et al., 2013) and neutrophils (Molero et al., 2002; Shindo, Moore, Flake, & Negishi, 2013) have been found to express estrogen receptors and the immune system is influenced by the hypothalamus. Also, there were a lack of phthalate, polybrominated diphenyl ethers (PBDE) and perfluorooctanoic acid (PFOA) studies, even though these ubiquitous chemicals have been associated with mood and anxiety disorders in humans such as ASD and ADHD (Crinnion, 2010; Gascon et al., 2011; Hoffman et al., 2010; S. M. Kim et al., 2010).

Hippocampus (Table 2)

The hippocampus is fundamental to learning and memory. Hippocampal cell proliferation and differentiation, as well as circuit development, are developmentally regulated by sex and thyroid hormones (Bourguignon et al., 2013); making it a potential target for endocrine disruption. In stark contrast to the studies on the hypothalamus, where the majority of studies reported outcomes based on sex, none of the hippocampal studies investigated sex-specific outcomes.

For the scope of this review, data was available from only two EDCs: BPA (exposures ranged from 40 µg/kg/day to 200 mg/kg/day) and PCBs (exposures ranged from 1mg/kg to 8 mg/kg). In all studies exposures began in gestation, and in all but two (K. Kim et al., 2009) extended into the postnatal period. Endpoints investigated included neurogenesis, phosphorylation, and protein and hormone-sensitive (sex or thyroid hormone)

gene expression. BPA was found to influence the formation of the dentate gyrus, causing it to delineate earlier in PND 1 mice (sex was not distinguished) compared to controls (K. Kim et al., 2009). BPA was also found to reduce synaptic density, enlarge the synaptic cleft, and shorten the active zone in PND 14 and 21 male mice in region 1 of the hippocampus proper (CA1) (X. Xu, Xie, et al., 2013). PCB mixture A1254 increased RC3/neurogranin mRNA expression in the dentate gyrus (DG) at PND 15 (sex not stated) compared to controls (Zoeller, Dowling, & Vas, 2000). Overall, evidence for disruptive effects in the hippocampus is strongest for the PCBs (Morreale de Escobar, Obregon, & Escobar del Rey, 2004). Impacted processes include early delineation of the dentate gyrus, altered synapses in the CA1 and altered neurogenesis, all consistent with reports on children exposed to PCBs exhibiting cognitive, memory, and learning deficits (Jacobson & Jacobson, 1996; Stewart et al., 2008).

Although BPA and PCBs can clearly alter hippocampal neurodevelopment, results were not reported by sex in most cases but instead conducted in males only (6 studies), females only (1 study), or with a group of animals where sex was not defined/distinguished in analysis (4 studies). Thus it is not possible to ascertain which sex may be more vulnerable to effects of EDCs in this region. This lack of focus on sexual dimorphisms in the hippocampus is reflective of the hippocampal neuroscience literature as a whole which, despite the identification of numerous structural and functional sex differences, has traditionally has focused on one sex within a single study (Barth, Villringer, & Sacher, 2015; Huang & Woolley, 2012; Shors, Chua, & Falduto, 2001; Woodruff et al., 2014). Establishing how EDCs modulate sex differences, particularly those associated with memory

deficits and other mental impairments, remains an area ripe for future investigation. Moreover, identifying specifically when the developing hippocampus is most susceptible to EDC exposures (most available studies exposed animals from mid-gestation to weaning), needs further assessment. Ideally, these studies would also include both sexes.

Cortex, Cerebellum, and Mid-brain (Table 3)

Compared to the hypothalamus and hippocampus, relatively few studies have examined developmental EDC related effects elsewhere in the brain, but we identified 3 relevant PCB studies (exposures ranging from 1-25 mg/kg), 7 BPA studies (exposures ranging from 2 - 20,000 µg/kg) and 4 dioxin studies (2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD; a class of highly persistent environmental compounds formed in industrial processes such as waste combustion and the primary component of Agent Orange; exposures ranging from 0.2 – 2 µg/kg bw/day). Seven of 14 studies differentiated results by sex, but three failed to identify which sex was used. All but one study used an exposure window that began in gestation and five used a longer perinatal exposure window.

Evidence for sex-specific vulnerability within this literature was uneven with one study finding that females were more sensitive to PCB exposure than males, showing decreased forebrain thyroxine concentrations as well as decreased deiodination, but only in females (Morse, Wehler, Wesseling, Koeman, & Brouwer, 1996), . Other studies found that both males and females were affected by TCDD or BPA. For example, dioxin was found to lower Bcl-2 expression in the female cortex and cerebellum at PND 0, but not in male. In contrast, dioxin was found to increase Bcl-xl expression at PND 0 in the cortex and cerebellum in the male, but not the female (Chang et al., 2005). BPA was found to alter

expression of genes such as *S100b*, *Slc6a4*, *Htr1b*, *Maoa*, *Scla3*, and *Gap43*, at PND 1, 4, 8 in sex and spatiotemporal manner in the cortex, thalamus, and pons (Itoh, Yaoi, & Fushiki, 2012). One study investigating BPA-related effects on extracellular signal-regulated kinase (ERK) 1/2 signaling in the cerebellum via intracerebellar injection, reported that BPA and ethinyl estradiol (EE) increased ERK cell numbers and signaling at low and high, but not intermediate exposures (Zsarnovszky, Le, Wang, & Belcher, 2005). This effect was not sex-specific, which is not entirely surprising given that the cerebellum is not considered a highly sexually dimorphic structure. Sex specific effects were observed as early as the late gestational period (Itoh et al., 2012; Morse et al., 1996; Nishizawa, Imanishi, & Manabe, 2005; Nishizawa et al., 2003; Nishizawa, Morita, Sugimoto, Imanishi, & Manabe, 2005) indicating that maternal exposure to EDCs can have a significant effect on fetal brain development, even in regions not typically considered to be sexually dimorphic

Collectively, the TCDD studies found decreased expression of myelin basic protein (Fernandez et al., 2010), increased expression of apoptotic genes (Chang et al., 2005), and inhibited acetylcholinesterase activity (Ahmed, 2011), suggesting impaired cerebellar development and function. These studies help reveal the neurologic sources of motor and learning disabilities seen in dioxin-exposed children (Vreugdenhil, Lanting, Mulder, Boersma, & Weisglas-Kuperus, 2002). An extensive literature outside the scope of this review has identified interactions with the aryl hydrocarbon receptor (AhR) as the primary mode of action for TCDD neurotoxicity (Mimura & Fujii-Kuriyama, 2003; Patel, Kim, Peters, & Perdew, 2006; Tijet et al., 2006). Dioxin is known to bind to AhR with high affinity and when AhR is hyperactivated during embryogenesis it can be teratogenic (Peters

et al., 1999). AhR is also thought to aid in the metabolism of xenobiotic compounds, especially polycyclic aromatic hydrocarbons. Interestingly none of the dioxin studies identified for this review examined effects on AhR activity or expression. By contrast, increased expression of AhR was reported in the BPA studies in both males and females, all investigating similar gestational periods of exposure in the ICR mouse (outbred strain selected for high fertility and used in cancer research) (Nishizawa, Imanishi, et al., 2005; Nishizawa et al., 2003; Nishizawa, Morita, et al., 2005). Although there was no report of birth defects in late gestation (when the animals were evaluated), like abnormal liver or kidney function, cleft palate, reduced litter size, etc. typically seen with altered AhR function in dioxin exposure, increased AhR expression could potentially result in altered neural differentiation, resulting in inappropriate catecholaminergic differentiation of Neuro2a cells (Akahoshi, Yoshimura, & Ishihara-Sugano, 2006).

The reviewed PCB studies primarily investigated thyroid hormone related endpoints in the forebrain and cerebellum, including brain thyroid hormone concentration and metabolism (Morse et al., 1996) as well as thyroid hormone responsive gene expression (Takahashi et al., 2009; Zoeller et al., 2000). Thyroid hormone is crucial for neurodevelopment and alteration of gene expression and hormone metabolism may contribute to the cognitive disorders seen in PCB exposed children (Jacobson & Jacobson, 1996; Stewart et al., 2008; Vreugdenhil et al., 2002).

In general these studies found spatiotemporal, sex, and exposure related effects on gene expression related to neurogenesis, aryl hydrocarbon receptor expression and related genes, and apoptosis regulation (Chang et al., 2005; Itoh et al., 2012; Nishizawa, Imanishi, et

al., 2005; Nishizawa et al., 2003; Nishizawa, Morita, et al., 2005). Altered neuronal differentiation, along with altered receptor signaling (Zsarnovszky et al., 2005) and changes in neuronal migration (Itoh et al., 2012) could be a mechanism by which EDCs, including BPA, alter hallmark behaviors of ADHD, ASD and other psychosocial disorders (de Cock et al., 2012; Eubig et al., 2010), as reported in children (J. M. Braun et al., 2011; Harley et al., 2013) and numerous animal studies (Grandjean & Landrigan, 2014; Landrigan, Lambertini, & Birnbaum, 2012; Wolstenholme, Rissman, & Connelly, 2011).

Whole Brain and Embryonic Brain Regions (Table 4)

Because it is logistically difficult to differentiate brain regions in the pre-term rodent brain, some analyses have focused on whole brain (4 of 9 studies) or large subregions including the ventricular zone, cortical plate, telencephalon or diencephalon (5 of 9 studies). Because sex differences emerge during embryonic development, examination of the fetal brain for EDC effects yields critical insight as to the mechanisms by which EDCs manipulate sexual differentiation, and the critical windows during which the developing brain is must vulnerable. Only 2 studies differentiated results by sex, and, of the remaining, only 1 reported the sex examined. In one of the studies that differentiated results by sex, a sex difference in the expression of *Gper* and *Esrrg* not present in controls was induced in whole brain gene expression on GD 18.5 in BPA exposed animals, with males having higher concentrations than females (Wolstenholme et al., 2012). These two genes are estrogen receptor related genes and may indicate that males are more sensitive to BPA exposure.

Expression of thyroid hormone responsive, estrogen receptor related, myelination related, and oxytocin and vasopressin related genes, as well as neurogenesis were the most

common endpoints investigated, with BPA (4 studies with exposures ranging from 20 μg/kg to 5 mg/kg), PCBs (3 studies with exposures ranging from 20 μg/kg – 6 mg/kg), and dioxin exposure (2 studies with exposure ranging from 0.7-20 μg/kg TCDD). Expression of myelination (Fernandez et al., 2010), organo- and neuro-genesis (Fujita et al., 2006; Komada et al., 2012), and thyroid hormone related genes (Gauger et al., 2004; Morse et al., 1993), as well as neuronal differentiation and migration (Itoh et al., 2012) and cell cycle exit (Nakamura et al., 2012; Naveau et al., 2014) were all found to be altered by EDC exposure. Some of these studies evaluated similar endpoints later in development, such as myelination levels in the cerebellum (Fernandez et al., 2010) and whole brain thyroxine deiodinase activity (Morse et al., 1993), and confirm that many of the effects may persist into adolescence and beyond. Collectively these findings reinforce that EDCs can have organizational effects on brain structure and suggest that gestational exposure alone has the ability to forever alter neurodevelopment including myelination.

Every study in this category found effects of EDC exposure on gene expression related to sex or thyroid hormone receptors or neurogenesis during the pre- or very early postnatal period. Maternal developmental exposure to EDCs can induce alterations in fetal neurodevelopment and sex difference organization. With every EDC investigated resulting in evidence of altered neurogenesis, then the next set of questions to be addressed are which EDC, at which dose, in which exposure window is likely to contribute to a particular neuropsychiatric disorder? With the barrage of exogenous chemicals that almost every pregnant woman or child is exposed to on a daily basis (Skakkebaek et al., 2011),

deciphering exactly when and how much of an EDC exposure is necessary to induce neuropsychological effects is a critical to the overall understanding of disease etiology.

Sex differences

Of the 46 studies included, 49% included both males and females and looked for potential sex differences as part of their analysis (Figure 1). 26% included males and/or females, but did not separate them in the analysis or did not state which sexes were included. 17% of studies only included males and 8% only included females. This is considerably better than the neuroscience literature in general. A survey of 1200 neuroscience papers from 2011 to 2012 (Woodruff et al., 2014) found outcome by sex was only reported 36% of the time, and studies listed the sex of the animals only 42% of the time. Thus, the number of studies identifying sex and the number of studies reporting outcome by sex was 32% and 13% higher, respectively, in the EDC literature reviewed here. The more frequent consideration of sex in the EDC literature, compared to the neuroscience literature, is logical considering their mechanisms of action. It was recognized quite early that EDCs have the potential to alter sex-specific physiology, and thus has historically been considered an important endpoint to report in the EDC literature. This work has significantly contributed to, and advanced basic knowledge of, the neuroendocrinology of sexual differentiation (A. C. Gore, 2008; Patisaul & Polston, 2008), and demonstrates that EDC researchers embraced the inclusion of sex in study design long before the NIH mandated inclusion of sex differences in preclinical research (Clayton & Collins, 2014). Just as sex may be a factor in calculating appropriate doses of drugs like zolpidem (used in Ambien) and acetaminophen (FDA, 2013;

Whitley & Lindsey, 2009), effects of EDCs are likely also sex-specific and should be taken into consideration when determining susceptibility and potential mechanisms of action.

Conclusion

The reviewed data reveal that developmental EDC exposure can alter neurodevelopment across the brain beginning early in fetal life, with sex specific effects observable throughout the brain even before puberty. The hypothalamus was found to be particularly affected by estrogenic EDCs in a sex, time, and exposure dependent manner; a logical and expected finding given the fundamental importance of estrogen for masculinizing the developing rodent brain. These data clearly demonstrate that EDCs are capable of interfering with neural estrogen signaling but whether or not these specific phenotypic effects will be observable humans is unclear. There are key species differences in the developing rodent and human brain. Most significantly, androgens appear to be fundamental to primate brain masculinization while the role of estrogen is less well understood. It is reasonable to conclude, however, that EDCs are capable of perturbing estrogen function in the developing human brain, particularly the hypothalamus. Accordingly, evidence from human literature indicates that EDC exposure may be contributing to escalating rates of mood and affective disorders in humans, especially children (de Cock et al., 2012; Grandjean & Landrigan, 2014). The hippocampus also appears particularly vulnerable to endocrine disruption by estrogenic compounds such as BPA and PCBs although there is not enough available evidence from this specific data set to make any conclusions about sex-specific effects. The mechanisms by which BPA and PCBs alter hippocampal organization likely differ from each other and include mechanisms other than disruption of estrogen signaling. PCBs are well

characterized thyroid hormone disruptors (Winneke, 2011), for example, and emerging evidence suggests BPA may be an androgen disrupter in the developing brain (Kinch, Ibhazehiebo, Jeong, Habibi, & Kurrasch, 2015). Because the hippocampus is critical for learning and memory, further investigation into EDC effects on the organization of this region may help to elucidate potential linkages between EDC exposure and sex-biased deficits in IQ, memory and related cognitive functions in humans (Grandjean & Landrigan, 2014).

Analyses of whole brain or large subregions in EDC-exposed embryos are limited but reveal evidence of effects primarily related to neurogenesis, and effects which may involve mechanisms other than disruption of classical endocrine disruption. Moreover, these data suggest that EDC exposure prior to established hormone-dependent critical windows of sexual differentiation could result in broad, fundamental changes to brain organization particularly in cortex and cerebellum. These changes may include alteration of neuronal connections fundamental to complex behaviors such as activity, sociality, and executive function (Diamanti-Kandarakis et al., 2009; Kinch et al., 2015; Wolstenholme, Goldsby, & Rissman, 2013). Evidence from the cortex, cerebellum, and mid brain emphasize that effects from gestational exposures might be confounded or exacerbated by additional exposures later in development, including the postnatal period. Information on how exposures to emerging EDCs including fire retardants, surfactants, and other persistent contaminants known to be ubiquitous in household environments (Mariussen, 2012; Stapleton et al., 2009) impacts synaptogenesis, neural migration, and other developmental processes within the cortex, cerebellum, and mid brain are critically needed and likely to reveal new mechanisms of sex-

specific vulnerability. Comparison of results based upon sex in rodents at ages near neurodevelopmental critical periods like prenatal and postnatal testosterone surge as well as the brain growth spurt at PND 10, are needed to provide further insight into the alteration of brain organization which can contribute to the developmental basis of diseases such as ADHD, schizophrenia, and autism.

Compared to the neuroscience field as a whole, the neurodevelopmental EDC literature reported information about sex and potential sex differences more frequently. That neurodevelopmental effects of EDC exposure included alteration of sex differences, or the creation of new ones, was a common finding, and these observations will ultimately result in a better understanding of how male and female brains differ. The results of this review demonstrate that the time and effort it takes to include and analyze both sexes is worthwhile, especially in the endocrine disruption field, and should be considered a model for the larger neuroscience community.

Acknowledgements

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Tables

Table 1.1: EDC Effects on the Hypothalamus

Authors, Date	Animal Species, Strain	Sex	Exposure Route, Vehicle	Time of Exposure	EDC Exposure group(s)	Endpoints Measured	Age at Testing
(Cao et al., 2012)	Rat, Long Evans	M/F	Subcutaneous injection	PND 0-2	10 μg EB 50 μg/kg, 50 mg/kg BPA	ERα, ERβ, and Kiss1 mRNA expression in the anterior and mediobasal hypothalamus	PND 4 and 10
(Cao et al., 2013)	Rat, Sprague Dawley	M/F	Oral gavage to dam	GD 6-21	2.5, 25 μg/kg bw/day BPA 5, 10 μg/kg bw/day EE	ER mRNA expression in the hypothalamus and amygdala	PND 1
(Ceccarelli et al., 2007)	Rat, Sprague Dawley	M/F	Oral	PND 23-30	40 μg/kg BPA 0.4 μg/kg EE	Hypothalamic ERα expression	PND 37 and 90
(Colciago et al., 2009)	Rat, Sprague Dawley	M/F	Subcutaneous injection to dam	GD 15-19	10 mg/kg/day mixture of equal parts PCB 138, 153, 180 and 10^4 lower concentration of PCB 126	Enzyme expression in hypothalamus via RT-PCR	GD 20, PND 12, 21, 60
(Dickerson et al., 2011)	Rat, Sprague Dawley	M/F	i.p. injection, 0.1 ml DMSO 99.5%	GD 16 and 18	1 mg/kg Aroclor 1221 (industrial PCB mixture) 50 μg/kg EB 1 mg/kg mixture of PCB138, 153, 180 at equimolar concentration	Hypothalamic ERα expression, apoptotic cell numbers, gene expression	PND 1

Table 1.1 continued

(Facciolo et al., 2002)	Rat, Sprague Dawley	M/F, not distinguished	Oral, to dam	GD 0 – PND 23	400 µg/kg/day BPA	stt_2 expression and binding in presence of α containing GABA type A receptor agonists in the hypothalamus and hippocampus	PND 10, 23
(Facciolo et al., 2005)	Rat, Sprague Dawley	F	Oral, to dam	GD 0 – PND 23	40 and 400 µg/kg/day BPA	stt_3 expression and binding in presence of α containing GABA type A receptor agonists in extrahypothalamic structures, hippocampus, and cortex	PND 7, 55
(Hays et al., 2002)	Rat, Holtzman Sprague Dawley	M/F	Oral, to dam	GD 15	1 µg/kg TCDD	GAD 67 expression and progestin receptor (PR) expression in the preoptic area of the hypothalamus AhR and GAD dual label in preoptic area	PND 3
(He, Paule, & Ferguson, 2012)	Rat, Sprague Dawley	M/F	Oral gavage, to dam	GD 6-21	2.5, 25 µg/kg BPA 5.0, 10.0 µg/kg EE	SDN-POA volume	PND 21
(Hobler et al., 2010)	Rat, Wistar	M/F	Oral, to dam	GD 6 – PND 58	2 mg/kg/day TPTCl	Aromatase activity in the preoptic area of the hypothalamus	PND 1, 21, 58
(Khurana, Ranmal, & Ben-Jonathan, 2000)	Rat, Fischer 344	M/F	Subcutaneous injection	PND 1-5	100 µg day BPA 100 µg day octylphenol 5 µg day DES	ER expression in MBH and anterior pituitary	PND 30

Table 1.1 continued

(Lichtensteiger et al., 2015)	Rat, Wistar	M/F	Oral gavage, to dam	GD 7 – PND 21 GD 13-19 for groups containing paracetamol	100, 200, 450 mg/kg/day mixtures A-mix (di-n-butylphthalate, diethylhexylphthalate, vinclozolin, prochloraz, procymidone, linuron, epoxiconazole, p,p'-DDE) E-mix (bisphenol A, 4-methylbenzylidene camphor, 2-ethylhexyl 4-methoxycinnamate, butylparaben) AEP-mix (A-mix and E-mix plus paracetamol) Paracetamol alone -For more details about amounts of each chemical, see paper	Gene expression in POA and VMN by exon-microarray and rt-PCR	PND 6
(Losa et al., 2011)	Rat, Long Evans	F	Subcutaneous injection	PND 0-3	1, 10 mg/kg bw Genistein 10 µg/kg bw EB	Kiss fiber density in the hypothalamus	PND 21, 24, 28, 33
(Navarro et al., 2009)	Rat, Wistar	M/F	Subcutaneous injection	PND 1 and PND 1-5	1, 10, 100, 500 µg EB to males 0.1, 1, 10, 100 µg EB to females 100, 500 µg BPA	Brain circulating hormone levels, hypothalamic mRNA expression of KiSS-1	PND 30
(Patisaul et al., 2006)	Rat, Sprague Dawley	M/F	Subcutaneous injection	PND 1, 1.5, 2, 2.5	50 µg EE 250 µg BPA 250 µg genistein	TH and ERα immunoreactivity in hypothalamic AVPV	PND 19

Table 1.1 continued

(Patisaul et al., 2007)	Rat, Sprague Dawley	M/F	Subcutaneous injection	PND 1, 1.5, 2, 2.5 PND 85	50 µg EE 250 µg BPA 250 µg genistein Gonadectomized adults with 10 µg EB and 48 hours later 500 µg progesterone	AVPV and SDN volume, calbindin labeled neuron numbers, GnRH neuronal activation	PND 19 and PND 98
(Rebuli et al., 2014)	Rat, Sprague Dawley	F	Oral gavage, to the dam	GD 6 – PND 90	2.5, 25, 260, or 2700 µg /kg bw/day BPA 0.5, 5.0 µg/kg bw/day EE	Hypothalamic estrogen receptor expression	PND 21, 90
(Rubin et al., 2006)	Mouse, CD-1	M/F	Osmotic pump to dam	GD 8 – PND 16	25, 250 ng/kg bw per day BPA	TH neuron number in preoptic area	PND 22-24, 27-29, post natal week 6-9
(Walker et al., 2014)	Rat, Sprague Dawley	M/F	Subcutaneous injection to dam	GD 16, 18	1 mg/kg A1221 50 µg/kg EB	Gene expression and DNA methylation in the AVPV and ARC	PND 15, 30, 45, 90

Table 1.2: EDC Effects on the Hippocampus

Authors, Date	Animal Species, Strain	Sex	Exposure Route, Vehicle	Time of Exposure	EDC Exposure group(s)	Endpoints Measured	Age at Testing
(Facciolo et al., 2002)	Rat, Sprague Dawley	M/F, not distinguished	Oral, to dam	GD 0 – PND 23	400 μg/kg/day BPA	stt_2 expression and binding in presence of α containing GABA type A receptor agonists in the hypothalamus and hippocampus	PND 10, 23
(Facciolo et al., 2005)	Rat, Sprague Dawley	F	Oral, to dam	GD 0 – PND 23	40 and 400 μg/kg/day BPA	stt_3 expression and binding in presence of α containing GABA type A receptor agonists in extrahypothalamic structures, hippocampus, and cortex	PND 7, 55
(K. Kim et al., 2009)	Mouse, ICR	M/F, not distinguished	Subcutaneous injection	GD 14.5-18.5 and Postnatal week 8	5, 10, 20 mg/kg BPA	neonatal hippocampal development and adult hippocampal neurogenesis	PND 1, 22, Postnatal week 8
(Kunz et al., 2011)	Rat, Sprague Dawley	M/F, not distinguished	Oral, to dam	GD 6 – PND 20	1 mg/L BPA	Hippocampal histology, growth, morphology (hippocampus, motor and somatosensory cortices), glial and neuronal development (cingulum), myelination (cortex and corpus callosum)	PND 20
(Takahashi et al., 2009)	Rat, F344/N	M	Subcutaneously injected osmotic pump to dam	GD 7 – PND 1	1 mg/kg PCB 106	Gene expression in cerebral cortex, hippocampus, and striatum	PND 1

Table 1.2 continued

(X. B. Xu et al., 2014)	Rat, Sprague Dawley	M	Oral, to dam	GD 21 – PND 21	0.1 mg/L BPA 500 μg/kg ER antagonist ICI 182,780	Hippocampal mRNA, phosphorylation, and total protein expression for ERα	PND 7, 11, 21
(X. H. Xu et al., 2010)	Rat, Sprague Dawley	M	Oral gavage, to dam	GD 7 – PND 21	0.05, 0.5, 5, 50, 200 mg/kg/day BPA	Hippocampal NMDAR subunit, ERβ and aromatase cytrochrome P450 protein expression	PND 4, 7, 14, 21, 56
(X. Xu, Xie, et al., 2013)	Mouse, ICR	M	Oral, to dam	GD 7 – PND 21	4, 0.4 or 0.04 mg/kg/day BPA	Pyramidal cell synaptic density in the CA1 region of the hippocampus, Synaptic structural modification in pyramidal cells of the CA1 region of the hippocampus, hippocampal western blot	PND 14, 21 and 56
(X. Xu, Xie, et al., 2013)	Mouse, ICR	M	Oral, to dam	GD 7 – PND 21	0.04, 0.4, 40 mg/kg/day BPA	Synaptic density and structural modification of pyramidal cells in CA1 region of hippocampus, synaptic protein and receptor expression	PND 14, 21, 56
(Zoeller, Bansal, & Parris, 2005)	Rat, Sprague Dawley	M	Oral, to dam	GD 6 – PND 15	1, 10, 50 mg/kg BPA	Hippocampal mRNA expression of TH responsive genes	PND 15
(Zoeller et al., 2000)	Rat, Sprague Dawley	M/F, not distinguished	Oral, to dam	GD 6 – PND 21	1, 4, 8 mg/kg A1254	Hippocampal and cerebellar mRNA expression of TH responsive genes	PND 5, 15, 30

Table 1.3: EDC Effects on Cortex, Cerebellum, and Mid-brain

Authors, Date	Animal Species, Strain	Sex	Exposure Route, Vehicle	Time of Exposure	EDC Exposure group(s)	Endpoints Measured	Age at Testing
(Ahmed, 2011)	Rat, Wistar	M/F, not distinguished	Gastric intubation, to dam	GD 1 – PND 30	0.2 or 0.4 μg/kg bw/ day TCDD	Cerebellar monoamine and GABA concentrations and acetylcholinesterase activity (AchE)	GD 16, 19, PND 10, 20, 30
(Chang et al., 2005)	Rat, Sprague Dawley	M/F	Oral, to dam	GD 15	2 μg/kg TCDD	Expression of cytochrome P450 1A1 (CYP1A1), Bax, Bcl-2, Bcl-xl using western blot and RT-PCR in cortex and cerebellum	PND 0 and PND115-125
(Facciolo et al., 2005)	Rat, Sprague Dawley	F	Oral, to dam	GD 0 – PND 23	40 and 400 μg/kg/day BPA	stt_3 expression and binding in presence of α containing GABA type A receptor agonists in extrahypothalamic structures, hippocampus, and cortex	PND 7, 55
(Fernandez et al., 2010)	Mouse, Dark-Agouti	M	Oral, to dam	GD 18	0.7 μg/kg TCDD	Myelin protein expression, morphological analysis of myelination, expression of myelination related genes in medulla oblongata, cerebellum, telencephalon, diencephalon	PND 2/3, 14, 30, 135

Table 1.3 continued

(Itoh et al., 2012)	Mouse, ICR/Jcl	M/F	Subcutaneous injection, to the dam	GD 0.5-postnatal week 3	20 µg/kg bw/day BPA	neuronal migration in the ventricular zone, gene expression changes in embryonic telencephalon, DiI labeled cortical neurons in somatosensory cortex at postnatal week 3 and 12, gene expression in cortex, thalamus and pons	GD 10.5-18.5, Postnatal weeks 3 and 12 and PND 1, 4, 8
(Kawai et al., 2007)	Mouse, ICR	M	Oral, to dam	GD 11-17	2 ng/g bw/day BPA	ERα, ERβ, and serotonin immunoreactivity in the dorsal raphe nucleus	PND 35, 63, 91
(Morse et al., 1996)	Rat, Wistar	M/F	Oral, to dam	GD 10-16	5, 25 mg/kg Aroclor 1254	Forebrain and cerebellum thyroid hormone concentrations and thyroid hormone metabolism	GD 20, PND 21 and 90
(Nayyar, Zawia, & Hood, 2002)	Rat, Sprague Dawley	M/F, not distinguished	Oral, to dam	GD 15	0.25, 0.5, 1.0 µg/kg bw dioxin	Electrophoretic mobility shift assay of nuclear extract from cortex and cerebellum	PND 3, 5, 10, 15, 20, 30
(Nishizawa, Imanishi, et al., 2005)	Mouse, ICR	M/F	Oral, to dam	GD 6.5-17.5	0.02, 2, 200, 20,000 µg/kg day BPA 5 µg/kg EE	AhR, AhRR, Arnt expression in cortex and cerebellum, regulation of CYP1A1 and GST	GD 14.5 and 18.5
(Nishizawa et al., 2003)	Mouse, ICR	M/F	Oral, to dam	GD 6.5-17.5	2 µg/kg day BPA	RARα, and RXRα expression in cortex and cerebellum	GD 12.5, 14.5, 16.5, and 18.5
(Nishizawa, Morita, et al., 2005)	Mouse, ICR	M/F	Oral, to dam	GD 6.5-17.5	0.02, 2, 200, 20,000 µg/kg day BPA	AhR, RARα, and RXRα expression in cortex and cerebellum	GD 14.5 and 18.5
(Takahashi et al., 2009)	Rat, F344/N	M	Subcutaneously injection, dam	GD 7 – PND 1	1 mg/kg PCB 106	Gene expression in cerebral cortex, hippocampus, and striatum	PND 1

Table 1.3 continued

(Zoeller et al., 2000)	Rat, Sprague Dawley	M/F, not distinguished	Oral, to dam	GD 6 – PND 21	1, 4, 8 mg/kg A1254	Hippocampal and cerebellar mRNA expression of TH responsive genes	PND 5, 15, 30
(Zsarnovszky et al., 2005)	Rat, Sprague Dawley	M/F	Intracerebellar injection	PND 4-12	10^{-12} - 10^{-10} M and 10^{-7} – 10^{-6} M BPA 10^{-10} M EE	Activated ERK1/2 signaling in cerebellum	PND 4-12

Table 1.4: EDC Effects on Whole Brain and Embryonic Brain Regions

Authors, Date	Animal Species, Strain	Sex	Exposure Route, Vehicle	Time of Exposure	EDC Exposure group(s)	Endpoints Measured	Age at Testing
(Fernandez et al., 2010)	Mouse, Dark-Agouti	M	Oral, to dam	GD 18	0.7 μg/kg TCDD	Myelin protein expression, morphological analysis of myelination, expression of myelination related genes in medulla oblongata, cerebellum, telencephalon, diencephalon	PND 2/3, 14, 30, 135
(Fujita et al., 2006)	Mouse, C57BL/6	M/F, not distinguished	Oral, to dam	GD 7	20 μg/kg TCDD	Genes expression in whole fetal brain	GD 12
(Gauger et al., 2004)	Rat, Sprague Dawley	M/F, not distinguished	Oral, to dam	GD 6-16	1, 4 mg/kg A1254	TH responsive gene expression in fetal cortex	GD 16
(Itoh et al., 2012)	Mouse, ICR/Jcl	M/F	Subcutaneous injection, to the dam	GD 0.5-postnatal week 3	20 μg/kg bw/day BPA	neuronal migration in the ventricular zone, gene expression changes in embryonic telencephalon, DiI labeled cortical neurons in somatosensory cortex at postnatal week 3 and 12, gene expression in cortex, thalamus and pons	GD 10.5-18.5, Postnatal weeks 3 and 12 and PND 1, 4, 8
(Komada et al., 2012)	Mouse, C57BL/6J	F and M/F-not distinguished	Oral	GD 8.5-13.5	20 and 200 μg/kg BPA	fetal neurogenesis of the cortical plate of the dorsal telencephalon	GD 14.5
(Morse et al., 1993)	Rat, Wistar	M/F, not distinguished	Oral, to dam	GD 1 or GD 1-18	0.2, 0.6, 1.8 mg/kg bw hexachlorobiphenyl (HCB) 0.6 mg/kg bw Tetrachlorobiphenyl (TCB)	Activity of type II thyroxine – 5' deiodinase in the whole brain	GD 20, PND 7 and 21

Table 1.4 continued

(Nakamura et al., 2006)	Mouse, ICR/Jcl	M/F, not distinguished	Subcutaneous injection to dam	GD 0-10.5, 12.5, 14.5, or 16.5	20 μg/kg BPA	cell proliferation, neuronal differentiation and migration in the ventricular zone and cortical plate, gene expression in embryonic telencephalon	GD 10.5, 12.5, 14.5 and 16.5
(Naveau et al., 2014)	Rat, Wistar	M/F, not distinguished	Oral to dam	GD 6-17, 19, or 20	6 mg/kg/day Aroclor 1254	cell proliferation, S phase and cell cycle exit in the cortex, ventricular zone, and cortical plate, cell death and neuronal differentiation rate, neuronal migration	GD 17, 19, 20
(Wolstenholme et al., 2012)	Mouse, C57BL/6J	M/F	Oral, to the dam	GD 0 – 18.5	5 mg BPA/kg diet	whole brain gene expression by microarray and rtPCR	GD 18.5

Figure

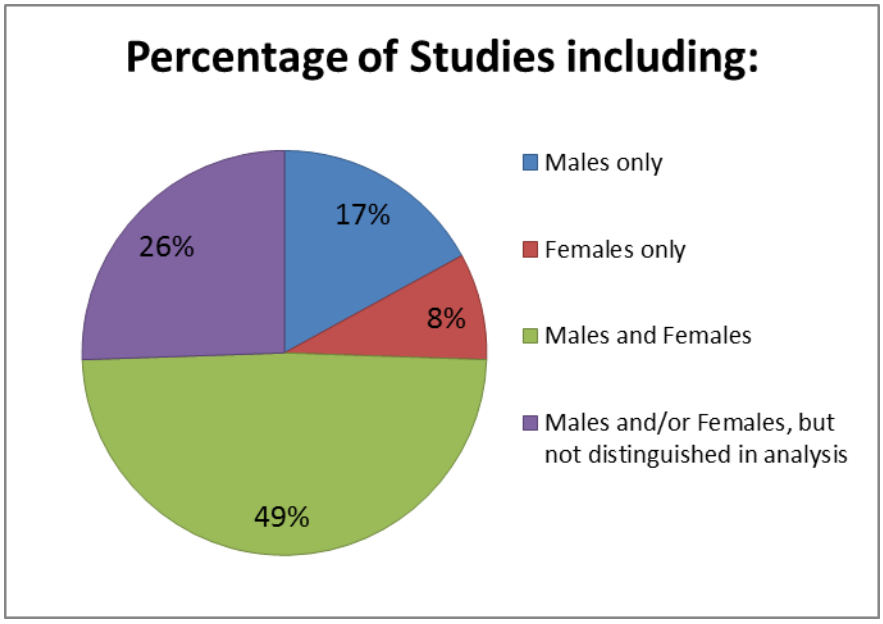

Figure 1.1: The Percentage of studies including sex in their analyses. Studies including males only, females only, males and females, and males and/or females, but were not distinguished in analysis were counted and compared to the number of studies included in the review to achieve a percentage for each category.

CHAPTER 2—Prenatal Bisphenol A Exposure Alters Sex-Specific Estrogen Receptor Expression in the Neonatal Rat Hypothalamus and Amygdala

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Abstract

Bisphenol A (BPA) exposure is ubiquitous, and in laboratory animals, early-life BPA exposure has been shown to alter sex specific neural organization, neuroendocrine physiology, and behavior. The specific mechanisms underlying these brain-related outcomes, however, remain largely unknown, constraining the capacity to ascertain the potential human relevance of neural effects observed in animal models. In the perinatal rat brain, estrogen is masculinizing, suggesting that BPA-induced perturbation of estrogen receptor (ESR) expression may underpin later in-life neuroendocrine effects. We hypothesized that prenatal BPA exposure alters sex-specific ESR1 (ERα) and ESR2 (ERβ) expression in postnatal limbic nuclei. Sprague Dawley rats were mated and gavaged on gestational days (GDs) 6–21 with vehicle, 2.5 or 25 μg/kg bw/day BPA, or 5 or 10 μg/kg bw/day ethinyl estradiol. An additional group was restrained but not gavaged (naïve control). Offspring were sacrificed the day after birth to quantify ESR gene expression throughout the hypothalamus

and amygdala by *in situ* hybridization. Relative to the vehicle group, significant effects of BPA were observed on ESR1 and ESR2 expression throughout the mediobasal hypothalamus and amygdala in both sexes. Significant differences in ESR expression were also observed in the mediobasal hypothalamus and amygdala of the naïve control group compared with the vehicle group, highlighting the potential for gavage to influence gene expression in the developing brain. These results indicate that ESR expression in the neonatal brain of both sexes can be altered by low-dose prenatal BPA exposure, with the caveat of the stress of gavage.

Key Words

brain; endocrine disruptor; endocrine disruption; hypothalamus; development; sexually dimorphic; ethinyl estradiol.

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Introduction

Human exposure to bisphenol A (BPA), a component of polycarbonate plastics, epoxy resins, dental sealants, thermal receipts, and other products, is indisputably low but widespread (Biedermann, Tschudin, & Grob, 2010; Cooper, Kendig, & Belcher, 2011;

FAO/WHO, 2011; Geens et al., 2012; Geens, Goeyens, & Covaci, 2011; Vandenberg, Hauser, Marcus, Olea, & Welshons, 2007). More than 90% of the U.S. population have measurable levels of BPA in bodily fluids, with children typically exhibiting higher internal levels than adults (Calafat, Ye, Wong, Reidy, & Needham, 2008; Vandenberg et al., 2010). In laboratory animal studies, early life exposure to BPA has been shown to impact the organization of numerous estrogen-sensitive neural endpoints including the sexual differentiation of hypothalamic regions important for sex-specific reproductive physiology and behavior (reviewed in Wolstenholme, Rissman, et al. (2011)). Those observations have elevated concerns regarding the potential for low-dose exposure to result in adverse neural health outcomes in humans (Beronius, Ruden, Hakansson, & Hanberg, 2010; Hengstler et al., 2011; Vandenberg, Maffini, Sonnenschein, Rubin, & Soto, 2009).

Understanding the specific molecular and cellular mechanisms through which BPA can alter the developing brain will help establish how such neural effects in rodents may be predictive of similar neural effects in humans (vom Saal et al., 2007; Wolstenholme, Rissman, et al., 2011). The goal of the present study was to quantify the effects of prenatal low-dose BPA exposure on sex-specific estrogen receptor (ESR) expression in the neonatal rat hypothalamus and amygdala (AMYG). Neonatal ESR expression was examined because the density of ESRs is regionally and sexually dimorphic at this age, and this differential sensitivity to neonatal estrogen ultimately confers permanent differences in neural structure and function (McCarthy, 2008; Simerly, 2002). Although there are critical species differences specific to how estrogen organizes the developing brain (McCarthy, 2008; Resko & Roselli, 1997), sex-specific ESR expression is present in perinatal rodents and primates

(including humans) (Gonzalez et al., 2007; Kato et al., 1998; Resko & Roselli, 1997; Wallen, 2005), the distribution of which is highly conserved in vertebrates (Brandenberger, Tee, Lee, Chao, & Jaffe, 1997; Cao & Patisaul, 2013; MacLusky, Chaptal, & McEwen, 1979; Walker, Juenger, & Gore, 2009). Thus, perturbation of neonatal ESR expression by BPA exposure could be a mechanism by which the steroid hormone–dependent organization of these subregions is altered. Moreover, very little is known regarding the impact of prenatal-only BPA exposure on brain sexual differentiation, so the results presented here yield new insights regarding effects resulting from this specific exposure window. Because BPA has long been regarded as estrogenic (Dodds, Goldberg, Larson; W., & Robinson, 1938), and prenatal estrogen exposure is masculinizing in female rodents (McCarthy, 2008), ethinyl estradiol (EE) was used as a reference estrogen. Understanding and elucidating the pathways and critical periods in which the vulnerabilities to, and consequences of, low-dose BPA exposure manifest in animal models will contribute mechanistic knowledge requisite to evaluate the potential risk of BPA exposure in humans.

Assessing consequences of exposure confined to a specific critical window may also help identify sensitive periods during which humans may be particularly vulnerable to the effects of BPA.

There are numerous sex differences throughout the mammalian brain, particularly within the hypothalamus and surrounding structures (Bonthuis et al., 2010; G. J. De Vries, 2004; Simerly, 2002), which underpin physiological and behavioral sexual dimorphisms. These differences are organized primarily by steroid hormones during critical windows of development throughout gestation, neonatal life, and adolescence (Schulz, Molenda-Figueira,

& Sisk, 2009; Simerly, 2002). Accordingly, the distribution of steroid hormone receptors, especially ESRs, during these critical windows of development is sex specific (e.g., in the neonatal rat (Cao & Patisaul, 2011)) and can be manipulated by exogenous administration of steroid hormones such as androgens and estrogens (McCarthy, 2008). Those early-life manipulations can have lifelong effects, including subfertility and behavioral changes (B. Cooke, Hegstrom, Villeneuve, & Breedlove, 1998; McCarthy, 2008; Morris, Jordan, & Breedlove, 2004; Simerly, 2002). Therefore, disruption of sex-specific ESR expression during the sexual differentiation process is one molecular mechanism by which developmental BPA exposure might confer lifelong effects on hypothalamic organization and function.

The hypothalamus is the apical coordinator of neuroendocrine activity, sending signals from the central nervous system to the pituitary gland and receiving feedback information from target endocrine glands (such as the gonads, pancreas, and thyroid) to organize, integrate, and maintain homeostatic endocrine function. The hypothalamus is an expansive region comprising numerous sexually dimorphic nuclei. We previously established that expression of the two nuclear ESR subtypes (ESR1, also known as ERα, and ESR2, also known as ERβ) is sexually dimorphic within these hypothalamic subregions of the neonatal rat (Cao & Patisaul, 2011). Moreover, we demonstrated that neonatal BPA exposure altered sex-specific ESR expression later in the neonatal period, particularly in the anteroventral periventricular nucleus of the hypothalamus (AVPV) and the medial preoptic area (MPOA) (Cao et al., 2012). These anterior hypothalamic regions are essential for ovulation in females and coordinate gonadotropin-releasing hormone activity in both sexes. Posterior regions,

including the arcuate nucleus (ARC) and the ventrolateral division of the ventromedial nucleus (VMNvl), were not meaningfully altered. Based on those prior studies, here we explored the hypothesis that BPA exposure, confined to the prenatal critical window, would impact ESR1 and ESR2 expression in these same hypothalamic subregions.

An additional region of interest for the present study was the AMYG, a structure lying lateral to the posterior realm of the hypothalamus. The AMYG comprised numerous subregions, each of which has differential and sex-specific expression levels of ESR1 and ESR2 in neonatal and adolescent rats (Cao et al., 2012; Kuhnemann, Brown, Hochberg, & MacLusky, 1994). Moreover, the cytoarchitecture of several amygdaloid subregions, such as total volume and neuronal soma size, is sexually dimorphic in adults and young rodents (B. M. Cooke, Stokas, & Woolley, 2007; B. M. Cooke, Tabibnia, & Breedlove, 1999; Hines, Allen, & Gorski, 1992; Johnson, Breedlove, & Jordan, 2008; Morris, Jordan, King, Northcutt, & Breedlove, 2008) and under the control of circulating sex hormones (B. M. Cooke, 2006; B. M. Cooke et al., 2007; B. M. Cooke et al., 1999). In rats, most of those sex differences are established during the perinatal period (Morris, Jordan, & Breedlove, 2008). The subregions within the amygdaloid complex are highly interconnected (Canteras, Simerly, & Swanson, 1992; B. M. Cooke & Simerly, 2005; Maras & Petrulis, 2010; Simerly, 2002) and integrate information sent and received from other brain regions to regulate a wide range of behaviors (Canteras et al., 1992; Simerly, 2002; Swanson & Petrovich, 1998), including sexual behavior (Harris & Sachs, 1975; Newman, 1999), aggression (Z. Wang, Hulihan, & Insel, 1997), anxiety behavior (Toufexis, 2007), and parental behavior (G. J. De Vries & Villalba, 1997; Fleming, Vaccarino, & Luebke, 1980). Subregions of interest for the present

study included the posterodorsal portion of the medial amygdala (MePD), the cortical amygdaloid nucleus, posterolateral (PLCo), the posteromedial (PMCo), and the amygdalo-hippocampal area (AHi), all of which play important roles in conferring behavioral sex differences.

Prior studies exploring the impact of early-life BPA exposure on brain development and gene expression have produced inconsistent data (Palanza et al., 2008; Richter et al., 2007; Wolstenholme, Rissman, et al., 2011), likely resulting from differences in exposure duration, dose, route of administration, and critical species differences in neural structure between rats and mice (Bonthuis et al., 2010). Because of those inconsistencies, study design–related recommendations for BPA research have been issued including statistical control for litter effects and the use of oral administration (Goodman et al., 2006; Hengstler et al., 2011; Hunt, Susiarjo, Rubio, & Hassold, 2009; Richter et al., 2007). In situations where BPA is thought to act as a weak estrogen, use of a concurrent reference estrogen and minimization of exogenous estrogen exposure are also suggested. All of those were incorporated into the current study, which was a component of a larger project, the methodological details of which have been published (Ferguson, Law, & Abshire, 2011; He et al., 2012). The BPA doses used (2.5 and 25 µg/kg bw/day) are well below the current no observable adverse effect level (NOAEL) of 50mg/kg bw/day and the current reference dose (tolerable daily intake) of 50 µg/kg bw/day (Chapin et al., 2008; Geens et al., 2012; National Toxicology Program, 1982). Despite well-recognized metabolic differences between rats and humans, the lowest BPA dose used here is near the top range of estimated daily BPA intake for human infants (FAO/WHO, 2011). Two doses of EE were included as the reference

estrogen because gestational estrogen exposure is well recognized to induce region-specific hypothalamic masculinization in female rats (McCarthy, 2008; Simerly, 2002).

Because prenatal stress can alter hypothalamic organization and contribute to behavioral abnormalities related to stress responsivity (Goel & Bale, 2009; Markham & Koenig, 2011), an additional point of interest in the present study was the potential neural impact of gavage. Gavage is historically the primary route of choice for oral exposure studies because it ensures consistent dosing; however, concern has been raised that numerous aspects of this procedure can be stressful to the animals (Balcombe, Barnard, & Sandusky, 2004). The impact of potential gavage-related stressors relative to the "stress" of the toxicant has not been explored for an EDC such as BPA. Thus, a naïve control group (restrained but not gavaged) was incorporated in the experimental design to investigate how gavage of the pregnant dams might alter ESR expression in the offspring. Because the AMYG coordinates responses to stress and fearful situations and maternal care (Cahill et al., 1996; G. J. De Vries & Villalba, 1997; Fleming et al., 1980; Harris & Sachs, 1975; Meaney, Dodge, & Beatty, 1981; Newman, 1999; Toufexis, 2007; Z. Wang et al., 1997), we hypothesized that any potential gavage effects would most likely be appreciable in amygdaloid subregions. Importantly, demonstration of gavage-associated effects on ESR expression would suggest that alternative, less stressful, routes of oral exposure may be more suitable for studies exploring the impact of early-life EDC exposure on sex-specific brain organization and gene expression.

Collectively, the results from this study yield insight into the mechanisms by which BPA might affect the sex-specific organization of hypothalamic and amygdaloid subnuclei.

These results also aid in establishing the degree to which early-life stress from gavage or other stressful procedures can induce significant effects on brain development, independent of the toxicant.

Materials and Methods

Animal care, prenatal BPA and EE exposure, brain collection, and section preparation.

Postnatal day 1 (PND1) pups were obtained from litters generated for a study described in detail earlier (Ferguson et al., 2011). Briefly, the National Center for Toxicological Research (NCTR) Breeding Colony supplied 364 female and 180 male Sprague Dawley rats (derived from Charles River Crl: COBS CD (SD) BR Rat, Outbred) at weaning (PND21). Upon reaching breeding age (see below), these rats became the dams and sires (i.e., F0 generation) of subsequent litters. Upon arrival to the vivarium at PND21, each rat was tail tattooed for identification, weighed, and group-housed (three same-sex/cage). Ad lib food (see below for diet information) and water were provided at all times. Housing rooms were maintained on a 12-/12-h light/dark cycle (6:00 a.m.–6:00 p.m.) at 22 ± 1° C (mean ± SE) and 45–55% humidity. All animals were maintained in accordance with the Guide for the Care and Use of Laboratory Animals from the 1996 National Research Council (NRC, 1996), and all studies were approved in advance by the NCTR Institutional Animal Care and Use Committee.

Upon arrival at PND21, rats were housed in polysulfone cages with polysulfone microfilter tops, both of which were purchased new at the beginning of the study (Ancare, Bellmore, NY). Polysulfone caging was chosen as no detectable in vivo or in vitro estrogenic effects from BPA extracted from new polysulfone cages have been observed (Howdeshell et

al., 2003). Glass water bottles with food-grade silicone stoppers (Fisher Scientific, St Louis, MO) and metal sipper tubes were used to further reduce potential environmental estrogen exposure. Metal mop buckets were used in the housing rooms, and all chow was stored in metal containers. This environment began for the F0 generation at PND21 and was maintained throughout the remainder of the study. Beginning upon arrival from the NCTR Breeding Colony at PND21 and throughout the study, all animals were maintained on a low phytoestrogen chow (TestDiet 5K96 (irradiated pellets), Verified Casein Diet 10 IF, TestDiet, Richmond, IN).

There were nine breeding rounds, each one consisting of a 24-h pairing of a male and female, with a 2-week interval between each round. Male and female breeders were those that were obtained at PND21, housed in polysulfone cages with glass water bottles, and fed low phytoestrogen chow as described above. Male/female pairings were random, subject to the constraint that no grandparent could be shared within a pair (i.e., no pairings were done between siblings, first, or half cousins). Beginning on PND87 or 90, each female was placed into a wire bottom cage with a PND90 male, which had been placed into the cage 72h earlier for habituation. The pair was housed together for 24h, after which the female and the cage bottom were visually examined for a sperm plug. If a sperm plug was detected, the female was removed, weighed, housed individually, and this day was termed gestational day (GD) 0. If no sperm plug was detected, the female was returned to her home cage and used in the next breeding round (2 weeks later). If no sperm plug was detected after two breeding rounds, the female was euthanized. Thus, litters in this study were derived from sperm plug positive females between PND88 (age at first breeding) and PND105 (age at second

breeding, if there was no sperm plug on the first breeding round). Males were used for a maximum of three breeding rounds after which they were euthanized. Because all breeding occurred prior to any treatment, breeding success or failure was unrelated to exposure.

Females for which a sperm plug was detected were randomly assigned to treatment within their body weight stratum. However, it became evident early in the study that probability of littering given sperm plug detection was positively related to body weight. Heavier sperm plug positive females were more likely to litter than lighter females. Therefore, sperm plug positive females were ranked by body weight and the ranked dams divided into four parts or quartiles. The rats in each quartile were randomly assigned to one of the six treatment groups (in as balanced a fashion as the counts permitted).

Bisphenol A (2,2-Bis(4-hydroxyphenyl)propane, Product no. B0494, TCI America) and EE (17α ethinyl estradiol, Product no. E4876; Sigma, St Louis, MO) were each dissolved in a 0.3% (by weight) aqueous solution of carboxymethylcellulose sodium salt (CMC, high viscosity) (Product no. C5013, Sigma). Because of the lipophilic nature of BPA, oils (e.g., corn oil, tocopherol-stripped corn oil, arachis oil) are often used as vehicle. However, suggestions that such oils could possess estrogenic activity (see Ashby and Lefevre (1997); Ryan (2005)) and their nutritive potential have discouraged their use (Ashby & Lefevre, 1997). Thus, an aqueous vehicle was chosen for use here, which also offered the advantage of using automated dosing pumps.

Beginning on the morning of GD6, dams were gavaged with 5.0ml 0.3% CMC/kg bw/day (vehicle control group), 2.5 µg BPA/kg bw/day (BPA 2.5 group), 25.0 µg BPA/kg bw/day (BPA 25 group), 5.0 µg EE/kg bw/day (EE 5 group), or 10.0 µg EE/kg bw/day (EE

10 group) using a Hamilton Microlab 500 system (Hamilton Company, Reno, NV) interfaced with an animal weight scale and animal data collection software developed at the NCTR. The Hamilton Microlab system ensures extremely accurate volume calculations and performs four common pipette modes: fill, dispense, autorefill, and prime. An automated algorithm calculated the necessary volume (5ml/kg) based on the daily body weight of each rat. Thus, the procedure was as follows: The home cage was removed from the housing rack and the dam placed into the body weight scale which then transferred the weight to the algorithm software. This prompted the Hamilton Microlab system to draw up the correct volume. The rat was removed from the body weight scale and restrained, and the technician inserted the gavage feeding needle (18 gauge, 76.2mm length, 3.0mm end ball, straight needle for pregnant dams; 24 gauge, 25.4mm length, 1.25mm end ball, straight needle for PND1–10 pups; 22 gauge, 38.1mm length, 1.25mm end ball, straight needle for PND11–21 pups) and dispensed the solution. The duration from removal from the body weight scale until replacement into the home cage was typically less than 45 s. Daily oral gavage of the dams continued through GD21 (the day prior to parturition). Dams in the naïve control group were removed from their home cage, weighed, and restrained in the gavage position for the same duration as would be used for a gavage but were not gavaged. Any potential stress associated with the restraint used for the gavage or the actual gavage itself is unlikely to be different from that produced by use of nonautomated gavage procedures. There were six treatment groups: (1) Naïve, (2) Vehicle, (3) BPA 2.5, (4) BPA 25, (5) EE 5, and (6) EE 10.

Dams were not treated on the day of parturition (typically, GD22) and were left undisturbed on that day. On the day after parturition (PND1), male and female pups (n = 5–

8/sex/group) were sacrificed by decapitation, the heads rapidly frozen on a flat block of dry ice, and stored at −80°C until cryosectioning at North Carolina State University. All brains were cryosectioned (Leica CM1900, Nussloch, Germany) into four serial sets of 20 µm coronal sections, mounted onto Superfrost plus slides (Fisher Scientific, Pittsburgh, PA), and stored at −80°C until *in situ* hybridization histochemistry (ISHH) processing.

ISHH.

For each gene, all sections containing the anterior hypothalamus or the mediobasal hypothalamus (MBH) with the surrounding AMYG subregions were processed simultaneously to avoid batch effects. Thus, two large batches of ISHH were performed for each gene. ISHH was performed using well-established procedures, the details of which (including transcriptional templates for ESR1 and ESR2, radiolabeling, the specificity of the antisense and sense probes, and the ISHH procedures) have been described in detail elsewhere (Cao & Patisaul, 2011, 2013).

Dried slides were exposed to Kodak Biomax MR X-ray film (Eastman Kodak, Rochester, NY), along with a ^{14}C autoradiographic microscale (Amersham Life Sciences, Arlington Heights, IL) for optical density curve generation. The films were then developed using a Konica SRX-101A film processor (Konica Corporation, Tokyo, Japan). Exposure time was 7 days for ESR1 in the POA, 15 days for ESR1 in the MBH and AMYG, and 27 days for ESR2 in all regions.

Image analysis and film quantification.

The MCID Core Image software program (InterFocus Imaging Ltd, Cambridge, England) was used to quantify ESR signal on the films using the digital densitometry

application and by following conventional procedures as reported previously (Cao et al., 2012; Cao & Patisaul, 2011, 2013). Regions of interest (ROIs) included the AVPV and MPOA within the anterior hypothalamus, the rostral and caudal portions of the VMNvl and ARC (rVMNvl, rARC, cVMNcl, cARC) within the MBH, and the MePD, PLCo, PMCo, and AHi subregions of the AMYG. The borders of each subregion were identified using well-established anatomical landmarks for neonatal rats (Cao & Patisaul, 2011, 2013) and the assistance of a brain atlas (Paxinos, Ashwell, & Tork, 1994).

ROI densities and background levels were measured from anatomically matched sections (two for each hypothalamic ROI and five for the AMYG). The resulting values for each brain section after background subtraction were then averaged to obtain a representative measurement (for that ROI) for each animal. Where there were two or more pups/sex/litter, the results were averaged to obtain a single representative value for that sex in that litter as suggested (Festing, 2006). Optical densities were converted to nCi/g tissue equivalents using a "best fit" curve (third degree polynomial) generated from the autoradiographic 14C microscales. In all cases, the signal was within the limits of the curve. For quality control, all measurements for the POA and MBH were completed by at least two investigators who were blind to the exposure groups. There was a high degree of concordance among the two data sets, and the results were averaged to obtain the final values for each ROI. AMYG measurements were then made by one of these investigators as described above (because the high degree of concordance between the two evaluators demonstrated with confidence that the remaining analyses could be completed by one person).

Statistics.

All statistical analyses were designed in consultation with statisticians at the NCTR and National Institute for Environmental Health and used the litter as the experimental unit for all analyses. In addition to determining whether exposure altered gene expression within each sex, establishing whether exposure altered sex differences in expression was also an experimental goal. Thus, two-way ANOVA with exposure, sex, and the interaction as the factors was used for all analyses. Preliminary inspection of the data indicated obvious differences between the vehicle control and naïve control groups. Thus, for each ROI and gene of interest (ESR1 or ESR2), a two-way ANOVA (with group, sex, and the interaction as factors) compared only the vehicle control and naïve control groups, exclusive of the other exposure groups. Subsequently, the vehicle control, BPA, and EE treatment groups (the naïve control group was excluded) were compared by two-way ANOVA, and Holm-Sidak multiple comparison tests used when a significant main effect of exposure group or sex was identified. Because ESR expression levels in some regions are known to be sexually dimorphic, if there was a significant main effect of sex (even if there was no significant interaction with exposure), Holm-Sidak comparisons were made within sex (i.e., the male vehicle control group was compared with each same-sex BPA and EE group, and the female vehicle control group was compared with each same-sex BPA and EE group). Additionally, to determine whether exposure impacted sexually dimorphic expression, for each gene where a significant main effect of sex was identified in the initial two-way ANOVA, Holm-Sidak comparisons were conducted within the vehicle control group to determine whether this group exhibited sexual dimorphism. If this comparison was not significant, no further between-sex

comparisons were done. However, if this comparison was significant, then each exposure group was evaluated individually for sexual dimorphism. All analyses were two-tailed, and results were considered significantly different when $p \leq 0.05$.

Results

Differences in ESR mRNA levels between the vehicle and naïve control groups.

For each ROI, the vehicle and naïve controls were compared independent of the other exposure groups to establish whether there was an impact of gavage on gene expression (Figures 2.1–2.7). There were few significant differences in the anterior hypothalamus. There were no significant effects of group on ESR1 or ESR2 levels in the AVPV or MPOA (Figures 2.1B and E, 2.2B and E). However, ESR2 levels in the MePD exhibited a significant group effect ($F(1,23) = 231.88$, $p \leq 0.001$) with expression levels lower in the vehicle control group. Significant main effects of group were found for both ESRs in the VMNvl (ESR1, rVMNvl ($F(1,23) = 53.11$, $p \leq 0.001$); ESR2, rVMNvl ($F(1,22) = 255.22$, $p \leq 0.001$); ESR1, cVMNvl, ($F(1,23) = 30.66$, $p \leq 0.001$); ESR2, cVMNvl, ($F(1,23) = 93.84$, $p \leq 0.001$)) (Figures 2.3D and I, 2.4B and E) and MePD (ESR1 ($F(1, 23) = 37.71$, $p \leq 0.001$); ESR2 ($F(1, 23) = 231.85$, $p \leq 0.001$)) (Figures 2.5B and 2.7B), with expression levels lower in the vehicle control group in each case. Significant main effects of group were found for ESR1 expression in the ARC (rARC ($F(1,23) = 25.04$, $p \leq 0.001$); cARC ($F(1,23) = 46.16$, $p \leq 0.001$)) (Figures. 2.3B and G) and all subregions of the AMYG (MePD ($F(1,23) = 37.71$, $p < 0.001$; PLCo ($F(1,23) = 50.84$, $p \leq 0.001$); PMCo ($F(1,23) = 62.54$, $p \leq 0.001$); AHi ($F(1, 23) = 67.70$, $p \leq 0.001$)) (Figures. 2.5D, 2.6B, and 2.6D), with expression levels lower in the vehicle control group in each comparison.

As expected, ESR expression was sexually dimorphic in several ROIs. Significant main effects of sex on ESR1 expression were identified in the MPOA ($F(1,22) = 15.80$, $p \leq 0.001$), rVMNvl ($F(1,23) = 12.25$, $p \leq 0.002$), cVMNvl $F(1,23) = 9.48$, $p \leq 0.005$), rARC ($F(1,23) = 7.85$, $p \leq 0.010$), and cARC ($F(1,23) = 15.18$, $p \leq 0.001$), with higher levels in females. A significant effect of sex on ESR2 expression was found in the AVPV ($F(1,22) = 13.30$, $p \leq 0.001$; Figure 2.2B) and MePD ($F(1,23) = 6.21$, $p \leq 0.02$), with higher expression levels in males. An additional sex difference was detected in the cVMNvl ($F(1,23) = 12.64$, $p \leq 0.002$; Figure 2.4E), with higher ESR2 expression levels in females. Compared with the naïve control group, these sex differences were generally conserved in the vehicle control group, but the magnitude of the difference was proportionally lower in accordance with the overall decrease in expression levels. Most notably, sex differences in ESR1 expression in the MPOA ($p = 0.07$) and ESR2 expression in the cVMNvl ($p = 0.097$) were marginal in the vehicle control groups compared with the naïve control groups where the sex differences were robust ($p \leq 0.001$ and $p \leq 0.003$, respectively). Additionally, expected sex differences in ESR1 expression in the cARC and VMNvl were present in the naïve controls ($p \leq 0.001$ and $p \leq 0.01$, respectively) but absent in the vehicle controls ($p = 0.17$ and $p = 0.15$, respectively).

Effects of prenatal BPA or EE exposure on POA and ESR1 and ESR2 Expression.

ESR1. No significant effects were found in the AVPV of either sex (Figure 2.1C and Table 2.1). A significant sex effect in the MPOA ($F(1,51) = 8.27$, $p \leq 0.006$) indicated higher ESR1 expression levels in females, whereas the main effect of exposure did not reach statistical significance ($F(4,51) = 2.07$, $p = 0.098$). Because there was a significant effect of

sex, ESR1 expression in the vehicle control MPOA was analyzed for sexual dimorphism but not found to be statistically significant ($p = 0.199$). Within-sex comparisons of expression levels in the MPOA found no differences between any BPA or EE group and the same-sex vehicle control group.

ESR2. In the AVPV, significant main effects of sex ($F(1,51) = 40.87$, $p \leq 0.001$) and exposure group ($F(4,51) = 3.33$, $p \leq 0.017$) were found in the analysis of ESR2 levels. ESR2 expression was higher in males (Figure 2.2C and Table 2.1). Because a significant effect of sex was detected, within the vehicle control group, expression was analyzed for sexual dimorphism and found to be significant ($p < 0.05$). This dimorphic expression was preserved in all BPA and EE groups ($p < 0.05$ for all). Within-sex analyses for exposure differences did not indicate any BPA or EE group differed significantly from its same-sex vehicle control group. In the MPOA, no significant effects were identified (Figure 2.2F).

Effects of prenatal BPA or EE exposure on MBH ESR1 and ESR2 expression.

ESR1. Results of the two-way ANOVA revealed significant main effects of exposure and sex on ESR1 expression in the rostral and caudal regions of the ARC and VMNvl but no significant interactions between sex and exposure (Figure 2.3) (Exposure effects: rARC ($F(4,53) = 10.87$, $p \leq 0.001$); cARC ($F(4,53) = 16.40$, $p \leq 0.001$); rVMNvl ($F(4,53) = 23.16$, $p \leq 0.001$); cVMNvl ($F(4,53) = 33.83$, $p \leq 0.001$)). Significant main effects of sex were found in the rARC ($F(1,53) = 6.70$, $p \leq 0.013$), cARC ($F(1,53) = 12.78$, $p \leq 0.001$), rVMNvl ($F(1,53) = 5.80$, $p \leq 0.020$), and cVMNvl ($F(1,53) = 12.45$, $p \leq 0.001$), with expression levels greater in females. In the rARC, the vehicle control group, ESR1

expression was sexually dimorphic ($p < 0.05$). This sex difference was lost in all but the EE 10 group ($p < 0.01$) (Figure 2.3C).

Within-sex analysis of ESR1 expression in the MBH ROIs revealed that prenatal EE and BPA exposure generally elevated expression in both sexes (Table 2.1). In the rARC, the male BPA 2.5, BPA 25, and the EE 10 groups exhibited significantly higher ESR1 expression levels than the male vehicle control group ($p < 0.01$ for all). Within females, however, only the EE 10 group was significantly higher than the vehicle control group ($p < 0.01$) (Figure 2.3C). In the cARC, within-sex analyses of ESR1 expression indicated that males in the BPA 2.5, BPA 25, and EE 10 groups exhibited significantly higher expression levels than the vehicle control group ($p < 0.02$ for all), whereas females in the the BPA 2.5 and EE 10 groups had significantly higher expression levels than the vehicle control group ($p < 0.01$ for both) (Figure 2.3H). Within males, the BPA 2.5, BPA 25, and EE 10 groups exhibited significantly higher ESR1 expression levels in the rVMNvl than the vehicle control group ($p < 0.02$ for all), whereas within females, the BPA 2.5 and EE 10 groups had significantly higher levels than the vehicle control group ($p < 0.01$ for both) (Figure 2.3E). Within males, expression levels of ESR1 in the cVMNvl were significantly higher in the BPA 2.5, BPA 25, and EE 10 groups relative to the same-sex vehicle control ($p < 0.01$ for all), whereas within females, only the BPA 2.5 and EE 10 groups were significantly higher than the same-sex vehicle control group ($p < 0.01$ for both) (Figure 2.3J).

ESR2. As expected, ESR2 expression was only detected in the VMNvl (Figure 2.4). ESR2 expression was absent in the neonatal ARC, an observation consistent with our previous description (Cao and Patisaul, 2011). The analyses revealed main effects of

exposure in the rVMNvl ($F(4,52) = 13.22$, $p \leq 0.001$) and cVMNvl ($F(4,53) = 15.79$, $p \leq 0.001$). A main effect of sex was found in the cVMNvl ($F(1,53) = 33.81$, $p \leq 0.001$), with greater expression in females, but the overall sex difference in the rVMNvl did not reach statistical significance ($F(1,52) = 3.64$, $p \leq 0.062$; Table 2.1 and Figures 2.4C and F). Within the cVMNvl vehicle controls, sex-specific expression did not reach statistical significance ($p < 0.06$) (Figures 2.4C and F). For the rVMNvl, comparisons of group differences within sex indicated significantly higher expression levels in males of the BPA 25 and EE 10 groups ($p < 0.01$ for both), and in females, there was an identical pattern ($p < 0.01$ for both) (Figure 2.4C). For the cVMNvl, comparisons of group differences within sex indicated significantly higher expression levels in males of the BPA 25 and EE 10 groups ($p < 0.01$ for both), whereas in females, there were significantly higher expression levels in the BPA 25, EE 5, and EE 10 groups relative to same sex vehicle controls ($p < 0.01$ for all) (Figure 2.4F).

Effect of prenatal BPA or EE exposure on AMYG ESR1 and ESR2 Expression.

As expected (Cao and Patisaul, 2012), ESR1 and ESR2 were coexpressed only in the MePD (Figures 2.5A and 2.7A). For all other amygdaloid ROIs, only ESR1 was detected.

ESR1. Significant main effects of exposure on ESR1 expression were observed in all amygdaloid subregions (MePD ($F(4,54) = 19.13$, $p \leq 0.001$); PLCo ($F(4,54) = 18.59$, $p \leq 0.001$); PMCo ($F(4,54) = 23.43$, $p \leq 0.001$); AHi ($F(4,55) = 29.72$, $p \leq 0.001$)) (Figures 2.5C and E, Figures 2.6C and E). A significant main effect of sex on ESR1 expression was observed in the MePD only ($F(1,54) = 4.82$, $p \leq 0.033$) (Figure 2.5C); however, expression was not significantly dimorphic in the vehicle control group. Within males, the BPA 2.5, BPA 25, and EE 10 groups exhibited significantly higher expression levels in the MePD than

same-sex vehicle controls ($p < 0.02$ for all), and an identical pattern was apparent for females ($p < 0.01$ for all). Comparisons within males of ESR1 expression levels in the PLCo, the PMCo, and the AHi indicated significant increases in the BPA 2.5, BPA 25, and EE 10 groups ($p \leq 0.031$ for all) compared with the vehicle control group (Figures 2.5C and E, Figures 2.6C and E). In females, there was a significant increase in ESR1 expression in the BPA 2.5, BPA 25, and EE 10 groups in the AHi and PMCo ($p \leq 0.001$), whereas in the PLCo, only the EE 10 group had increased expression ($p \leq 0.001$).

ESR2. ESR2 signal was robust only in the MePD (Figure 2.7A). Significant main effects of sex ($F(1,53) = 27.35$, $p \leq 0.001$) and group ($F(4,53) = 42.07$, $p \leq 0.001$) were found but no significant interaction ($F(4,53) = 2.29$, $p = 0.072$; Figure 2.7C). There was no significant sexual dimorphism in the vehicle control group. Within males, all BPA and EE groups exhibited significantly higher expression levels than the same-sex vehicle control group ($p < 0.01$ for all), whereas within females, only the BPA 25, EE 5, and EE 10 groups were significantly higher than the same-sex vehicle control group ($p < 0.04$ for all).

Discussion

The present results show region-specific alterations in ESR expression within the developing rat brain resulting from prenatal, low-dose, oral BPA exposure. Although prior studies, including three related studies using siblings of the animals described here (Ferguson, Law, & Abshire, 2012; Ferguson et al., 2011; He et al., 2012), have described BPA exposure effects on sexually dimorphic brain morphology and behavior, exposure occurred during both the prenatal and postnatal periods (Bai et al., 2011; Ferguson et al., 2012, 2011; He et al., 2012; Kubo et al., 2003; Kwon, Stedman, Elswick, Cattley, & Welsch,

2000; Rubin et al., 2006; Xi et al., 2011) or the postnatal period only (Monje, Varayoud, Munoz-de-Toro, Luque, & Ramos, 2010; Patisaul et al., 2006). Additionally, all but the three related studies (Ferguson et al., 2012, 2011; He et al., 2012) used higher doses and different exposure routes. Thus, the data presented here represent the most comprehensive and detailed evaluation of prenatal BPA exposure effects on ESR expression levels in limbic subnuclei of the neonatal rat. Importantly, this is the first study to provide region-specific information on the sensitivity of ESR expression in the neonatal amygdaloid complex to low-dose prenatal BPA exposure. In general, BPA effects were localized to the MBH and specific subregions of the AMYG. These regions were also sensitive to gavage, suggesting that ESR expression within these areas is particularly responsive to environmental stressors.

To our knowledge, this is the first study to provide data on ESR expression changes potentially related to orogastric gavage. The reported differences between the naïve controls and those animals gavaged with the vehicle were unexpected and could be attributable to one of two primary factors related to gavage: the stress of the gavage procedure itself or unanticipated biological activity of the vehicle. Orogastric gavage is useful because it ensures precise and accurate oral dosing, but potentially stressful to the animals as aspects of it (including restraint technique, vehicle type, and volume) can elevate stress hormones (Balcombe et al., 2004). The volume of gavage used here (5ml/kg) was below that which has been described to increase stress hormone levels (reviewed in Balcombe et al. (2004)). Further, the aqueous nature of the vehicle (0.3% carboxymethylcellulose) is reported to induce less of a stress response after gavage compared with lipid vehicles (A. P. Brown, Dinger, & Levine, 2000). Thus, the ESR expression differences between the naïve and

vehicle controls seem unlikely to have resulted from vehicle composition or volume. Additionally, because all subjects, including the naïve controls, were physically restrained, restraint stress is also unlikely to have been a contributing factor. Thus, the ESR expression changes likely resulted from the stress associated with the physical insertion of the feeding needle. Further work specifically focused on this question is needed.

Interpreting the BPA and EE results in regions where there were statistically significant differences in ESR expression between the naïve and vehicle controls requires caution because the BPA- or EE-related effects on expression may have been influenced by prenatal stress (from gavage). Comparisons of BPA- or EE-exposed groups with the naïve control group are not entirely appropriate because the naïve controls were not administered the vehicle and are thus not a true negative control. Notably, the region with the most pronounced differences in ESR expression between the naïve and vehicle-gavaged controls was the AMYG, a complex sexually dimorphic limbic structure that coordinates physiological and behavioral stress responses. Because the data suggest that, for some neural endpoints, gavage stress may be influential, alternative oral dosing strategies such as incorporating BPA in the diet (Jasarevic et al., 2011; Sieli et al., 2011; Wolstenholme et al., 2012), drinking water (Fujimoto, Kubo, & Aou, 2006; Kabuto, Amakawa, & Shishibori, 2004; Miyawaki, Sakayama, Kato, Yamamoto, & Masuno, 2007; Patisaul et al., 2012) or administration of BPA-laced food treats (Patisaul et al., 2013; Zoeller et al., 2005), could be considered in those specific cases. Each of these alternative approaches has its own limitations, which would require evaluation for generation of a suitable experimental design.

We have previously shown that ESRs are expressed in the subregions of the hypothalamus and AMYG in an age- and sex specific manner in the neonatal rat (Cao & Patisaul, 2011, 2013). The sex differences identified here were consistent with those prior observations although statistical significance was not attained in every region, most notably ESR1 expression in the AVPV. In the ARC and MPOA, the expected sex differences in ESR1 expression were observed in the naïve, but not the vehicle controls, suggesting that the effect of vehicle gavage on ESR gene expression may have suppressed expected sex differences. In general, sex differences were not significantly altered by prenatal exposure to either EE or BPA, a result which was unexpected given the longstanding recognition that estrogen can be masculinizing in the developing rodent brain (McCarthy, Wright, & Schwarz, 2009; Simerly, 2002). In the anterior hypothalamus, this is likely attributable to timing of exposure, as the early postnatal period (rather than the prenatal period) appears to be the "critical window" for estrogen-dependent masculinization in this region (Cao et al., 2012; Simerly, 2002). Data obtained from female siblings of the pups described here demonstrated that both EE doses significantly increased the volume of the sexually dimorphic nucleus of the preoptic area (SDN-POA) at weaning (He et al., 2012). In that companion study, however, oral dosing (direct to the pup) continued through weaning. This critical difference in exposure duration supports the position that postnatal exposure is required to masculinize gene expression and morphological sex differences in the anterior hypothalamus. Interestingly, although female SDN-POA volumes were significantly increased in the EE-exposed groups, they did not reach male-typical levels. Collectively, the data emphasize the importance of specifically identifying and defining exposure windows for

interpretation of BPA effects in the developing brain. In humans, the period encompassing the rodent perinatal period is believed to occur in mid to late gestation (Abbott, Zhou, Bird, Dumesic, & Conley, 2008; Aksglaede, Juul, Leffers, Skakkebaek, & Andersson, 2006; Selevan, Kimmel, & Mendola, 2000; Simerly, 2002); thus, the rat perinatal "critical window" is likely to be entirely prenatal in humans.

Within the MBH, although EE and BPA enhanced ESR1 expression in both sexes, the magnitude of the effect was not proportional to the dose administered for either compound. The lowest dose of BPA had statistically significant effects but not the lowest dose of EE. This could indicate that BPA is not simply acting as a weak estrogen but rather acting via an alternative pathway (or pathways) to alter ESR expression levels. The specific mechanisms by which low, but not high, doses of BPA can alter region-specific gene expression, and brain morphology remain to be elucidated. Those which occur in the absence of an EE effect suggest that it does not involve classic estrogen signaling but rather may occur via interactions with membrane ESRs, epigenetic changes, or other alternative pathways (Wolstenholme, Rissman, et al., 2011).

The gene expression changes observed here could reflect either a change in cellular levels of ESRs within each region or a change in the number of cells expressing ESR1 and ESR2. Although transcription levels can be transient (Cao & Patisaul, 2011, 2013), changes in cell numbers would suggest that the effect is likely permanent (McCarthy, 2008). Whether these changes in limbic ESR expression persist beyond the neonatal window is the subject of ongoing studies using related, older cohorts of animals. In the present study, exposure halted approximately 48 h before the brains were collected, which suggests that altered expression

persists for at least a few days. This is consistent with what has been reported for EE and the selective ESR modulator, tamoxifen, the effects of which on ESR2 and related gene expression persist as long as 2 weeks after treatment cessation (Patisaul, Aultman, Bielsky, Young, & Wilson, 2003). Thus, it is plausible that the overall sex-specific, temporal pattern of perinatal ESR expression as perturbed by BPA continues after exposure is terminated.

A potential concern stemming from the results reported here is the possibility that changes in early-life ESR expression following prenatal BPA exposure ultimately contribute to later in life effects on neuroendocrine function and behavior (Patisaul et al., 2012; Wolstenholme et al., 2012; Wolstenholme, Rissman, et al., 2011). One way by which this could occur is via altered sensitivity of these regions to endogenous estrogens as a result of BPA-disrupted ESR expression levels. Because those limbic nuclei undergo steroid hormone–directed sexual differentiation during this period (Baum, 2009; B. M. Cooke & Woolley, 2005; McCarthy et al., 2009; Simerly, 2002), altered endogenous hormone sensitivity may confer lifelong effects on neuroendocrine physiology and behavior. A long history of detailed work has shown that even an acute exposure to estrogen during critical windows of perinatal life can have permanent morphological and functional effects on neuroendocrine systems coordinating reproductive physiology and behavior (McCarthy, 2008; Simerly, 2002). How disruption of each ESR subtype individually contributes to potential neuroendocrine and behavioral alteration remains the subject of intense investigation. Although the specific functional role of ESR2 remains unclear, there is growing consensus that it is important for modulating affective and mood-related behaviors, including anxiety, aggression, and social interactions

(Handa, Ogawa, Wang, & Herbison, 2012; Patisaul & Bateman, 2008). Our results reveal that, throughout the limbic system, the expression of ESR2 appeared to be more sensitive to disruption by BPA exposure than ESR1 and was generally diminished by BPA exposure, particularly in the AMYG. We have recently shown in rats that this decreased expression persists through adolescence and is associated with elevated juvenile anxiety (Patisaul et al., 2012), an outcome which is consistent with the emerging consensus regarding the functional role of ESR2 in the brain (Fan, Xu, Warner, & Gustafsson, 2010; Handa et al., 2012).

Importantly, BPA exposure during development has also been shown to impact mood-related behaviors in other species, including mice and deer mice ((Jasarevic et al., 2011) and reviewed in Wolstenholme, Rissman, et al. (2011)) and at least one study has associated exposure with hyperactivity and elevated anxiety in young girls (J. M. Braun et al., 2011; J. M. Braun et al., 2009). Although humans and rodents undoubtedly perceive and express stress and anxiety differently, important core elements of the genetic and neurobiological basis of anxiety phenotypes are evolutionarily conserved across species, particularly in the AMYG and hypothalamus (Donner et al., 2008; Hohoff, 2009; Hovatta & Barlow, 2008; Koolhaas, de Boer, Buwalda, & van Reenen, 2007; Landgraf & Wigger, 2002; Millan, 2003). By contrast, other behaviors appear more resilient to BPA exposure. For example, a companion study using siblings of animals in the present study (and directly orally dosed through weaning) found no effects of BPA on righting reflex or slant board behaviors (Ferguson et al., 2011), a not unexpected finding as neither is known to be sensitive to early-life exposure to estrogens, although performances for both assessments are sexually dimorphic. A second companion study identified no consistent or dose-related BPA

effects on novelty preference, motor coordination, or spatial learning/memory (Ferguson et al., 2012). Thus, the potential long-term effects of the results reported here remain unclear. The present data, however, contribute critical information regarding the molecular changes to the limbic system, which may underpin previously identified behavioral effects attributed to BPA exposure. Future studies will be needed to more definitively establish the functional and physiological consequences of neonatal gene expression changes within limbic structures, including the AMYG and hypothalamus.

Conclusions

ESR expression in critical components of the neonatal rat limbic system was sensitive to prenatal BPA at exposures as low as 2.5 µg/kg bw, which is lower than the currently established NOAEL of 50mg/kg bw. In some limbic structures, however, gavage-related effects on ESR expression were observed, suggesting that expression differences in those specific regions may reflect the interaction of prenatal BPA exposure and stress. The functional significance of these changes in ESR receptor expression remains to be definitively established, but we hypothesize that they contribute to hormone-sensitive behavioral changes already attributed to early-life BPA exposure, including mood- and activity-related behaviors (Patisaul et al., 2012; Wolstenholme et al., 2012; Wolstenholme, Rissman, et al., 2011), because ESRs in the limbic structures examined here play important roles in mediating these behaviors. These data provide novel information regarding mechanisms by which early-life BPA exposure impacts the developing brain and may underpin related health effects in later life.

Funding

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Acknowledgements

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Table

Table 2.1: Summary of sex-specific ESR1 and ESR2 expression in ROIs examined. Comparative expression levels between the sexes (Female [F] and Male [M]) for each gene within a subregion can be read horizontally across the table, whereas expression levels for each exposure group can be read vertically. For each comparison, "=" indicates no statistically significant difference, ">" indicates statistically greater, and "<" indicates statistically lower. [a]p value trends toward significance, p = 0.055.

Effects of Prenatal BPA Exposure on Sexually Dimorphic ESR Expression in the Neonatal Rat Brain

Regions	Genes	Subregions	Naïve	Vehicle	EE 5	EE 10	BPA 2.5	BPA 25
Anterior hypothalamus	ESR1	AVPV	F = M	F = M	F = M	F = M	F = M	F = M
		MPOA	F > M	F = M	F = M	F > M	F = M	F = M
	ESR2	AVPV	F < M	F < M	F < M	F < M	F < M	F < M
		MPOA	F = M	F = M	F = M	F = M	F = M	F = M
Mediobasal hypothalamus	ESR1	rARC	F = M	F > M	F = M	F > M	F = M	F = M
		cARC	F > M	F = M	F = M	F > M	F = M	F = M
		rVMNvl	F > M	F = M	F = M	F = M	F = M	F = M
		cVMNvl	F > M	F = M	F = M	F > M	F = M	F = M
	ESR2	rVMNvl	F = M	F = M	F = M	F = M	F = M	F > M
		cVMNvl	F > M	F > M[a]	F > M	F > M	F > M	F = M
Amygdala	ESR1	MePD	F = M	F = M	F = M	F = M	F = M	F = M
		PLCo	F = M	F = M	F = M	F = M	F = M	F = M
		PMCo	F = M	F = M	F = M	F = M	F = M	F = M
		AHi	F = M	F = M	F = M	F = M	F = M	F = M
	ESR2	MePD	F = M	F = M	F < M	F < M	F < M	F = M

Figures

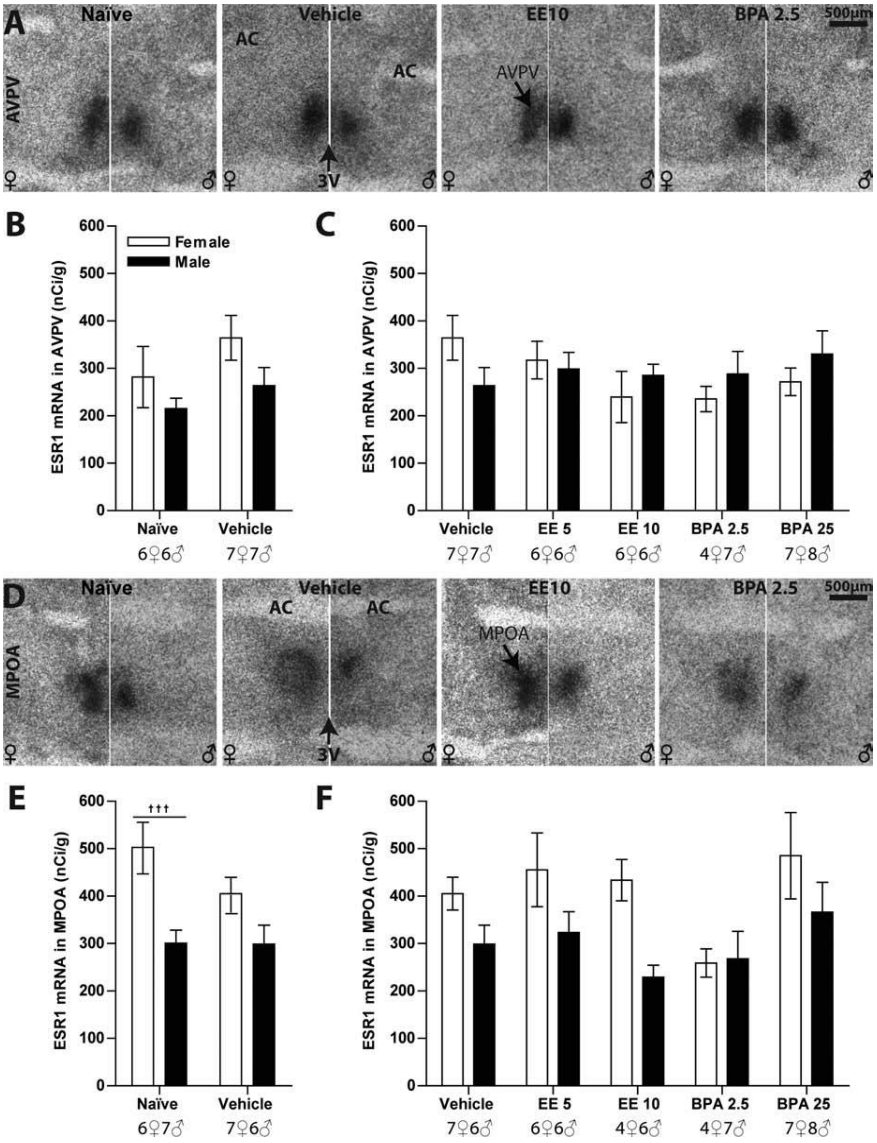

Figure 2.1: ESR1 mRNA expression in the AVPV and MPOA. Representative autoradiographs depicting ESR1 mRNA in the AVPV (A) and MPOA (D) from naïve, vehicle, EE 10, and BPA 2.5 exposure groups (from left to right in both A and D, females on the left side of each panel, males on the right). Optical density of ESR1 expression was analyzed in the AVPV (B and C) and MPOA (E and F). A main effect of sex in ESR1 expression was observed in the MPOA by two-way ANOVA, but no effect of exposure was found for either region. A significant sex difference was observed in the naïve control (represented by †††$p \leq 0.001$) but not in other groups. Graphs depict mean ± SEM, and sample size is shown under each group; scale bar = 500 µm for all panels in A and D; 3V = third ventricle; AC = anterior commissure.

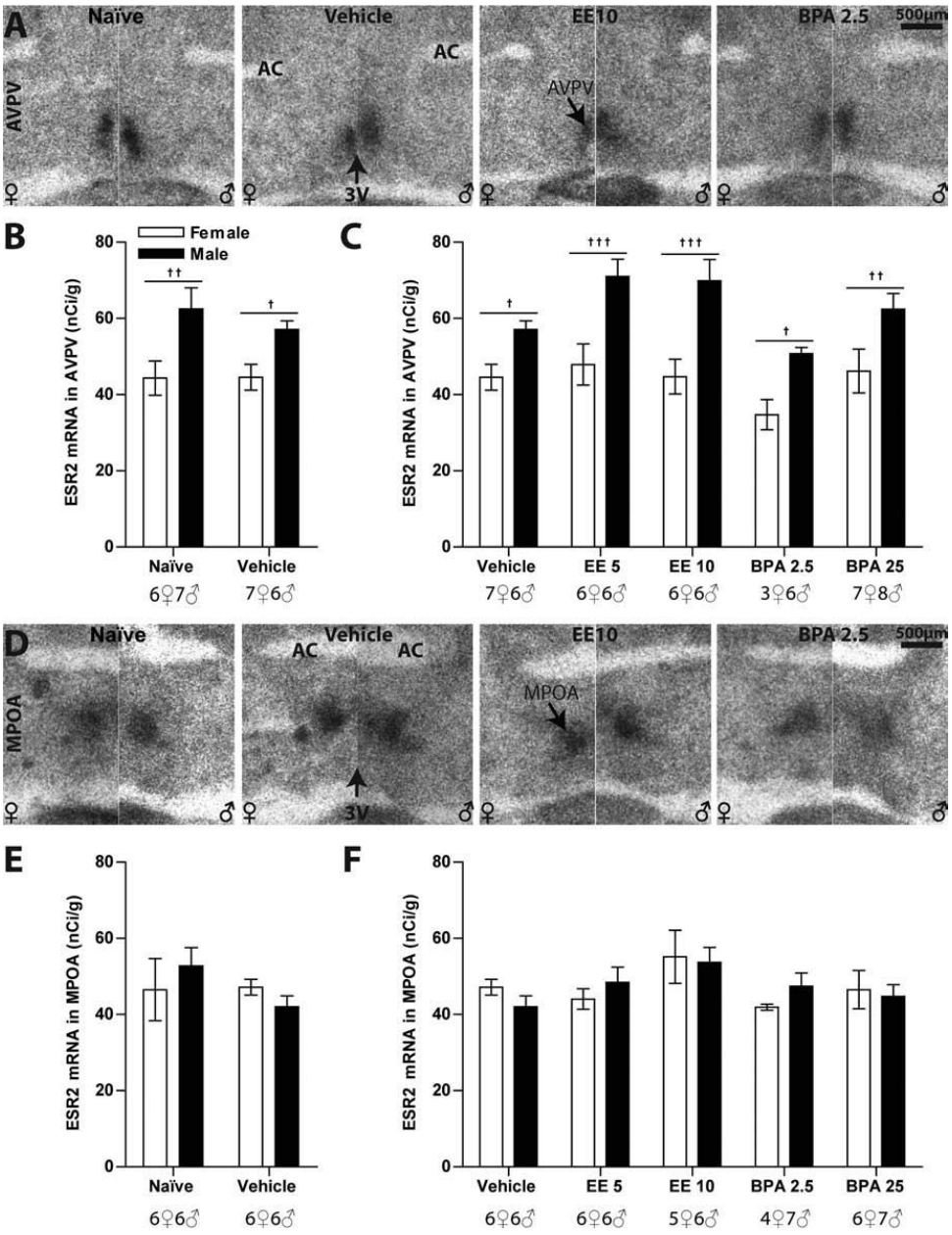

Figure 2.2: ESR2 mRNA expression in the AVPV and MPOA. Representative autoradiographs depicting ESR2 signal in the AVPV (A) and MPOA (D). Optical density analysis of ESR2 mRNA in the AVPV (B and C) and MPOA (E and F) indicated that the levels of ESR2 mRNA were sexually dimorphic in the AVPV, with higher levels in males in all groups. No significant effect of EE or BPA was observed in the AVPV or MPOA. Graphs depict mean ± SEM, and sample size is shown under each group. Sex differences in expression are represented by †$p \leq 0.05$, ††$p \leq 0.01$, and †††$p \leq 0.001$; scale bar = 500 µm for all panels in A and D; 3V = third ventricle; AC = anterior commissure.

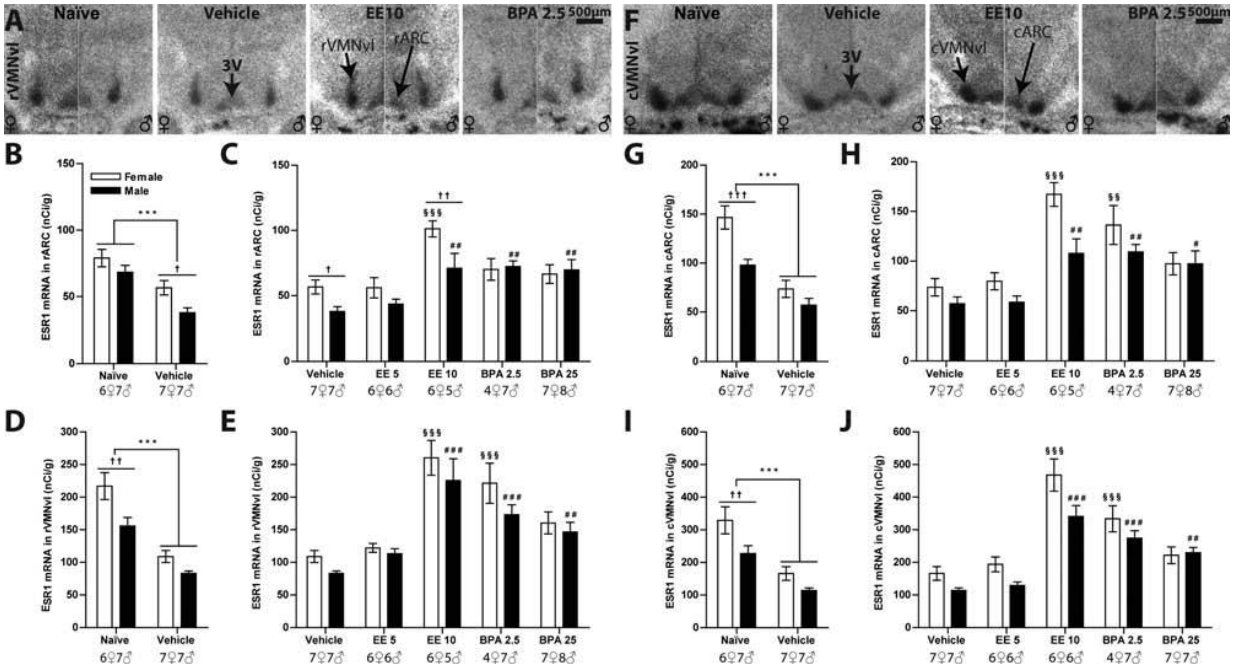

Figure 2.3: ESR1 mRNA expression in the MBH. Representative autoradiographs depicting ESR1 signal in the rostral (A) and caudal (F) MBH. Optical density analysis in the rARC (B and C) and rVMNvl (D and E) showed that ESR1 mRNA signal in the naïve controls was significantly higher than in the vehicle controls. ESR1 expression in the rARC and rVMNvl was significantly increased in all exposure groups in males, except for EE 5. In females, EE 10 increased ESR1 in rARC and rVMNvl, and BPA 2.5 exposure also significantly increased ESR1 mRNA levels in the female rVMNvl compared with the vehicle control. In the cARC (G and H) and cVMNvl (I and J), ESR1 mRNA levels were significantly higher in the naïve controls than in the vehicle controls. ESR1 expression in the male caudal MBH was significantly increased in all exposure groups, except for EE 5, compared with the vehicle controls. In females, the ESR1 mRNA was significantly increased only in the EE 10 and BPA 2.5 exposure groups. Graphs depict mean ± SEM, and sample size is shown under each group; differences in expression between the naïve and vehicle controls are represented by ***$p \leq 0.001$; differences between exposure groups and to their same-sex vehicle controls are indicated by #$p \leq 0.05$, ##$p \leq 0.01$, and ###$p \leq 0.001$ in males and §§$p \leq 0.01$ and §§§$p \leq 0.001$ in females. Sex differences in expression are represented by †$p \leq 0.05$ and ††$p \leq 0.01$; scale bar = 500 µm for all panels in A and F; 3V= third ventricle.

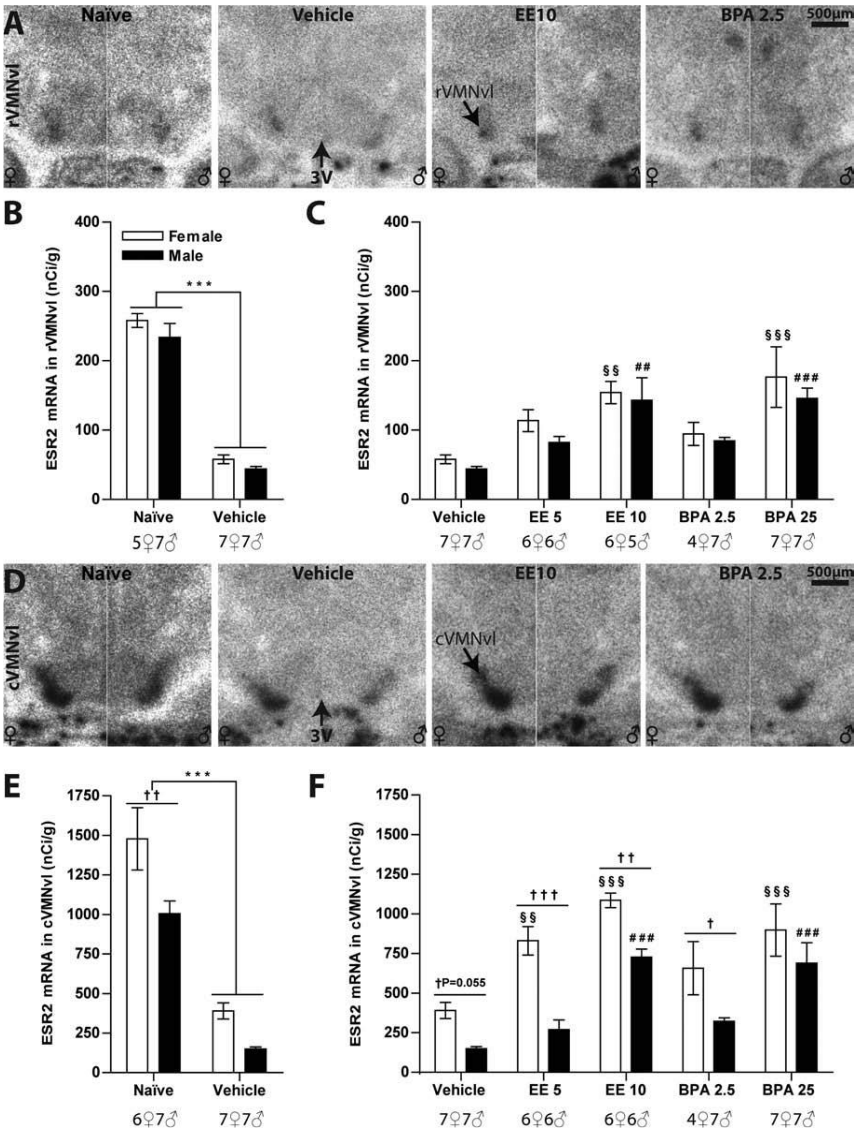

Figure 2.4: ESR2 mRNA expression in the MBH. Representative autoradiographs depicting ESR2 mRNA labeling in the rVMNvl (A) and cVMNvl (D). ESR2 signal was absent in the ARC (A and D). Optical density analysis of ESR2 expression (B, C, E, and F) showed that ESR2 signal in the naïve controls was significantly higher than in the vehicle controls. Compared with the same-sex vehicle controls, ESR2 expression in the rVMNvl and cVMNvl was significantly increased in the EE 10 and BPA 25 exposure groups in both sexes and in the EE 5 group in females (C and F). Graphs depict mean ± SEM and sample size is shown under each group; differences in expression between the naïve and vehicle controls are represented by ***$p \leq 0.001$ and between exposure groups and same-sex vehicle controls by ##$p \leq 0.01$ and ###$p \leq 0.001$ in males, and §§$p \leq 0.01$ and §§§$p \leq 0.001$ in females; †$p \leq 0.05$, ††$p \leq 0.01$, and †††$p \leq 0.001$ represent sex differences in ESR2 expression; scale bar= 500 µm; 3V= third ventricle.

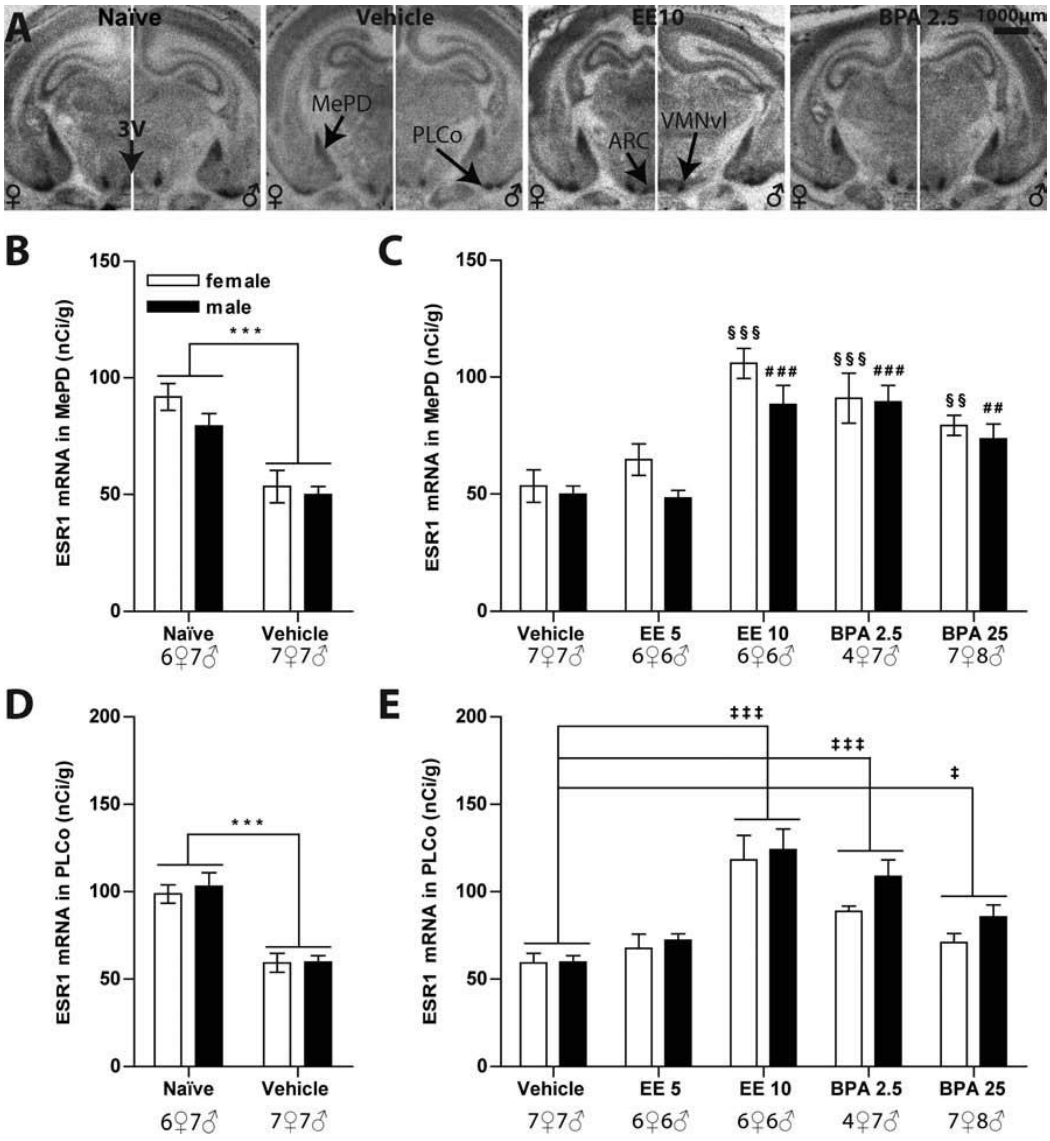

Figure 2.5: ESR1 mRNA expression in the MePD and PLCo. Representative autoradiographs show ESR1 signal in the MePD and PLCo (A). Optical density analysis of ESR1 expression in the MePD (B and C) and PLCo (D and E) revealed that ESR1 mRNA in the naïve controls was significantly higher than in the vehicle controls in both regions. ESR1 expression in both regions was significantly increased in all exposure groups except for EE 5 compared with the vehicle controls. Sexually dimorphic ESR1 expression was not observed in any exposure group in either the MePD or the PLCo. Graphs depict mean ± SEM, and sample size is shown under each group; differences in expression between the naïve and vehicle controls are represented by ***$p \leq 0.001$ and between exposure groups and vehicle controls by ##$p \leq 0.01$ and ###$p \leq 0.001$ in males and §§$p \leq 0.01$ and §§§$p \leq 0.001$ in females in MePD. In PLCo, differences in expression between the exposure groups and vehicle controls are represented by ‡$p \leq 0.05$ and ‡‡‡$p \leq 0.001$; scale bar = 1000 µm; 3V = third ventricle.

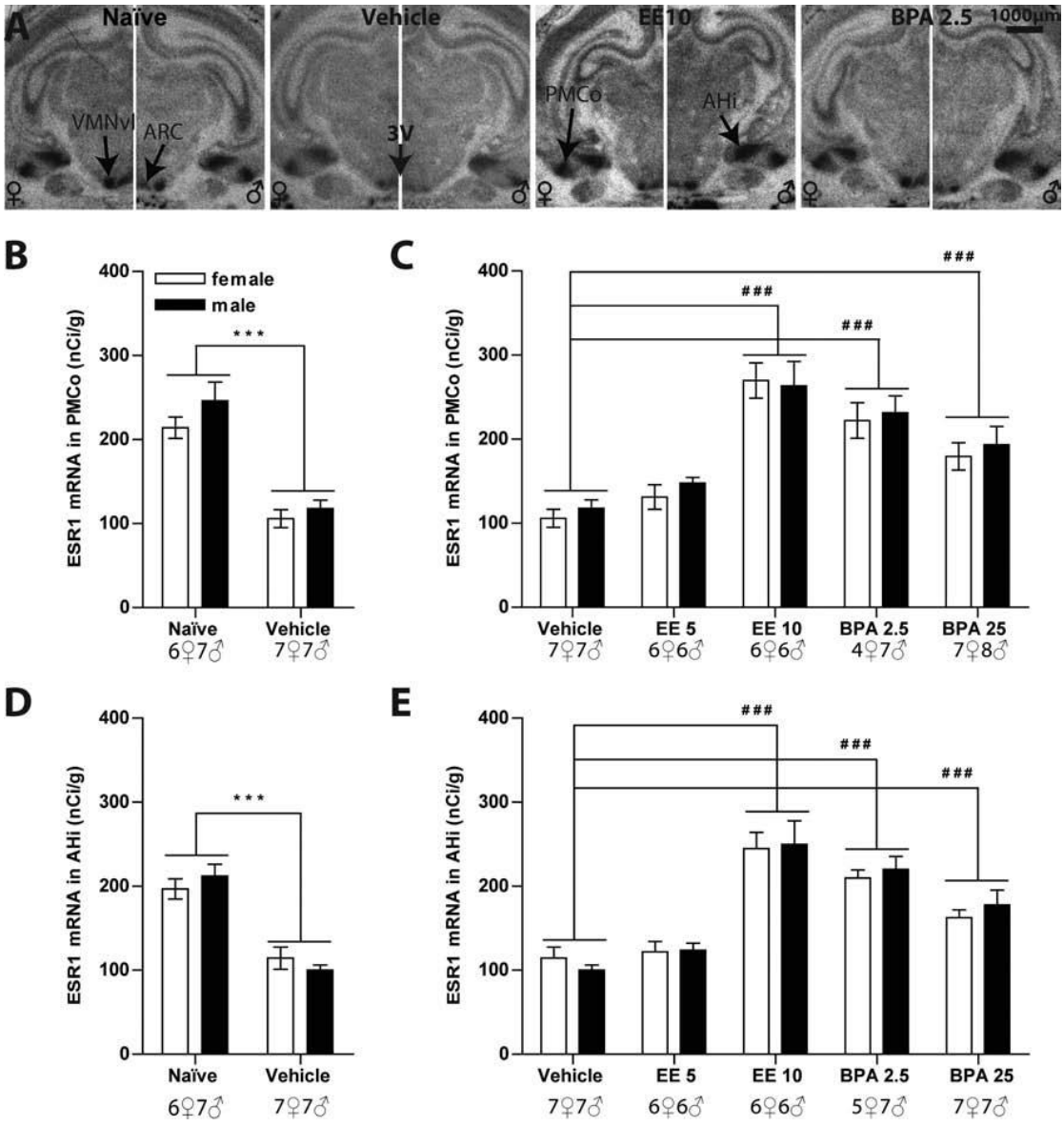

Figure 2.6: ESR1 mRNA expression in the PMCo and AHi. Representative autoradiographs showing ESR1 signal in the PMCo and AHi (A). Optical density analysis of ESR1 expression in the PMCo (B and C) and AHi (D and E) revealed that ESR1 mRNA signal in the naïve controls was significantly higher than in the vehicle controls. ESR1 expression in both regions was significantly increased in all groups, except for EE 5, compared with the vehicle controls. Sexually dimorphic ESR1 expression was not observed in any exposure group in either the PMCo or the AHi. Graphs depict mean ± SEM, and sample size is shown under each group; differences in expression between the naïve and vehicle controls are represented by ***$p \leq 0.001$ and between exposure groups and vehicle controls by ###$p \leq 0.001$; scale bar = 1000 µm; 3V = third ventricle.

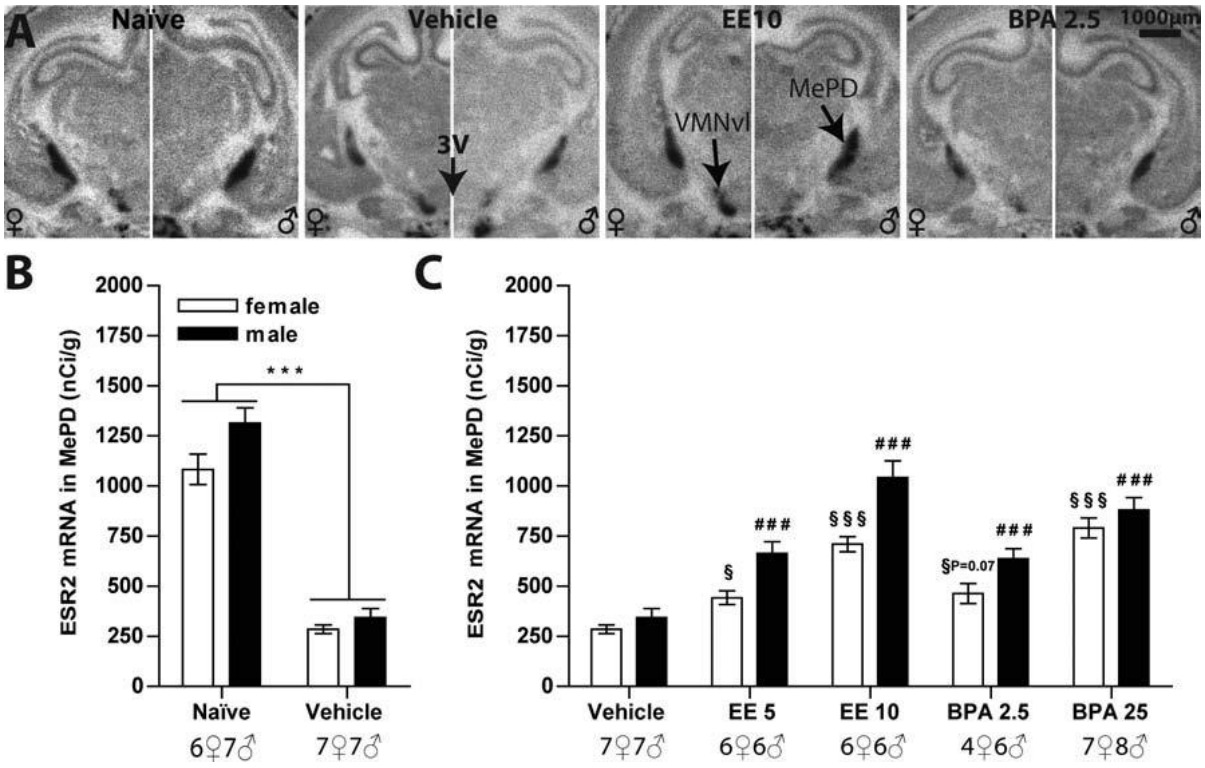

Figure 2.7: ESR2 mRNA expression in the MePD. Representative autoradiographs depicting ESR2 signal in the MePD (A). Optical density analysis of ESR2 expression in the MePD (B and C) showed that ESR2 mRNA levels in the naïve controls were markedly higher than in the vehicle controls. ESR2 expression was significantly increased in all exposure groups compared with same-sex vehicle controls in males and was significantly increased in EE and BPA 25 groups in females, and a p value of 0.07 in the BPA 2.5 group. Graphs depict mean ± SEM, and sample size is shown under each group; differences in expression between the naïve and vehicle controls are represented by ***$p \leq 0.001$ and between the exposure groups and vehicle controls by ###$p \leq 0.001$ in males and §$p \leq 0.05$ and §§§$p \leq 0.001$ in females; scale bar = 1000 µm for all panels in A; 3V = third ventricle.

CHAPTER 3— Investigation of the Effects of Subchronic Low Dose Oral Exposure to Bisphenol A (BPA) and Ethinyl Estradiol (EE) on Estrogen Receptor Expression in the Juvenile and Adult Female Rat Hypothalamus

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Abstract

Concerns have been raised regarding the long-term impacts of early life exposure to the ubiquitous environmental contaminant bisphenol A (BPA) on brain organization. Because BPA has been reported to affect estrogen signaling, and steroid hormones play a critical role in brain sexual differentiation, there is also concern that BPA exposure could alter neural sex differences. Here, we examine the impact of subchronic exposure from gestation to adulthood to oral doses of BPA below the current no-observed-adverse effect level (NOAEL) of 5 mg/kg body weight (bw)/day on estrogen receptor (ESR) expression in sexually dimorphic brain regions of prepubertal and adult female rats. The dams were gavaged daily with vehicle (0.3% carboxymethylcellulose), 2.5, 25, 260, or 2700 μg BPA/kg bw/day, or 0.5 or 5.0 μg ethinyl estradiol (EE)/kg bw/day from gestational day 6 until labor

began. Offspring were then gavaged directly from the day after birth until the day before scheduled sacrifice on postnatal days 21 or 90. Using *in situ* hybridization, one or more BPA doses produced significant decreases in *Esr1* expression in the juvenile female rat anteroventral periventricular nucleus (AVPV) of the hypothalamus and significant decreases in *Esr2* expression in the adult female rat AVPV and medial preoptic area (MPOA), relative to vehicle controls. BPA did not simply reproduce EE effects, indicating that BPA is not acting solely as an estrogen mimic. Also, the caveat of the discovered BPA-G in the serum of the vehicle controls (at the level of the 2.5 BPA group) must be taken into consideration when making conclusions about these results. The possible consequences of long term changes in hypothalamic ESR expression resulting from subchronic low dose BPA exposure on neuroendocrine effects are discussed and being addressed in ongoing, related work.

Key Words

brain; endocrine disruptor; endocrine disruption; hypothalamus; development; subchronic exposure; sexually dimorphic; ethinyl estradiol; bisphenol A

Disclaimer

Introduction

Exposure to bisphenol A (BPA), a common component of epoxy resins and polycarbonate plastics, is nearly ubiquitous with >90% of the United States population having traces of BPA in their urine (Calafat et al., 2008; Vandenberg et al., 2010). Exposure occurs primarily by consuming food and beverage products (estimated by the World Health Organization (WHO) to be in the range of 0.01–0.05 μg/kg bw/day for adults and 0.02–0.12 μg/kg bw/day for children) into which BPA has leached from the plastic or the resin-based coatings lining the interior of the container (Calafat, 2011; FAO/WHO, 2011). Dermal contact with thermal receipts and inhalation of airborne particles are additional suspected sources of exposure (Biedermann et al., 2010; Cooper et al., 2011; FAO/WHO, 2011; Geens et al., 2012; Geens et al., 2011). In their 2008 evaluation of developmental and reproductive effects of BPA exposure, the National Toxicology Program (NTP) concluded that there was 'some concern for effects on the brain and behavior' (Shelby, 2008). In a 2010 statement, the FDA indicated that they shared these concerns, although the current FDA assessment is that 'BPA is safe at the very low levels that occur in some foods' (http://www.fda.gov/newsevents/publichealthfocus/ucm064437.htm, updated March 2013). Here, we tested the hypothesis that subchronic low dose BPA exposure alters estrogen receptor (ESR) expression in the female rat hypothalamus.

Sexually dimorphic brain ESR distribution and activity fundamentally contribute to the organization of steroid hormone directed morphological and functional sex differences in the developing brain (McCarthy, 2008; Rissman, 2008; Schwarz & McCarthy, 2008). Sex-specific neural organization is requisite for physiological and behavioral sex differences that

emerge later in life (G. J. De Vries, 2004; Simerly, 2002). Studies in a variety of species, including rats and mice, have shown that early life exposure to BPA perturbs the organization of numerous estrogen-sensitive neural endpoints including the sexual differentiation of hypothalamic subnuclei seminal to sex-specific reproductive physiology and behavior (Kundakovic et al., 2013; Patisaul et al., 2012; Wolstenholme et al., 2012) (and reviewed in Rosenfeld (2012); Wolstenholme, Rissman, et al. (2011)). The classical, estrogenic mode of action for BPA has been challenged because it has relatively low binding affinities for nuclear ESRs (10,000- to 100,000-fold lower than estradiol) (Andersen et al., 1999; Barkhem et al., 1998; Kuiper et al., 1998). Disruption of ESR expression, particularly in regions with pronounced sex differences, may be an alternative mechanism by which BPA alters ESR-dependent sex-specific brain organization. This may occur via epigenetic changes (Kundakovic et al., 2013; Nugent, Schwarz, & McCarthy, 2011) or alternative mechanisms which ultimately result in altered ESR expression (La Rosa, Pellegrini, Totta, Acconcia, & Marino, 2014; Wright, Schwarz, Dean, & McCarthy, 2010). Understanding the specific molecular and cellular mechanisms through which BPA can alter the developing brain will help address how neural effects in rodents might be predictive of similar effects in humans.

It is well established that even transient sex differences in gene expression during critical windows of brain development can cause permanent differences in brain structure and, consequently, neuroendocrine physiology and behavior (B. Cooke et al., 1998; G. J. De Vries, 2004; McCarthy, 2008; Morris et al., 2004; Simerly, 2002). In a related prior study, we showed that prenatal exposure to 2.5 and/or 25 μg/kg bw/day BPA (via orogastric gavage to the dam) increases the distribution and density of estrogen receptor alpha (*Esr1*) and estrogen

receptor beta (*Esr2*) gene expression in the mediobasal hypothalamus and amygdala of newborn rats (Cao et al., 2013). We and others have also demonstrated that neonatal BPA exposure at dosages ranging from 50 μg/kg bw/day to 50 mg/kg bw/day (via subcutaneous injection to the pup) can alter *Esr1* and *Esr2* gene expression and ESR1 immunoreactivity in the anterior hypothalamus of peripubertal female rats (Adewale, Todd, Mickens, & Patisaul, 2011; Cao et al., 2014; Cao et al., 2012; Kundakovic et al., 2013; Monje, Varayoud, Luque, & Ramos, 2007; Monje et al., 2010), suggesting that perturbed ESR mRNA levels may persist across the lifespan (direction and magnitude of the effect is region and age specific). This hypothesis is supported by the observation that oral exposure to BPA in late adolescence (40 μg/kg bw/day) can also result in altered hypothalamic ESR1 immunoreactivity in adult rats of both sexes (Ceccarelli et al., 2007). Establishing how ESR expression patterns are changed across adolescence and into adulthood was a primary goal of the present study. Additionally, although human exposure is low and chronic, rather than confined to one critical period, most prior studies examining the neural impacts of BPA have confined exposure to a specific critical window of development. To better model human exposure in the current study, BPA was administered orally and subchronically within a range that included doses below the 5 mg/kg/day NOAEL established from guideline toxicological studies. These studies, therefore, provide critical information regarding potential effects of lifetime BPA exposure at doses that meet the NTP's definition of 'low dose' (Melnick et al., 2002).

We have previously generated a detailed profile of sexually dimorphic expression pattern of *Esr1* and *Esr2* across several hypothalamic subregions of the prepubertal rat (Cao

& Patisaul, 2011). Nuclei showing pronounced sex differences were selected as the regions of interest (ROIs) for the present study. Hypothalamic ROIs included the anteroventral periventricular nucleus (AVPV) and medial preoptic area (MPOA). Both are structurally and functionally sexually dimorphic and essential for initiating ovulation in females and coordinating gonadotropin releasing hormone (GnRH) activity in both sexes. Work from our group and others have examined BPA-related effects on ESR content and gene expression in the AVPV and MPOA in peripubertal and adult rodents (Adewale et al., 2011; Monje et al., 2007; Patisaul, 2013; Patisaul et al., 2006; Rubin et al., 2006); data which enhance their relevance as focal areas for the present studies. Areas of interest in the mediobasal hypothalamus included the ventrolateral division of the ventromedial hypothalamic nucleus (VMNvl) and the arcuate nucleus (ARC). These regions contribute to a wide range of neuroendocrine activities including growth, feeding, and reproductive behavior. Collectively, these regions were also assessed in our prior studies (Adewale et al., 2011) including the related study (using different animals but derived from the same National Center for Toxicological Research (NCTR) colony), where ESR expression was quantified on PND 1 following gestational exposure to the two lowest BPA doses used for the present studies (2.5 and 25 μg/kg bw/day) (Cao et al., 2013).

To maximize the potential to detect effects with human relevance and minimize interexperimental inconsistencies likely resulting from experimental design differences (e.g., exposure duration, dose, route of administration) and species differences in neural structure and responsivity to steroid hormones in early development (Bonthuis et al., 2010), study design related recommendations for BPA research have been published by several groups

(Goodman et al., 2006; Hengstler et al., 2011; Hunt et al., 2009; Richter et al., 2007). These include minimization of xenoestrogen exposure, statistical control for litter effects, oral dosing over a wide and closely spaced dose range which include doses below the NOAEL and, in situations where BPA is thought to act as a weak estrogen, the inclusion of a concurrent reference estrogen. All of these recommendations were incorporated into the current study. Two of the four BPA doses used (2.5 and 25 μg/kg bw/day) are well below the current reference dose (tolerable daily intake) of 50 μg/kg bw/day (Chapin et al., 2008; National Toxicology Program, 1982), and the two highest doses (260 and 2700 μg/kg bw/day) are below the lowest-observed adverse-effect level (LOAEL) of 50 mg/kg bw/day. Two doses of ethinyl estradiol (EE) were included as the reference estrogen because gestational estrogen exposure is well recognized to induce region-specific hypothalamic masculinization in female rats (McCarthy, 2008; Simerly, 2002). A group of vehicle exposed male conspecifics was included to specifically test for BPA-related impacts on sex-specific ESR expression. The animals used for the present studies were siblings of animals used for a larger, more comprehensive toxicity study, the experimental design details and results of which were published previously (Churchwell et al., 2014; Delclos et al., 2014). Those studies did not contain neural endpoints thus the results from the present study are novel in that they yield insight into the mechanisms by which BPA exposure impacts the sex-specific organization of hypothalamic subnuclei over a wide range of doses (2.5–2700 μg/kg bw/day).

Materials and Methods

Animal care, BPA and EE exposure, brain collection, and section preparation.

All animals were obtained from an Association for Assessment and Accreditation of Laboratory Animal Care (AALAC)-accredited National Center for Toxicological Research (NCTR) facility and all procedures were approved by the NCTR Laboratory Animal Care and Use Committee. The animals used in the current study were part of a larger study, conducted in compliance with good laboratory practices (GLP); the full description of animal care and dosing procedures can be found in Churchwell et al. (2014) and Delclos et al. (2014). Briefly, weanling male and female Sprague Dawley (SD) rats, obtained from the NCTR breeding colony, were maintained on a soy- and alfalfa-free diet (Test Diets 5K96 irradiated pellets; Purina Mills, Richmond, IN), and extracts of all housing materials (polysulfone caging (Ancare Corp., Bellmore, NY), hardwood chip bedding (P.J. Murphy, Montville, NJ), silicone water bottle stoppers (Plasticoid Co., Elkton, MD), and glass drinking water bottles) were screened to quantify BPA levels in leachates (Delclos et al., 2014). None of the materials had BPA levels above the average analytical blanks. Diet extracts were also assayed for BPA and did show BPA levels above those in analytical blanks in all six lots of diet used in the study. The mean BPA level in the diets used in the study was 2.6 ± 0.8 (standard deviation) ppb. Based on food intake measurements, the calculated ingested dose of BPA from the diet was ~0.25 μg/kg bw/day, that is, ~10-fold lower than the lowest dose used in the study (Delclos et al., 2014).

Two weeks prior to mating, female breeders were randomized to exposure groups stratified by body weight to give approximately equivalent body weights by exposure group.

Male breeders were assigned such that breeding between siblings or first cousins did not occur. Females were checked daily for the presence of an *in situ* copulation plug or sperm in the vaginal smear. The day when an *in situ* plug or a sperm-positive smear was found was considered gestation day (GD) 0, at which point the male was removed and euthanized. Breeding occurred in four rounds spaced 3 weeks apart.

Daily gavage dosing of the pregnant dams began on GD 6 and continued until the onset of labor. BPA (TCI America, Portland, OR; lot no. 111909/AOHOK (air-milled)) and EE (Sigma-Aldrich, St Louis, MO; lot no. 028K1411) doses were prepared in the vehicle, 0.3% carboxymethylcellulose (CMC; Sigma-Aldrich; catalogue no. C5013, lot no. 048K0023) in water and administered in 5 ml/kg bw using a modified Hamilton Microlab 500 series pump system (Lewis et al., 2010). The main study used seven levels of BPA with half-log spacing between 2.5 and 2700 μg BPA/kg bw/day, two high doses of BPA (100,000 and 300,000 μg/kg bw/day), two doses of EE (0.5 and 5.0 μg/kg bw/day), a vehicle control, and a naïve control. Separate pump systems were used for vehicle control, BPA, and EE groups with each day's dosing conducted from low to high dose. Due to the specific interest in the potential for low dose effects, the labor intensity of the procedures employed, and the hypothesis that results observed in our prior, related study (conducted in different animals unrelated to those in the present study but derived from the same NCTR SD rat colony) may persist across the life span (Cao et al., 2013), exposure groups selected for analysis of the ESR expression levels in brain were vehicle, 2.5 μg BPA/kg bw/day (BPA 2.5), 25 μg BPA/kg bw/day (BPA 25), 260 μg BPA/kg bw/day (BPA 260), 2700 μg BPA/kg bw/day (BPA 2700), 0.5 μg EE/kg bw/day (EE 0.5), and 5.0 μg EE/kg bw/day (EE 5). The range of

BPA groups was chosen to analyze effects of exposure both above and below the EPA reference dose of 50 μg BPA/kg bw/day. Detailed information regarding internal dosimetry is available in Churchwell et al. (2014). Notably, and despite substantial efforts to minimize the environmental levels of BPA in the animal rooms, the predominant phase II metabolite, BPA-glucuronide (BPA-G), was detected in the serum of naïve (not included in the present study) and vehicle control animals (LOD 0.4–0.6 nM (~0.15–0.25 ng/ml); detected levels varied from ~2 to 40 times the LOD), in both 21- and 80-day-old animals. Although the measured levels of BPA-G are consistent with unintentional exposure of control animals to BPA at levels approximating the lowest dose of BPA in the study (2.5 μg/kg bw/day) (Churchwell et al., 2014), the exact source of the environmental BPA is unconfirmed. This caveat must be considered in evaluating the apparent effects at the lowest BPA dose level.

Neither dams nor pups were dosed on the day of birth (PND 0). On PND 1, litters were culled to a maximum of five pups per sex per litter (and a minimum of three pups per sex per litter), and daily weighing and direct gavage of the pups began. Dosing stopped on the day before scheduled sacrifice (PND 21 or 90 ± 5). Pups were individually housed after weaning (PND 21; a practice consistent with the FDA guidance in place at the time the experiment was initiated) in the same conditions as their parents. The only males used for this study were vehicle exposed males. BPA and EE exposed animals were all females (one per litter). Adult females (PND 90 ± 5) were sacrificed on the predicted day of estrus to minimize the influence of variable endogenous sex steroid hormone levels on ESR expression levels. All cycling females were sacrificed on estrus, with the exception of five animals, three in the low and two in the high EE groups, which were in an intermediate stage

(estrus/diestrus). As described in Delclos et al. (2014), a high proportion of the EE animals were not cycling normally. All animals were sacrificed by CO2 asphyxiation, the brains removed and rapidly frozen on a flat block of dry ice, and stored at −80◦C until shipping on dry ice to North Carolina State University (NCSU). There were 18–23 litters per exposure group in the main study and no more than one pup per sex per litter was evaluated. For the present study, 10 brains per exposure group (up to one per sex per litter) were randomly selected at NCTR prior to shipment to NCSU. Although there were no same sex littermates evaluated, there were male and female vehicle control littermates evaluated (four at PND 21 and five at PND 90). All tissues were coded prior to shipping and all work at NCSU was done blinded to sex and exposure groups. The brains were cryosectioned (Leica CM1900, Nussloch, Germany) into four serial sets of 20 μm coronal sections, mounted on Superfrost plus slides (Fisher Scientific, Pittsburgh, PA), and stored at −80◦C until *in situ* hybridization histochemistry (ISHH) processing.

In situ hybridization histochemistry.

Riboprobe-based ISHH was performed using probes designed for similar studies, and procedures are described in detail elsewhere (Cao et al., 2014; Cao & Patisaul, 2011, 2013; Cao et al., 2013). ISHH was performed in eight total batches, one for each time point (PND 21 and PND 90), gene (*Esr1* and *Esr2*), and region of interest (ROI; anterior and mediobasal hypothalamus) to minimize interbatch variance. Immediately following ISHH, the slides were dried and exposed to Kodak Biomax MR X-ray film (Eastman Kodak, Rochester,

NY). A 14C autoradiographic microscale (Amersham Life Sciences, Arlington Heights, IL) was included to generate a standard curve for the optical density calculations. Exposure time was 11 days for *Esr1* (all regions) and 17 days for *Esr2* (all regions).

Image analysis and film quantification.

ROIs for both the juvenile and adult rats were identified using the same criteria and landmarks extensively described in our prior papers using similar approaches (Cao et al., 2014; Cao et al., 2012; Cao & Patisaul, 2011) with the guidance of a brain atlas (Paxinos & Watson, 2007) and (as needed) our in-house library of NISSL stained sections spanning a range of ages in both sexes. Figure 3.1 and Supplementary figure 3.1 depict several of the landmarks used for section selection. ROIs in the anterior hypothalamus were the AVPV and MPOA (for additional information on landmark identification using different techniques see Herbison (2008); Losa et al. (2011); Patisaul et al. (2006), and the rostral and caudal portions of the VMNvl and ARC (rVMNvl, rARC, cVMNvl, cARC) in the mediobasal hypothalamus). Intensity of ESR signal on the autoradiograms was quantified using the digital densitometry application of the MCID Core Image software program (Inter-Focus Imaging Ltd, Cambridge, UK) using routine procedures (Cao et al., 2013; Patisaul, Whitten, & Young, 1999). All analyses were done blinded to sex and exposure groups.

ROI densities and background levels were measured from three anatomically matched sections per animal. The resulting values for each brain section after background subtraction were then averaged to obtain a representative measurement (for that ROI) for each animal. Densities were then converted to nCi/g tissue equivalents using a best-fit curve (cubic spline), derived from the autoradiographic ^{14}C microscales included with each ISHH

experiment. For all measurements, signal was within curve limits. All measurements were obtained by two investigators, both blind to exposure groups, to ensure repeatability in densitometry. The data sets were in high concordance with each other, and thus averaged to obtain final values for each gene and ROI.

Statistics.

Data analysis was performed using published guidelines established for assessing low-dose endocrine disruptor data (Haseman, Bailer, Kodell, Morris, & Portier, 2001). Within each exposure group, no same-sex litter mates were included, so potential litter effects did not need to be statistically accounted for within sex. In the vehicle control litters, four male/female litter mates were used at PND 21 and five were used at PND 90. Potential sibling effects were not taken into account in the analysis because all comparisons regarding impacts of BPA and EE exposure were made within females. Any derivation from the n = 10 animals/sex/group received resulted from an insufficient number of quantifiable sections in the ROI and the removal of statistically significant outliers via studentized residual (PND 21: two less samples in the AVPV, two in the rVMN, and one in the cVMN of the ESR1 batch; one in the cVMN of the ESR2 batch; PND 90: two outliers in the AVPV, one in the rVMN, and one in the rARC of the ESR1 batch; one in the MPOA and six in the rVMN of the ESR2 batch). Exclusion did not affect study outcome. The study had multiple, related but independent hypotheses embedded in the design, which necessitated a statistical approach where each hypothesis was independently considered, and included only the relevant groups for addressing that specific hypothesis. The primary goal was to establish if BPA impacts ESR expression in the female hypothalamus. Vehicle males were used for the purposes of

establishing if any BPA related effects in females resulted in the loss of expected sex difference in expression. The two EE groups were used for the purposes of determining if statistically significant BPA-related effects were consistent with an 'estrogenic' effect. Thus, it was first established with t-tests (pooled variance) if sex differences between the vehicle male and female controls were present, this is represented with a thin line across graphs, where sex differences were found at the male vehicle expression level, in order to visually compare differences in expression levels to the male vehicle group (Figures 3.2–3.4 and Supplementary figures 3.2–3.4). This confirmed assay sensitivity to known sex differences in ESR expression. Next, a one-way ANOVA was run to compare the female vehicle and four BPA exposure groups, followed by a Holm-Sidak post hoc test to establish whether any of the BPA exposure groups differed from the female vehicle group. The EE groups were not included in this ANOVA because inclusion of known 'positive controls' biases the analysis toward astatistically significant outcome (Haseman et al., 2001). If no significance was found by one-way ANOVA, no further statistical testing was conducted. BPA groups found to differ significantly from the female vehicle group were then compared via t-test to each EE group and the male vehicle group to establish if the effect was consistent with estrogenic activity and/or masculinization. Because the effects of EE on gene expression were considered informative, but a separate experiment, the effect of

EE exposure on ESR expression was then considered by comparing the two EE groups to the vehicle controls by a Dunnett's post hoc test (Haseman et al., 2001). All analyses were two-tailed and results were considered significantly different when $p \leq 0.05$.

Results

PND 21 Esr1 and Esr2 Expression in the POA

Esr1. Representative autoradiograph images for each experimental group and their expression levels are found in Figure 3.2A and an image of larger area and identified landmarks is found in Figure 3.1. AVPV *Esr1* expression in the female vehicle group (mean ± SEM = 156.315 ± 13.937 nCi/g) was significantly higher than the male vehicle group (88.591 ± 16.291 nCi/g; $p \leq 0.006$; Figures 3.2B and 3.2C) and masculinized by EE, but only at the highest dose (97.360 ± 12.727 nCi/g; $p \leq 0.004$; Figure 3.2C). Of the BPA exposed groups, expression was only significantly altered in the BPA 25 exposure group (84.713 ± 12.236 nCi/g; $p \leq 0.02$) and not significantly different from the male controls, indicative of masculinization (Figure 3.2B and 3.2C). In the MPOA, a sex difference in expression was observed as anticipated (357.820 ± 37.588 nCi/g, females and 227.643 ± 23.539 nCi/g, males; $p \leq 0.02$) but no significant effects of BPA or EE exposure were found (Supplementary figures 3.2A and 3.2B).

Esr2. For both the AVPV and MPOA, female expression (107.206 ± 18.497 nCi/g, AVPV and 123.847 ± 21.193 nCi/g, MPOA) was significantly higher than the male expression (45.104 ± 8.772 nCi/g, AVPV and 62.249 ± 9.2 nCi/g, MPOA; $p \leq 0.01$ and $p \leq 0.02$, respectively), but no effect of BPA or EE exposure was identified (Supplementary figure 3.3).

PND 21 Esr1 and Esr2 Expression in the MBH

Esr1. *Esr1* expression was significantly higher in the female rVMNvl (202.376 ± 15.522 nCi/g) compared with the male rVMNvl (107.774 ± 11.779 nCi/g; $p \leq 0.001$), but no

sex difference was observed in the cVMNvl (Supplementary figures 3.2C and 3.2D). Female *Esr1* levels were masculinized by EE in both the rVMNvl (EE 5, 106.767 ± 6.88 nCi/g) and the cVMNvl (female vehicle, 174.171 ± 17.032 nCi/g and EE 5, 115.820 ± 12.951 nCi/g), but only at the highest dose (Supplementary figures 3.2D and 3.2F; $p \leq 0.001$ and $p \leq 0.038$, respectively). No significant effects of BPA exposure were found in either subregion of the VMNvl (Supplementary figures 3.2C and 3.2E). In the ARC (rARC and cARC), *Esr1* expression was not sexually dimorphic, and no significant effects of BPA or EE exposure were observed (Supplementary figures 3.2G–J).

Esr2. Expression of *Esr2* in the VMNvl was not sexually dimorphic, and no significant effects of BPA or EE exposure were observed (Supplementary figures 3.3E–H). As expected, no appreciable *Esr2* expression was found in the ARC in any of the experimental groups (Cao & Patisaul, 2011).

PND 90 Esr1 and Esr2 Expression in the POA

Esr1. *Esr1* expression was sexually dimorphic in the MPOA (326.819 ± 32.923 nCi/g, females and 191.175 ± 25.743 nCi/g, males) ($p \leq 0.007$), but not the AVPV (Supplementary figures 3.4A–D). Both doses of EE masculinized MPOA *Esr1* expression (EE 0.5, 181.872 ± 16.702 nCi/g; $p \leq 0.006$ and EE 5, 208.376 ± 23.827 nCi/g; $p \leq 0.001$), but neither dose had a significant effect on AVPV *Esr1* expression (Supplementary figures 3.4A–D). No significant effects of BPA exposure were found in either theMPOA or AVPV (Supplementary figures 3.4A and 3.4C).

Esr2. Representative autoradiograph images for each experimental group and their expression levels are found in Figures 3.3A and 3.4A and an image of larger area and

identified landmarks is found in Figure 3.1. AVPV *Esr2* expression was significantly higher in females (69.582 ± 6.563 nCi/g, females and 37.251 ± 7.835 nCi/g, males; $p \leq 0.005$) and significantly reduced in the EE groups (EE 0.5, 39.383 ± 5.904 nCi/g; $p \leq 0.008$ and EE 5, 44.904 ± 7.762 nCi/g; $p \leq 0.03$), indicative of masculinization (Figures 3.3B and 3.3C). Compared with vehicle conspecifics, *Esr2* expression was significantly lower in the BPA 2.5, BPA 25, and BPA 260 groups (39.143 ± 6.608 nCi/g; $p \leq 0.005$, 35.556 ± 5.385 nCi/g; $p \leq 0.005$, and 35.034 ± 4.724 nCi/g; $p \leq 0.02$, respectively). Expression levels in these BPA-exposure groups did not significantly vary from the male vehicle group, indicative of masculinization (Figures 3.3B and 3.3C). In the MPOA, *Esr2* expression was significantly higher in females (155.919 ± 6.304 nCi/g, females and 55.217 ± 6.059 nCi/g, males; $p \leq 0.001$) and significantly reduced by both doses of EE (EE 0.5, 106.555 ± 15.734 nCi/g; $p \leq 0.009$ and EE 5, 95.025 ± 10.034 nCi/g; $p \leq 0.002$) (Figures 3.4B and 3.4C). Among the BPA exposure groups, expression was only significantly altered in the BPA 2.5 exposure group (95.715 ± 10.781 nCi/g; $p \leq 0.01$) with levels comparable to the EE groups but significantly higher than the male group (Figures 3.4B and 3.4C; $p \leq 0.01$).

PND 90 Esr1 and Esr2 Expression in the MBH

Esr1. *Esr1* expression was significantly higher in the female rVMNvl (256.379 ± 37.774 nCi/g, females and 123.115 ± 15.468 nCi/g, males; $p \leq 0.005$) and cVMNvl (276.734 ± 46.437 nCi/g, females and 126.289±14.712 nCi/g, males; $p \leq 0.013$), but not masculinized by either dose of EE (Supplementary figures 3.4E–H). No significant effects of BPA exposure were found in either VMNvl subregion (Supplementary figures 3.4E and 3.4G). In the ARC, female expression of *Esr1* was significantly higher than male expression in the

rARC (199.561 ± 21.284 nCi/g, females and 132.069 ± 13.568 nCi/g, males; $p \leq 0.02$), but not the cARC, and no significant effects of EE or BPA were identified in either subregion (Supplementary figures 3.4I–L).

Esr2. In the VMN, no significant sex differences were identified and no significant effects of EE or BPA were observed (Supplementary figure 3.5). *Esr2* expression was absent in the ARC, as previously reported (Cao & Patisaul, 2011).

Discussion

The present study represents the most comprehensive and region-specific evaluation of BPA and EE exposure effects on ESR expression levels in limbic subnuclei of the female rat at weaning and early adulthood. These results suggest that subchronic, low dose BPA or EE exposure can induce age- and region-specific effects on hypothalamic ESR expression in female rats. Expression changes induced by BPA and EE were primarily confined to the anterior hypothalamus, an estrogen-sensitive region required for ovulation and aspects of female reproductive behavior (Semaan & Kauffman, 2010; Simerly, 2002). AVPV *Esr1* expression was reduced on PND 21 (at 25 μg BPA/kg bw/day), but not PND 90, and *Esr2* expression was reduced in both the AVPV (2.5, 25, and 260 μg BPA/kg bw/day) and MPOA (2.5 μg BPA/kg bw/day) at PND 90. Significant effects of BPA were observed only at doses of 260 μg/kg bw/day and lower, demonstrating that consistent and statistically significant effects of subchronic BPA exposure on ESR expression can manifest at doses equivalent to, or below, the current oral reference dose of 50 μg/kg bw per day.

A notable caveat, as described in Delclos et al. (2014) and Churchwell et al. (2014), is that BPA-G was found in the serum of naïve (not included in the present study) and vehicle

control animals littermates to the ones used in the current study at levels that were statistically indistinguishable from the 2.5 μgBPA/kg bw/day exposure group. Although the source of the background BPA exposure in the naïve and vehicle groups remains undetermined, the presence of BPA-G in negative control serum potentially confounds the interpretation of the changes observed in the lowest (2.5 μg BPA/kg bw/day) BPA exposure group, but not those in higher dose groups. In the parent toxicity study (from which the animals for the present study were obtained), Delclos et al. (2014) concluded that 'Our interpretation of the results of the present study is that BPA in the 'low dose' region from 2.5 to 2700 μg/kg bw/day did not produce effects in the evaluated endpoints that differ from normal background biological variation.' No neural endpoints were included in those evaluated endpoints, thus the present data suggest that neural effects within that low dose range are plausible. This assertion is supported by prior data from similar studies using different animals (Cao et al., 2013) and different routes of administration (Cao et al., 2014; Cao et al., 2012).

Ongoing studies in the CLARITY-BPA research program (Schug et al., 2013), which incorporate a similar (2.5–2500 μg BPA/kg bw/day) dose range, assessment of BPA-G serum levels, and combine behavior, neuroanatomical, and molecular endpoints, should provide resolution regarding confirmation of ESR expression changes and the functional significance of observed ESR expression changes. This important follow-up study will be able to examine the potential linkage of the transcriptional changes observed here to neuroendocrine and behavioral outcomes. Recent behavioral studies in this rat model using 2.5 and 25 μg BPA/kg bw/day have failed to detect significant behavioral effects in juveniles or adults (Ferguson et

al., 2011; Ferguson, Law, & Kissling, 2014), but a range of behavioral effects have been reported by other groups (reviewed in (Rochester, 2013; Wolstenholme, Rissman, et al., 2011)). In addition, the main study from which the animals were obtained, detected effects on the female reproductive tract only at very high doses of BPA (100,000 and 300,000 μg/kg bw/day; Delclos et al. (2014)). The present data suggest, however, that reproductive effects reported in numerous prior studies, including altered ovarian morphology, estrus cyclicity, and subfertility ((Calhoun et al., 2014; Souter et al., 2013) and reviewed in (FAO/WHO, 2011; Rochester, 2013)) may be attributable to changes in ESR content within the anterior hypothalamus.

Inclusion of a wide range of BPA doses, ranging from 2.5 to 2700μg/kg bw/day (with 10-fold spacing between consecutive doses) allowed the characterization of the dose-response and assessment of impacts below the current NOAEL. The shape of the dose-response relationships for the BPA-related effects differed among the regions examined. For example, AVPV *Esr2* levels were modulated by three BPA doses at PND 90, including the lowest dose group, and the magnitude of the effect did not appreciably differ between doses. At PND 21, AVPV *Esr1* levels were modulated by 25 μg BPA/kg bw/day only. Inclusion of the intermediate and high BPA dose groups available from the main study (Delclos et al., 2014) may have provided additional resolution of dose-response relationships, but the data presented here reveal the potential for significant effects in the low BPA dose range. As noted elsewhere, this is being further investigated in this same model.

These experiments serve as an important follow-up to our prior, related study showing that limbic ESR expression just after birth (PND 1) is altered by gestational

exposure to BPA (2.5 and 25 μg/kg bw/day) (Cao et al., 2013). For that study, the animals were derived from the same colony of NCTR SD rats but bred, housed, dosed, and handled in a separate building so they are not siblings or otherwise related to the animals in the present study. Although no direct measurements of serum BPA or BPA-G were made in the Cao et al. (2013) study, our unpublished data suggest that the unintentional exposure to BPA in the present study is related to the use of very high BPA doses (100 and 300 mg/kg bw day, Delclos et al. (2014)). The highest dose in the Cao et al. (2013) study was 25 μg BPA/kg bw/day; hence the low dose range expression effects reported in those animals are not believed to be confounded by unintentional exposure to BPA. One of the primary hypotheses tested in the present study was if the ESR expression changes observed on PND 1 persist across the lifespan. Although ESR expression was found to be altered at puberty and early adulthood, the impacted brain regions and the direction of BPA-related ESR expression changes reported here differ from those observed in the complementary study examining neonates. In the PND 1 animals, BPA-related ESR expression level differences were observed in the MBH, rather than the anterior hypothalamus. Additionally, in the neonates, BPA was found to increase *Esr1* and *Esr2* expression, an effect which is directionally opposite to the expression changes reported here for older animals. Age at assessment and exposure duration likely primarily account for these differences. Importantly, endogenous steroid hormone levels are higher in males than females on PND 1, a difference which fundamentally contributes to brain sexual differentiation (McCarthy, 2008; Simerly, 2002), and thus may also confer sex and age-related differences in BPA sensitivity (FAO/WHO, 2011). Notably, our prior study (Cao et al., 2013) found a significant impact of gavage on

PND 1 ESR expression levels, suggesting a critical interaction between gestational BPA exposure and prenatal stress on brain ESR expression. Although the main study from which the animals used in the present study were obtained (Delclos et al., 2014) did include a naïve control group, the PND 1 results were not available at the time that the present study was designed. Thus, unfortunately the naïve control group was not incorporated to address the separate issue of gavage-related effects on ESR expression.

The ESR subtype impacted by BPA differed on PNDs 21 and 90, suggesting that *Esr1* and *Esr2* may be differentially sensitive to BPA depending on gonadal state. In the prepubertal animals, when endogenous estrogen levels are naturally low, Esr1 was found to be decreased while, in the adults, when circulating estrogen levels are elevated, *Esr2* expression was reduced. Consequently, the sexually dimorphic pattern of *Esr1* and *Esr2* expression normally seen in the AVPV and MPOA was lost in BPA exposed animals, specifically, AVPV Esr1 expression in the BPA 25 group at PND 21, AVPV *Esr2* expression in the BPA 2.5, 25, and 260 groups at PND 90, and MPOA *Esr2* expression in the BPA 2.5 group at PND 90. These age- and region-specific results are not entirely unexpected because ESR expression naturally varies with age in limbic nuclei, particularly those which are morphologically and functionally sexually dimorphic, including the hypothalamic regions investigated here (Cao & Patisaul, 2011; Chakraborty, Hof, Ng, & Gore, 2003; Chakraborty, Ng, & Gore, 2003; Ikeda, Nagai, Ikeda, & Hayashi, 2003; Walker, Kirson, Perez, & Gore, 2012; Wilson et al., 2002). This temporal variability in ESR expression is crucial for organizing and maintaining sex differences across the life span; thus disruption, even a temporary disruption, during critical windows of development could alter sex-specific brain

structure and function (G. J. De Vries, 2004). The present data suggest that perturbation of region- and age-specific ESR expression may underlie previously reported BPA-related morphological and ESR protein level differences in sexually dimorphic brain regions such as the sexually dimorphic nucleus (SDN) and the AVPV (Adewale et al., 2011; Cao et al., 2014; He et al., 2012; McCaffrey et al., 2013; Patisaul et al., 2006, 2007; Rubin et al., 2006; Viberg, Fredriksson, Buratovic, & Eriksson, 2011).

Although there are well recognized species differences in terms of how and where the brain is sexually dimorphic, steroid hormones are known to be fundamental to the orchestration and maintenance of these differences (Bonthuis et al., 2010; Swaab, 2007; Wallen, 2005). Reports published by the NTP, WHO, and others have expressed concerns that BPA exposure, at levels below the current NOAEL, might alter sex-specific neural organization and thereby pose a risk to developing fetuses, infants, and young children (Chapin et al., 2008; Gontier-Latonnelle et al., 2007; Rochester, 2013; Shelby, 2008; Shelnutt, Kind, & Allaben, 2013), although a direct demonstration of such effects in this model has not yet been assessed. Further work is ongoing (Schug et al., 2013) to address these concerns. In humans, the period encompassing the rodent perinatal period is believed to occur in mid to late gestation (Abbott et al., 2008; Aksglaede et al., 2006; Selevan et al., 2000; Simerly, 2002); thus, the rat perinatal 'critical window' is likely to be entirely prenatal in humans. Additionally, although estrogen derived from local aromatization of testicular androgens is well known to be required for masculinization in the rat brain, the role estrogen plays in the sex-specific organization of the human brain remains unclear (Giedd, Castellanos, Rajapakse, Vaituzis, & Rapoport, 1997; Herman, Jones, Mann, & Wallen, 2000;

Swaab, 2007). Thus, it is difficult to predict with certainty how disruption of ESR expression in the human brain may manifest.

The male vehicle group was specifically included to ensure that expected sex differences in sexually dimorphic ESR expression were present in vehicle control animals, and to evaluate BPA-related changes on these differences. The observed sex differences reported here at PND 21 are largely consistent with prior work characterizing brain ESR distribution (Cao & Patisaul, 2011). Importantly, *Esr1* and *Esr2* expression was higher in the female MPOA, as expected. On PND 21, AVPV *Esr1* expression was observed to be significantly higher in females than in males, but previous work from our lab using Long Evans rats found no statistically significant sex difference on PND 19 (Cao & Patisaul, 2011), suggesting that the magnitude of this difference is enhanced at weaning or differs by strain. Our data are consistent with the findings of Kuhnemann et al. which reported that, in Wistar rats, sex differences in ESR binding persist from PNDs 28–49 in the POA (Kuhnemann et al., 1994). Similarly, it has also been reported that Esr1 expression is greater in SD females than in males at PND 14, an observation that is consistent with our findings in the prepubertal animals (Orikasa, Kondo, Hayashi, McEwen, & Sakuma, 2002). Overall, in the present study, the sex differences abrogated by BPA were primarily restricted to the preoptic area.

Inclusion of the two EE exposure groups served to (1) confirm that these animals are sensitive to estrogen-induced masculinization and (2) establish if any BPA-related effects were consistent with an estrogenic mode of action. Our study examining ESR expression in PND 1 animals of the same SD strain, found no significant evidence that EE, at 5 and 10

μg/kg bw/day, masculinizes female *Esr1* and *Esr2* expression in either the anterior or the mediobasal hypothalamus (Cao et al., 2013). However, littermates examined at PND 21 showed a significant increase in the volume of the female sexually dimorphic nucleus of the preoptic area (SDN-POA), although the magnitude of the change did not reach male-typical levels at either dose (He et al., 2012). A study from a different lab group using Long Evans rats investigated the impact of EE exposure ranging from 0.05 to 50 μg/kg bw/day on a variety of neurobehavioral endpoints and suggested that exposure in the 5–50 μg/kg bw/day range is the most effective to masculinize reproductive behaviors and advance female pubertal onset. No endpoints were affected by the 0.5 μg/kg bw/day dose of EE (Ryan, Hotchkiss, Crofton, & Gray, 2010); however, neonatal exposure in that study was lactational rather than direct as in the present study. In the current study, exposure to EE decreased ESR expression in regions where expression was sexually dimorphic and affected by BPA exposure, suggesting that BPA has masculinizing effects in these regions. At PND 21, masculinization of Esr1 expression by EE was found in the female AVPV, but only at the higher dose (5 μg/kg bw/day). At PND 90, masculinization of *Esr2* expression by EE in the AVPV and MPOA was achieved with both exposures of EE (0.5 and 5 μg/kg bw/day). Collectively, the data suggest that the minimally effective exposure level for inducing hypothalamic masculinization of ESR expression by EE in SD rats (when administered by gavage) is at or above 0.5 μg/kg bw, though sensitivity may vary with age and route of administration.

Although BPA has a 10,000-fold lower binding affinity for ESRs, in some cases where ESR expression was significantly altered by BPA and EE, the effect occurred at a

lower relative dose of BPA than EE. This observation is consistent with what we previously reported in PND 1 animals from a companion study (Cao et al., 2013) and suggests that, although BPA has historically been characterized as a weak estrogen, it may act through alternative pathway(s) to perturb ESR expression. Possibilities other than classic estrogen signaling include activity through membrane receptors (including GPR30 (Ge et al., 2014; Thomas & Dong, 2006)), epigenetic changes, or interactions with other steroid hormone receptors (Gentilcore et al., 2013; La Rosa et al., 2014; Wolstenholme, Rissman, et al., 2011). For example, emerging evidence suggests that BPA binds to estrogen related receptor gamma (ERRγ), a nuclear receptor whose natural ligand is not known and is thought to play a role in the differentiation and maturation of the fetal brain (Hermans-Borgmeyer, Susens, & Borgmeyer, 2000; Lorke, Susens, Borgmeyer, & Hermans-Borgmeyer, 2000; Matsushima et al., 2007; Takayanagi et al., 2006). Although the present studies were not designed to specifically examine the specific mechanisms by which BPA impacts cell-specific ESR expression, subsequent studies should address this data gap.

The BPA-related decreases in ESR expression reported here could reflect either a decrease in cellular levels of *Esr1* and *Esr2* or reduced numbers of cells transcribing *Esr1* and *Esr2*. A change in cell number would suggest that permanent, organizational changes within the impacted brain region have occurred (McCarthy, 2008). Because BPA-related effects on ESR expression differed with age, this appears to be unlikely, but compensation for cell loss via increased expression in existing cells cannot be ruled out (G. J. De Vries, 2004). Presumably, decreased ESR expression is indicative of reduced ESR protein levels; a relationship established in prior studies (Monje et al., 2007).

Conclusions

The data provided here contribute novel information regarding mechanisms by which subchronic, low dose exposure to BPA may influence sex-specific brain development. Effects of BPA on hypothalamic ESR expression were primarily observed at exposure levels less than the current oral reference dose for BPA of 50 μg/kg bw/day, but not at the highest dose (2700 μg/kg bw/day) employed. The functional significance of perturbed hypothalamic ESR expression during prepuberty and early adulthood remains to be definitively established, but the data presented here suggest the potential for apical effects on neuroendocrine systems below the currently established NOAEL of 5 mg/kg bw/day. Ongoing studies will further address the relationships between transcriptional changes in the brain and physiological/behavioral effects (Schug et al., 2013).

Funding

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Figures

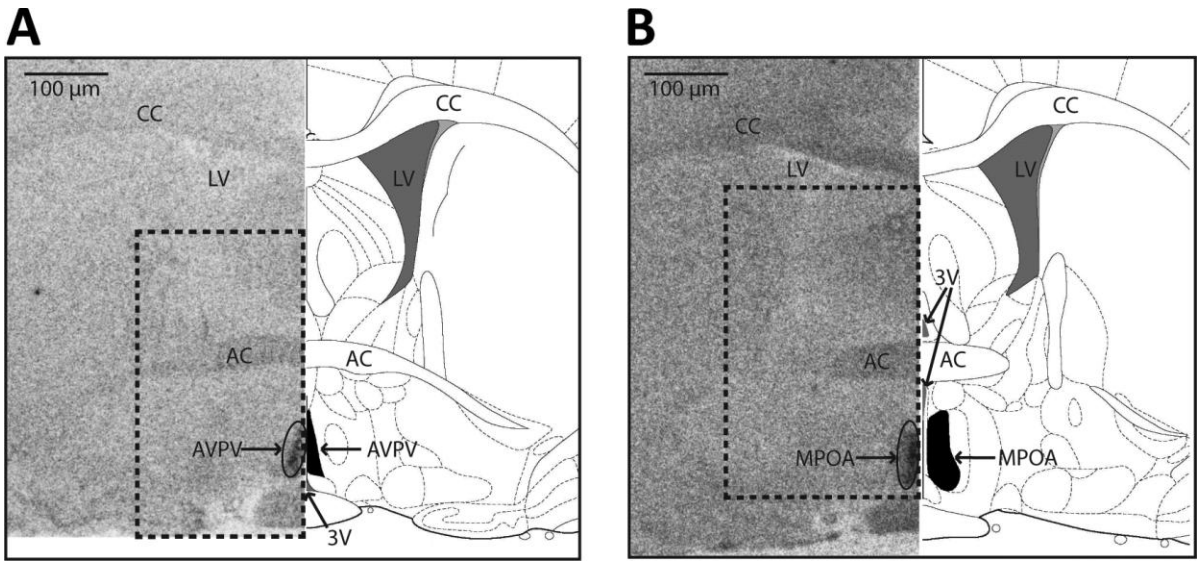


Figure 3.1: Autoradiograms and atlas depictions of AVPV and MPOA. (A and B) Depiction of primary landmarks and regions of interest on the autoradiograms (left panel) and corresponding rat atlas images modified from Paxinos and Watson (2007) (right panel). (A) AVPV and surrounding landmarks. (B) MPOA and surrounding landmarks. The AVPV and MPOA are encircled on the autoradiograph image, and shaded in black on the atlas image (corresponding to bregma −0.12 mm for the AVPV and bregma −0.36 mm for the MPOA). The dotted box encapsulates the region depicted in the images presented in Figures 3.2A, 3.3A, and 3.4A. (3V = third ventricle, AC = anterior commissure, CC = corpus callosum, LV = lateral ventricle.)

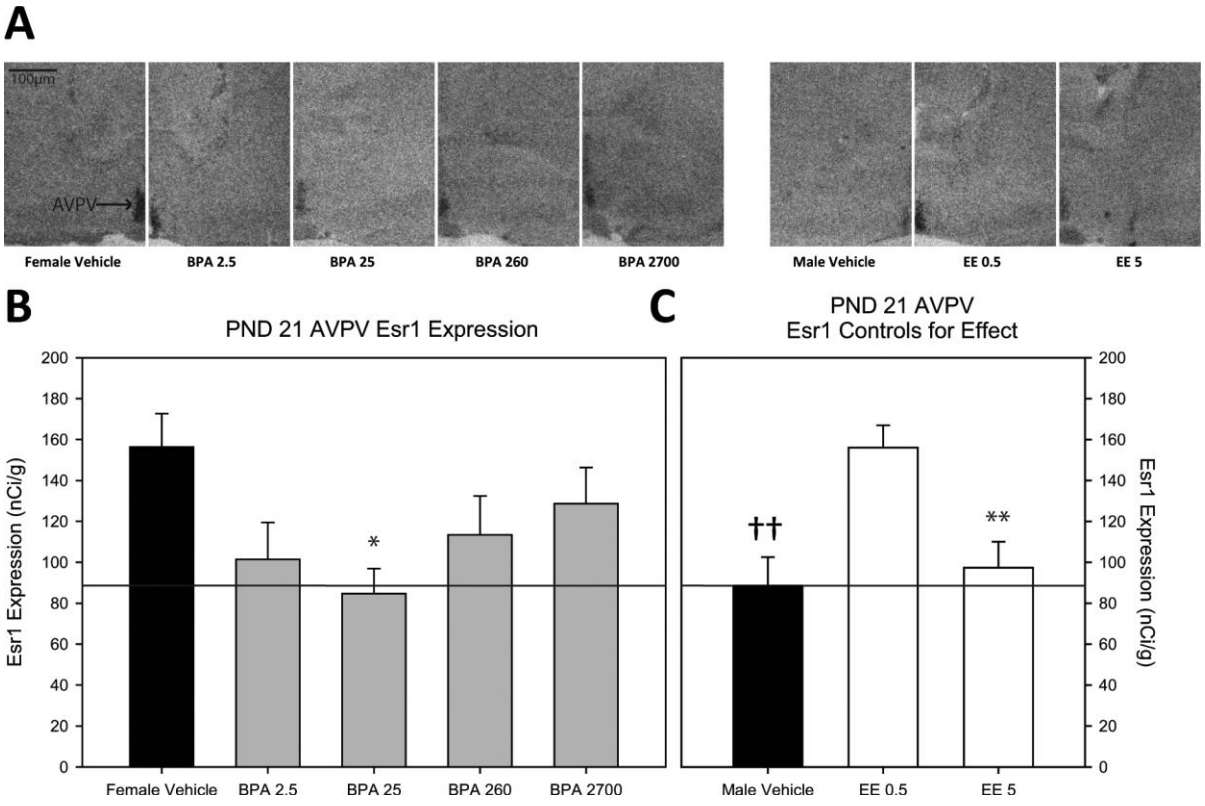


Figure 3.2: Juvenile *Esr1* mRNA expression in the AVPV. (A) Representative autoradiograph images of *Esr1* expression in the AVPV (indicated by arrow in female vehicle image) of each exposure group. (B) Impact of BPA exposure on *Esr1* expression in the PND 21 female AVPV. Only the BPA 25 group significantly differed from the female vehicle group. The positive control groups are depicted in (C). *Esr1* expression was significantly lower in the male vehicle group compared with the female vehicle group and this sex difference is indicated by the thin line traversing across panels (B) and (C). Expression in the BPA 25 group was reduced to male-typical levels. Among the EE groups, *Esr1* expression was only significantly reduced in the EE 5 group compared with female vehicle controls (n = 9–10 per group; *$p \leq 0.05$, **$p \leq 0.01$ compared with female vehicle group; sex differences denoted with ††$p \leq 0.01$; graphs depict mean ± SEM).

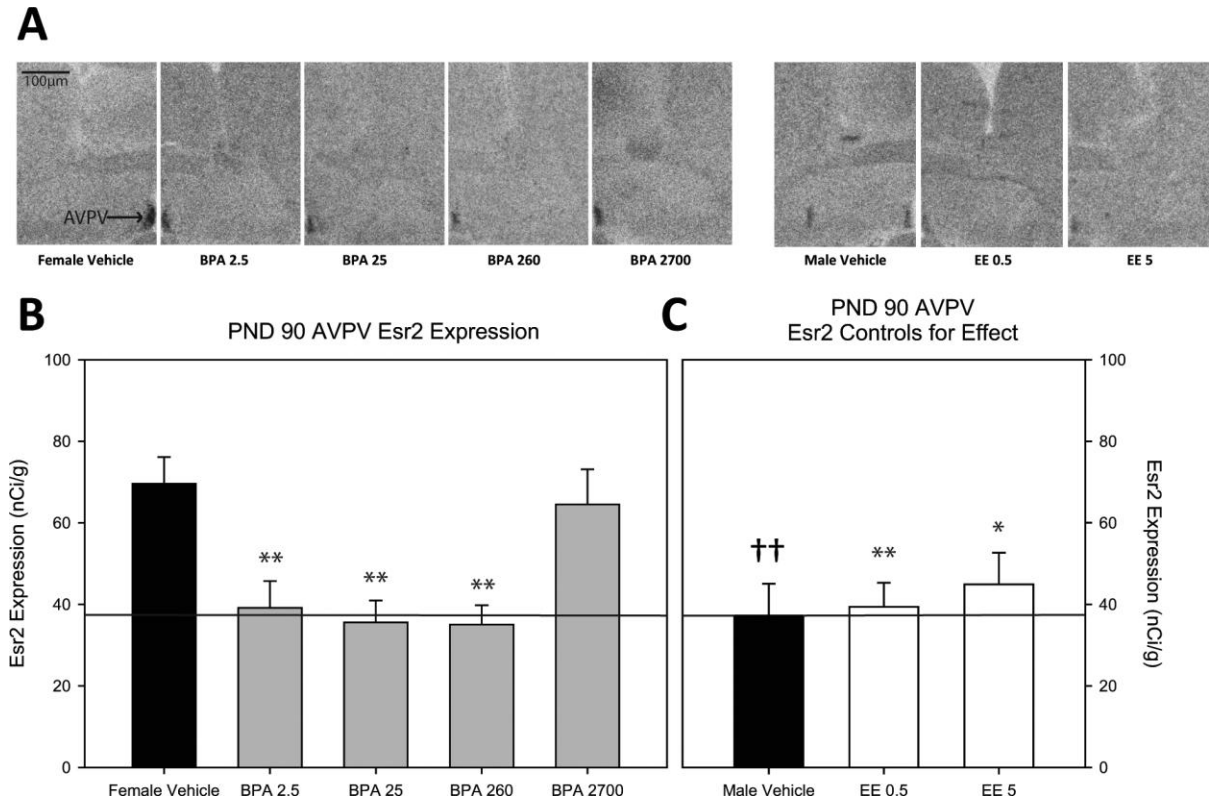


Figure 3.3: Adult *Esr2* mRNA expression in the AVPV. (A) Representative autoradiograph images of *Esr2* in the AVPV (indicated by arrow in female vehicle image) of each exposure group. (B) Impact of BPA exposure on *Esr2* expression in the PND 90 female AVPV. *Esr2* expression was significantly lower in all of the BPA exposed groups except the BPA 2700 group compared with the female vehicle controls. The positive control groups are depicted in (C). *Esr2* expression was significantly lower in the male vehicle group compared with the female vehicle group and this sex difference is indicated by the thin line traversing across panels (B) and (C). Expression the three significantly impacted BPA groups was reduced to male-typical levels. Among the EE groups, *Esr2* expression was significantly reduced in both the EE 0.5 and EE 5 groups compared with female vehicle controls, with levels approximating those seen in the male vehicle group (n = 10 for all groups; *$p \leq 0.05$, **$p \leq 0.01$ compared with female vehicle group; sex differences denoted with ††$p \leq 0.01$; graph depicts mean ± SEM).

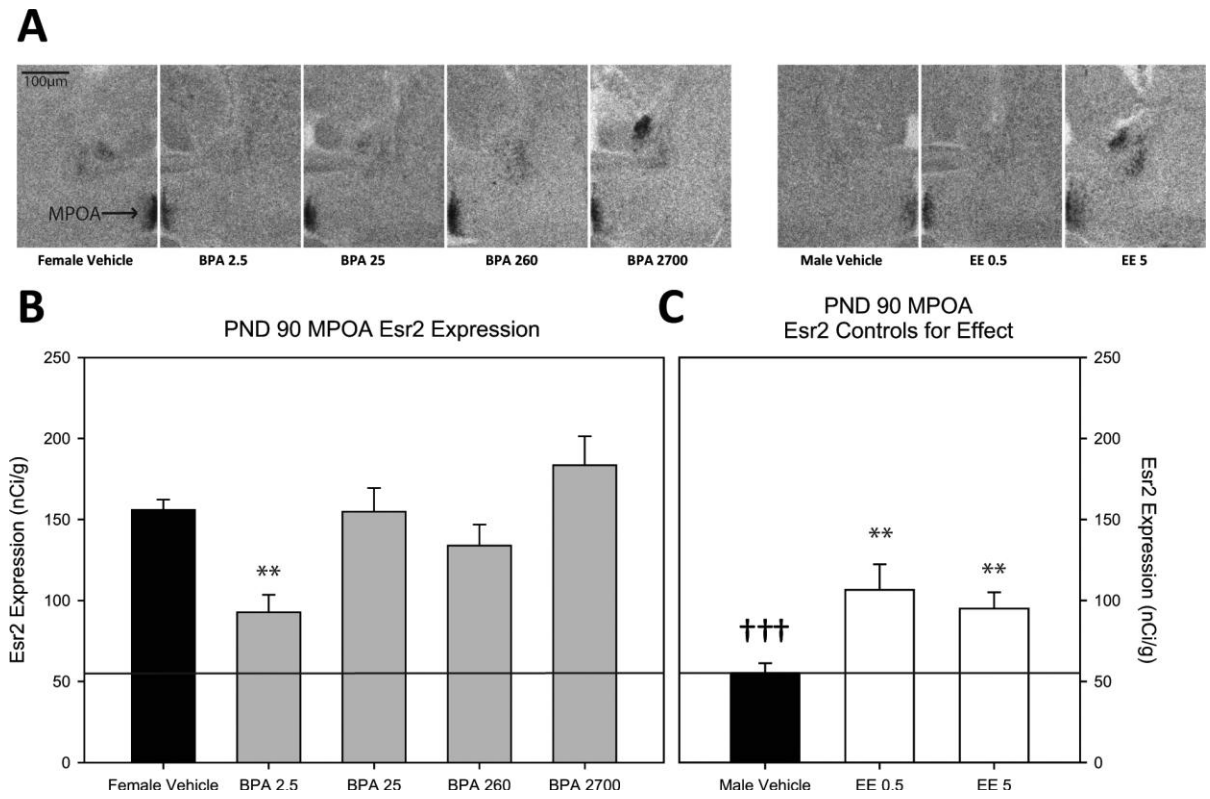
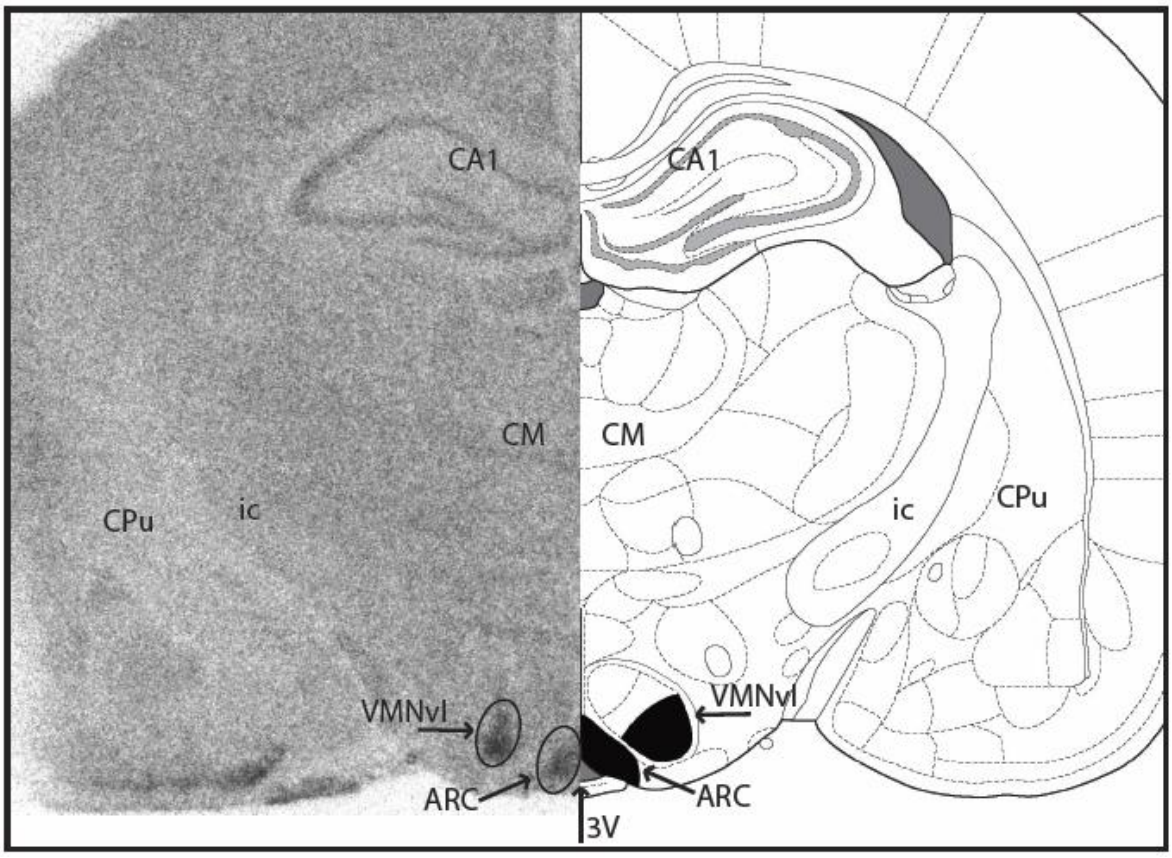
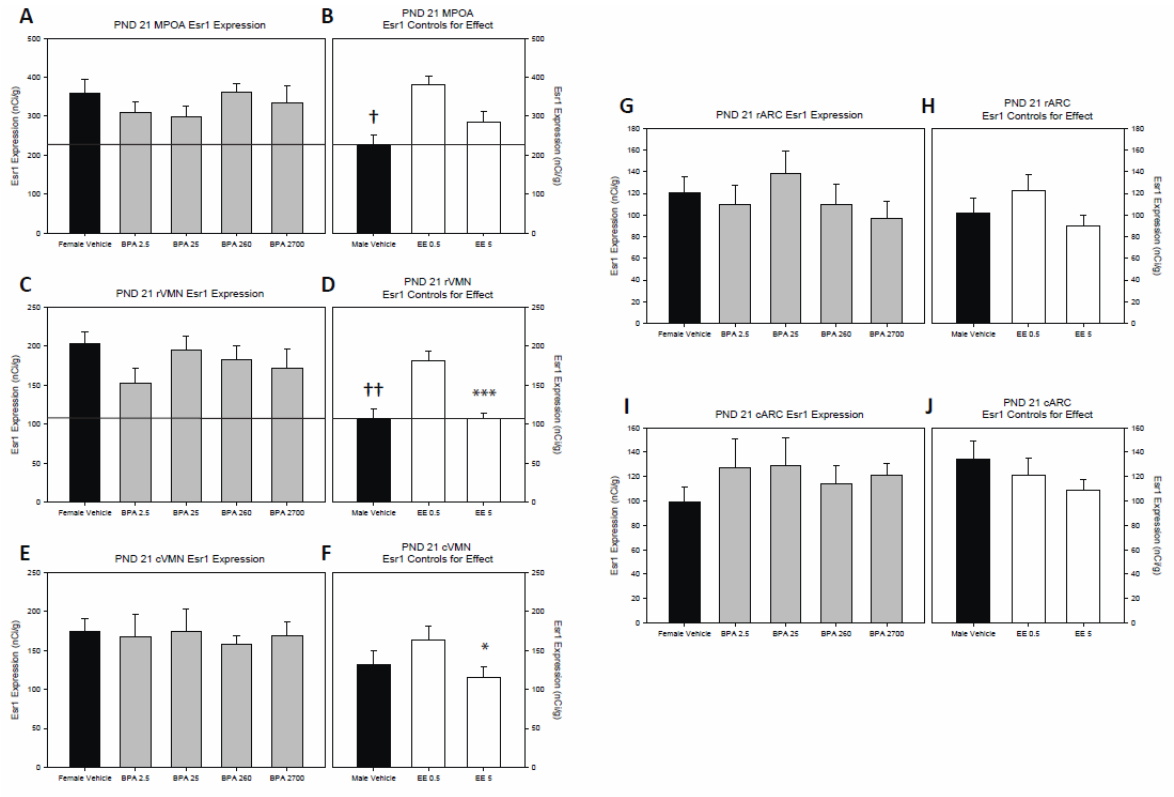


Figure 3.4: Adult *Esr2* mRNA expression in the MPOA. (A) Representative autoradiograph images of *Esr2* expression in MPOA (indicated by arrow in female vehicle image) of each exposure group. (B) Impact of BPA exposure on *Esr2* expression in the PND 90 female MPOA. Expression was significantly reduced in the BPA 2.5 group compared with the female vehicle controls. The positive control groups are depicted in (C). *Esr2* expression was significantly lower in the male vehicle group compared with the female vehicle group and this sex difference is indicated by the thin line traversing across panels (B) and (C). Among the EE groups, *Esr2* expression was significantly reduced in both the EE 0.5 and EE 5 groups compared with female vehicle controls. Although the magnitude of decreased *Esr2* expression was approximately equivalent among the BPA 2.5, EE 0.5, and EE 5 groups, expression was higher than typical for unexposed males, so the sex difference in *Esr2* expression was only partially abrogated (n = 8–10 per group; **$p \leq 0.01$; sex differences denoted with †††$p \leq 0.001$; graph depicts mean ± SEM).

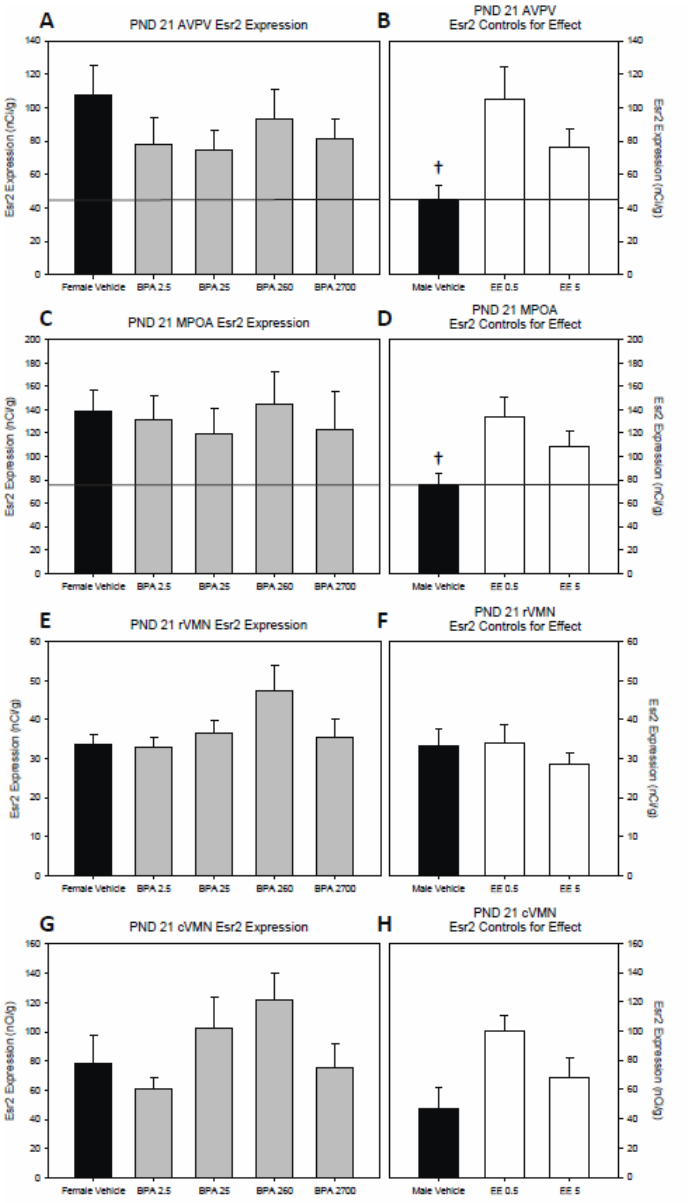
Supplementary Figures



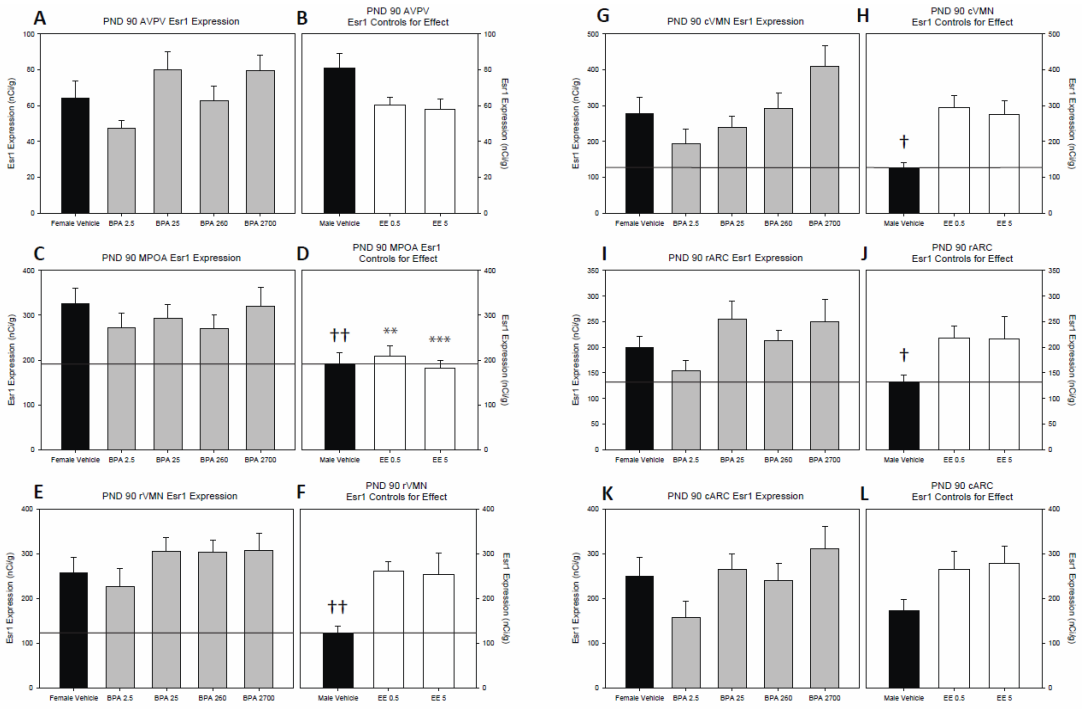
Supplementary Figure 3.1: Autoradiograms and atlas depictions of VMNvl and ARC. Depiction of primary landmarks and regions of interest on the autoradiograms (left panels) and corresponding rat atlas images modified from Paxinos and Watson (2007) (right panel). The images are representative of the region classified rVMNvl in the present studies with the cVMNvl further caudal. The VMN and ARC are encircled on the autoradiograph image, and shaded in black on the atlas image (corresponding to Bregma -2.52 mm for both. (3V=third ventricle, CA1= CA1 region of the hippocampus, CM=central medial thalamic nucleus, CPu=caudate putamen, ic=internal capsule.)



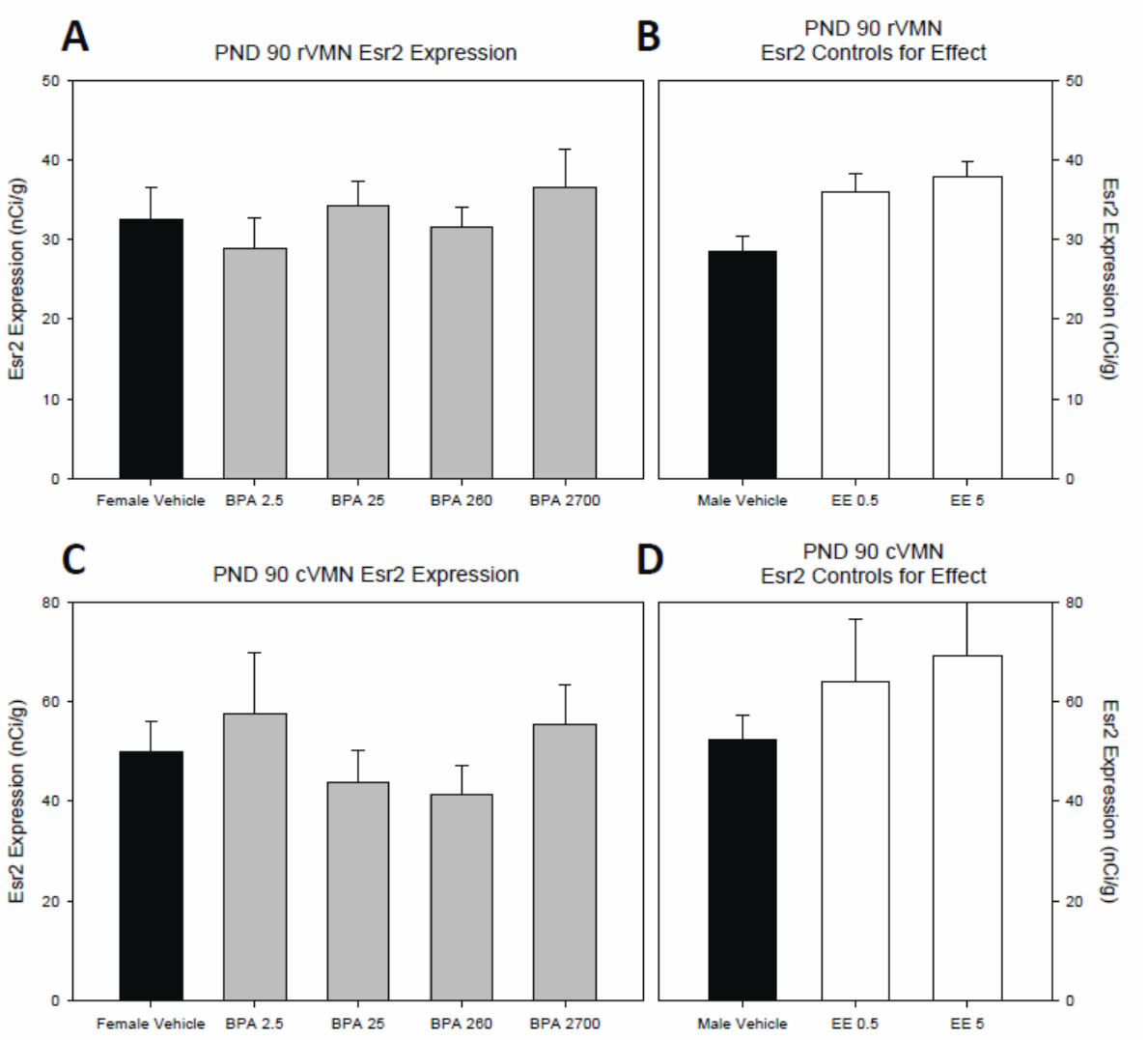
Supplementary Figure 3.2: Juvenile *Esr1* mRNA expression in the POA and MBH. (A)(C)(E)(G)(I) BPA had no significant impact on *Esr1* expression in the PND 21 female rat MPOA, rVMN, cVMN, rARC, and cARC, respectively. The positive control groups are depicted in (B)(D)(F)(H) and (J). *Esr1* expression was sexually dimorphic in the MPOA (A and B) and rVMN (C and D) with expression significantly lower in the Male Vehicle group compared to the Female Vehicle group. This sex difference is indicated by the thin line traversing across panels A and B, and C and D. Among the EE groups, *Esr1* expression was significantly reduced in the rVMN and cVMV of the EE5 group compared to the Female Vehicle group, with levels in the rVMN approximating those seen in the Male Vehicle controls. (n = 6-10 per group; *$p \leq 0.05$; ***$p \leq 0.001$ compared to Female Vehicle group; sex differences denoted with †$p \leq 0.05$, and ††$p \leq 0.01$; Graphs depict mean ± SEM).



Supplementary Figure 3.3: Juvenile *Esr2* mRNA expression in the POA and MBH. (A)(C)(E)(G) BPA had no significant impact on *Esr2* expression in the PND 21 female rat AVPV, MPOA, rVMN, and cVMN, respectively. The positive control groups are depicted in (B)(D)(F) and (H). *Esr2* expression was sexually dimorphic in the AVPV (A and B) and MOPA (C and D) with expression significantly lower in the Male Vehicle group compared to the Female Vehicle group. This sex difference is indicated by the thin line traversing across panels A and B, and C and D. EE had no significant effect on *Esr2* expression (B)(D)(F)(H). (n = 5-10 per group; sex differences denoted with †$p \leq 0.05$; Graphs depict mean ± SEM).



Supplementary Figure 3.4: Adult *Esr1* mRNA expression in the POA and MBH. (A)(C)(E)(G)(I)(K) BPA had no significant impact on *Esr1* expression in the PND 90 female rat AVPV, MPOA, rVMN, cVMN, rARC, and cARC, respectively. The positive control groups are depicted in (B)(D)(F)(H)(J) and (L). *Esr1* expression was sexually dimorphic in MPOA (C and D), rVMN (E and F), cVMN (G and H) and rARC (I and J) with expression significantly lower in the Male Vehicle group compared to the Female Vehicle group. This sex difference is indicated by the thin line traversing across panels C and D, and E and F, G and H, and I and J. Among the EE groups, in the PND 90 MPOA *Esr1* expression was significantly reduced in the EE0.5 and EE5 groups compared to the Female Vehicle group, to levels that are male-typical range (C and D). (n = 7-10 per group; **p ≤ 0.01, ***p ≤ 0.001 compared to the Female Vehicle group; sex differences denoted with †p ≤ 0.05, and ††p ≤ 0.01; Graphs depict mean ± SEM)



Supplementary Figure 3.5: Adult *Esr2* mRNA expression in the POA and MBH. (A)(C) BPA had no significant impact on *Esr2* expression in th ePND 90 female rat rVMN, and cVMN, respectively. The positive control groups are depicted in (B) and (D). No significant sex differences were observed nor any significant effects of EE. (n = 6-10 per group; Graphs depict mean ± SEM).

CHAPTER 4— Impact of Low Dose Oral Exposure to Bisphenol A (BPA) on Juvenile and Adult Rat Exploratory and Anxiety Behavior: A CLARITY-BPA Consortium Study

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Abstract

Bisphenol A (BPA) is a high volume production chemical and has been identified as an endocrine disruptor, prompting concern that developmental exposure could impact brain development and behavior. Prior rodent and human epidemiologic studies suggest that early life exposure to BPA may result in an anxious, hyperactive phenotype later in life, but results are conflicting and data from studies incorporating guideline study elements using doses below the no-observed-adverse-effect level (NOAEL) are limited. To address this, the present studies were conducted as part of the CLARITY-BPA (Consortium Linking Academic and Regulatory Insights on BPA Toxicity) program. The impact of perinatal BPA exposure (2.5, 25, or 2500 µg/kg body weight/day) on behaviors related to anxiety and

exploratory activity was assessed in juvenile (pre-pubertal) and adult NCTR Sprague-Dawley rats of both sexes. Vehicle and ethinyl estradiol (EE; 0.5 µg/kg body weight/day) were used as controls. Dams were gavaged from gestational day 6 until the start of labor, and offspring were directly gavaged on postnatal days 1- 21 (i.e., through weaning). Behavioral assessments included open field, elevated plus maze, and zero maze. Anticipated sex differences in behavior were statistically identified or suggested in most, but not all, cases. No consistent effects of BPA were observed for any endpoint, in either sex, at either age. Significant differences between BPA-exposed and EE-exposed groups were identified for some endpoints, emphasizing that BPA may not act strictly as an "estrogenic" compound. Limitations of the present study are discussed include lower than anticipated statistical power arising from large inter-individual variation on behavioral measures and low concordance across behavioral tasks.

Key Words

Bisphenol A, CLARITY, behavior, anxiety, exploratory activity

Disclaimer

This document has been reviewed in accordance with United States Food and Drug Administration (FDA) policy and approved for publication. Approval does not signify that the contents necessarily reflect the position or opinions of the FDA nor does mention of trade names or commercial products constitute endorsement or recommendation for use. The findings and conclusions in this report are those of the authors and do not necessarily represent the views of the FDA.

Introduction

Bisphenol A (BPA) is a high volume production, industrial chemical now ubiquitous in the environment and used to create numerous products including polycarbonate plastics and epoxy resins. BPA has been extensively evaluated for potential adverse effects, but overall results have been mixed and consensus on the potential health risks at human-relevant exposure levels has not been reached. Over the past decade, the National Toxicology Program (NTP), World Health Organization (WHO), and Food and Agricultural Organization (FAO) have expressed concern for effects on the brain and behavior and issued recommendations to fill key data gaps (Beronius et al., 2010; Chapin et al., 2008; FAO/WHO, 2011; FDA, 2012; National Toxicology Program, 1982). Notably, the WHO/FAO report highlighted concerns regarding the potential for BPA exposure to increase anxiety and specifically recommended use of multiple, concordant assays for anxiety testing in laboratory animals, including the elevated plus and zero mazes (EPM and ZM). The report also recommended testing both sexes at multiple ages and the incorporation of multiple BPA doses. In 2010, the FDA expressed similar concern for effects on the developing fetus, infants, and young children, but more recently asserted that "BPA is safe at the current levels occurring in foods" while advocating for and engaging in further research on neural endpoints with the goal of obtaining new data to enhance risk assessment (http://www.fda.gov/newsevents/publichealthfocus/ucm064437.htm, updated March 2013). In a December 5, 2014 announcement, the FDA further concluded that "The available information continues to support the safety of BPA for the currently approved uses in food containers and packaging."

(http://www.fda.gov/Food/IngredientsPackagingLabeling/FoodAdditivesIngredients/ucm064437.htm)

The present study was conducted as part of the CLARITY-BPA (Consortium Linking Academic and Regulatory Insights on BPA Toxicity) program (Birnbaum et al., 2012; Schug et al., 2013), a collaborative effort between academic and regulatory scientists, coordinated by the National Toxicology Program (NTP), the National Institute of Environmental Health Sciences (NIEHS), and the U.S. Food and Drug Administration (FDA) National Center for Toxicological Research (NCTR) to draw on the strengths of both guideline and academic studies to fill data gaps and address research recommendations identified by the WHO and others (Beronius et al., 2010; Chapin et al., 2008; FAO/WHO, 2011; FDA, 2012; National Toxicology Program, 1982). Using sibling rats from an ongoing guideline-compliant chronic two year study, which follows standard protocols and endpoints typically considered by regulatory agencies in hazard identification and risk assessment (Birnbaum et al., 2012; Schug et al., 2013), the CLARITY-BPA program incorporates novel endpoints routinely used in academic labs but not typically considered in traditional guideline studies (Myers et al., 2009). Through a similar collaborative effort, we showed that NCTR Sprague-Dawley rats dosed with BPA during the same developmental period as the current study exhibited altered estrogen receptor (ER) expression in hypothalamic structures fundamental for the manifestation of anxiety and related behaviors (Rebuli et al., 2014). The brains used in that study were collected from siblings of animals used in the NCTR 90-day subchronic study, which assessed the effects of BPA on body and organ weights, puberty onset, histopathology, sperm parameters, and internal dosimetry across life stages (Churchwell et al., 2014; Delclos

et al., 2014). These results highlight the advantages of a multi-agency research approach and provide support for the hypothesis that developmental BPA exposure may result in anxiety and exploratory-related behavioral alterations.

Numerous epidemiological and experimental animal studies have assessed behavioral outcomes following developmental BPA exposure and some report heightened anxiety (relevant studies published around and after the 2011 FAO/WHO report on BPA up through December, 2014 are summarized in Supplementary Table 4.1) and hyperactivity (summarized in Supplementary Table 4.2). Anxiogenic behaviors following BPA exposure have been observed across a wide range of doses (0.25-40,000 µg/kg bw in mice and 5-5,000 µg/kg bw in rats) (see Supplementary Table 4.1 for references) and recent epidemiological studies have associated BPA exposure with hyperactivity in boys and girls (J. M. Braun et al., 2011; Harley et al., 2013; Perera et al., 2012). This behavior is a hallmark of Attention Deficit Hyperactivity Disorder (ADHD); a link which has stimulated discussion regarding the potential for BPA and other endocrine disrupting compounds (EDCs) to contribute to ADHD and other behavioral disorders with sexually dimorphic incidences (Aguiar, Eubig, & Schantz, 2010; Kiguchi et al., 2008). Evidence for BPA-related hyperactivity in animal models is limited and mixed (Supplementary Table 4.2). Effects across age and sex on the brain and behavior are unclear as few studies incorporated multiple ages and both sexes. Minimization or alteration of behavioral sex differences in anxiety and exploratory activity have emerged as a specific area of focus. For example, exploration of novel spaces by adult rodents, including regions designed to be "threatening" and thus anxiety-provoking, is typically higher in females in behavioral estrus relative to females at other phases of the

estrous cycle and males (E. E. Anderson, 1940; Archer, 1975; Diaz-Veliz, Alarcon, Espinoza, Dussaubat, & Mora, 1997; Frye, Petralia, & Rhodes, 2000; Mora, Dussaubat, & Diaz-Veliz, 1996; Patisaul, Blum, Luskin, & Wilson, 2005). Similarly juveniles are typically more active than adults. BPA may minimize or reverse these age and sex-dependent differences (examples in Supplementary Table 4.1).

Aspects of study design and endpoint selection may partially account for inconsistent outcomes in the available literature on the behavioral effects of BPA. For example, BPA does not appear to effectively lactationally transfer in rats due to rapid deconjugation by the dam (Doerge, Vanlandingham, Twaddle, & Delclos, 2010), so studies relying on this exposure route may not have achieved adequate exposure (or the intended internal dose) in the suckling offspring. Importantly, use of a soy-based diet in BPA studies may be a confounder and obfuscate behavioral sex differences because such diets contain hormonally active isoflavones (Patisaul et al., 2005; Patisaul et al., 2012; Thigpen et al., 2013; Thigpen et al., 2007).

The CLARITY-BPA study was designed to incorporate recommended experimental design elements for BPA research (Beronius et al., 2010; Chapin et al., 2008; FAO/WHO, 2011; FDA, 2012; National Toxicology Program, 1982), including use of a low phytoestrogen diet, multiple BPA doses to allow characterization of the dose-response curve when possible, a reference estrogen control group, and well-established behavioral tests including the EPM and ZM (Hogg, 1996; Johnston & File, 1991; Wohr & Scattoni, 2013). Exposure began to the dam during gestation and, following parturition, direct dosing of pups was employed to circumvent the poor lactational transfer of BPA and ensure adequate

perinatal exposure. Male and female rats were developmentally exposed through weaning on postnatal day (PND) 21 to one of three BPA doses (2.5, 25, 2,500 µg/kg bw) and anxiety and activity levels were measured as juveniles or adults using the EPM and the open field (OF). Adults were also assessed using the ZM (Cryan & Sweeney, 2011). Inclusion of a pre-pubertal cohort fills a critical gap in the existing literature regarding the impact of developmental BPA exposure because most published data were obtained from adult animals. Ultimately, gene expression and other neural endpoints will be measured in the rats used here (forthcoming), allowing for correlations to be made between behavioral and neural effects.

Materials and Methods

This study is a component of the CLARITY-BPA program (Birnbaum et al., 2012; Schug et al., 2013). Comprehensive study design details are fully described in Heindel et al. (in preparation) and only applicable portions are summarized here. All procedures involving animals were approved in advance by the NCTR Institutional Animal Care and Use Committee and conducted in an Association for Assessment and Accreditation of Laboratory Animal Care (AALAC)-accredited facility. Throughout the study, animal rooms were maintained at 23 ± 3°C with a relative humidity of 50 ± 20% and a 12:12 hr light/dark cycle. Food and water were available ad libitum. Dams and pre-weaned pups were housed with lights on at 6:00 AM; animals were moved at weaning to a different building with a shifted light cycle (off at 11:00, on at 23:00) to accommodate behavioral testing in the dark phase. The overall experimental design and timeline is depicted in Figure 4.1.

Animal husbandry and dose selection

Housing and diet were selected to minimize unintended exposure to BPA and other endocrine disruptors. The diet was soy- and alfalfa-free to minimize phytoestrogen content (5K96 verified casein diet 10 IF, round pellets, γ-irradiated (catalogue # 1810069), Test Diets, Purina Mills, Richmond, IN). Diet lots and other study materials were monitored for BPA by liquid chromatography/mass spectrometry as described previously (Delclos et al., 2014; Rebuli et al., 2014). No assayed lot of diet contained BPA above the protocol-specified limit of 5 ppb (Heindel et al. (in preparation)), a level that would result in an ingested dose approximately 10-fold below the lowest BPA dose tested. No study materials were found to have BPA detectable above the analytical method blank (data not shown), consistent with data reported previously (Delclos et al., 2014; Rebuli et al., 2014). Each lot of diet was further certified to contain less than 1 ppm genistein and daidzein, and less than 0.5 ppm zearalenone and coumestrol (data not shown).

The exposure groups for the CLARITY-BPA consortium and the parallel guideline-compliant chronic study included a vehicle control group (0.3% aqueous carboxymethylcellulose (CMC)), five BPA doses: 2.5, 25, 250, 2500, and 25,000 μg/kg bw/day, and two ethinyl estradiol (EE) groups: 0.05 and 0.5 μg/kg bw/day. Each of these eight groups was then subdivided into two exposure period groups: a continuously exposed group and a "stop dose" group. For all groups, dosing began on gestational day (GD) 6. Dosing was terminated at weaning (PND 21) for the "stop dose" group. For the present study, a subset of animals was obtained from the "stop dose" groups, and thus exposed from GD 6 until PND 21. Because it was not feasible to behaviorally assess all available animals

in all dose groups, twelve per sex were tested in five groups: vehicle, BPA 2.5, 25, and 2500 μg/kg bw/day and EE 0.5 μg/kg bw/day. Results from previous studies using the same strain and similar dosing paradigms were used to guide dose selection and sample size (Cao et al., 2013; Ferguson et al., 2012, 2011; Ferguson et al., 2014; Rebuli et al., 2014).

Dose preparation and administration

BPA (CAS # 80-05-7, TCI America Lot # 111909/AOHOK [air-milled], >99.9% purity) and EE (CAS # 57-63-6, Sigma Lot # 071M1492V, >99.9% purity) were prepared in the vehicle, 0.3% aqueous CMC (Sigma-Aldrich, St. Louis, MO; catalogue # C5013, Lot # 041M0105V) in water, and administered by gavage daily at a volume of 5 ml/kg bw using a modified Hamilton Microlab® ML511C programmable 115V pump (Hamilton Co., Reno, NV; (Lewis et al., 2010)).

Approximately two weeks prior to mating, female NCTR Sprague-Dawley breeders were randomized to exposure groups stratified by body weight to give approximately equivalent mean body weights in each group. No sibling or first cousin mating was permitted. Rats were mated in five loads or cohorts spaced four weeks apart. Animals for the present study resulted from loads 4 and 5. Mating was conducted as previously described (Delclos et al., 2014), but solid-bottomed polysulfone caging with hardwood chip bedding was used in place of wire bottom cages. Daily gavage dosing for dams was done immediately after body weight collection (dose volume determined by that day´s body weight) from GD 6 and continued until parturition began (neither dams nor pups were dosed on the day of birth (PND 0)). Direct gavage of the pups began on PND 1 after the litter was culled. For pups younger than PND 5, the gavage needle did not enter the esophagus. Pups

were weighed and gavaged daily until PND 21 (weaning). This pre-weaning part of the study was GLP-compliant.

Weaning and transfer of subjects

Offspring were weaned on PND 21, after their last daily gavage, and tattooed on the tail with a unique identification number. Animals used for the present study were then transported to a different building for housing and behavioral testing (termed "behavioral building" in Figure 4.1). The post-weaning housing rooms were held under identical environmental conditions as the pre-weaning housing room described above, except for the light cycle (23:00 – 11:00), in order to accommodate testing in the dark phase. Only pups from litters with at least 9 live pups on PND 0 (for all subjects, there were 9-17 pups/litter in load 4 and 10-17 pups/litter in load 5) and a balanced sex ratio at birth (for all subjects, load 4: min/max males was 4 and 9; min/max females was 4 and 10; load 5: min/max males was 4 and 9; min/max females was 3 and 9; no more than a 4 pup sex difference, except two litters in load 5, which had a 5 pup sex difference, 9 males and 4 females) were used in this study. Juvenile testing began on PND 25, allowing the animals from PND 21 to PND 25 to habituate to the new building. Juvenile and adult test subjects were siblings; that is, one/sex/litter was assessed as juveniles and another one/sex/litter was assessed in adulthood. At weaning, each subject was housed with one or two conspecifics (same-exposure group, same-sex, same-age, non-siblings). Where needed, treatment-naïve "companion" rats were used to provide cagemates for those study subjects that could not be housed with a conspecific (i.e., those in which only one litter of that exposure group was born on that day). No data were collected from these "companion" rats.

Behavioral testing

Rats were assessed either as juveniles on PND 25-27 or at adulthood (Figure 4.1). After behavioral testing was completed, animals were weighed (reported here) and sacrificed for tissue collection (analyses pending). Juveniles were assessed using the elevated plus maze (EPM) and open field (OF). Adults were first assessed for 7 consecutive days using a Barnes Maze (Johnson et al., manuscript in preparation), then on the EPM, OF, and zero maze (ZM). Testing procedures conformed to commonly used standards previously reported and used by us and others (A. A. Braun, Skelton, Vorhees, & Williams, 2011; Cao et al., 2013; Ferguson & Boctor, 2010; Ferguson et al., 2012; Hogg, 1996; Patisaul et al., 2012; Pellow, Chopin, File, & Briley, 1985; Shepherd, Grewal, Fletcher, Bill, & Dourish, 1994). Behavioral testing rooms (each containing only one type of maze) contained a white noise generator (producing ~66 dB; Marpac Dohm, Rocky Point, NC), and multiple apparatuses, half designated for males and half designated for females. All apparatuses were cleaned with 70% ethanol after each testing session. Subjects were pre-assigned to an apparatus such that approximately equal numbers of each exposure group were tested in each. When possible, cagemates were tested on the same day, but if not feasible (e.g., when estrus cycle did not match testing protocol), cagemates were tested in sequential sessions (days between testing of cagemates ranged from 1-8). All assessments commenced after housing room lights were off (approximately 11:00) and were completed within four hours. For testing, all subjects were transported to the nearby test room in their home cages on a rolling cart, and remained on the cart until testing. The hallway between the housing room and test rooms was illuminated with red light. The open field was a beam break assay (PAS-Open Field, San

Diego Instruments, San Diego, CA). All other tests were video recorded from overhead cameras under dim red lighting and analyzed from the video by TopScan software (Clever Sys Inc., Reston, VA).

Juvenile testing spanned PNDs 25-27 to minimize the likelihood that females would be tested after vaginal opening (pubertal onset). For adult behavioral testing, subjects from the two loads (4 and 5) were subdivided into testing intervals for logistical reasons. Both sexes (beginning at PND 77 for half of each load and PND 91 for the remainder) were handled daily to become habituated to human contact. Because behavior varies across the estrous cycle, monitoring and controlling for estrous cycle to the best degree possible is crucial for decreasing biological variability that could result from different estrous cycle phases at the time of the assessment and ensuring testing consistency (E. E. Anderson, 1940; Archer, 1975; Diaz-Veliz et al., 1997; Frye et al., 2000; Mora et al., 1996; Patisaul et al., 2005). Vaginal lavage began on PND 84 (for half of each load) or 98 (remaining animals) and continued daily until sacrifice. Estrous cycle stage was assessed each morning (between 7:30 and 8:00, or 3-3.5 hours before testing) via vaginal lavage. Slides were categorized by two experienced testers blind to treatment. Rats were tested on the EPM and ZM on the day they were categorized to be in proestrus or any stage of estrus (early to late). OF testing was conducted the day after EPM testing, regardless of estrous stage. Thus, the testing sequence for females was: 1) EPM during proestrus or estrus, 2) OF on the subsequent two days, and 3) ZM during proestrus or estrus. The testing sequence for males was 4 consecutive days (i.e., EPM, two days of OF, and ZM). Adult testing spanned 11 days maximally from PND 97 (for half of the subjects) or PND 111 (for the remainder).

All juveniles were weighed and sacrificed on PND 28. On the day after ZM testing, adult males were weighed and sacrificed for tissue collection (tissue analyses pending). To minimize estrous cycle variability at time of sacrifice, adult females continued vaginal lavage after ZM testing until it was predicted that proestrus or any stage of estrus would occur on the subsequent day, at which time they were weighed and sacrificed (tissue analyses pending). Because all of the behavioral tests require that the animals be active and exploratory, body weight was assessed for the narrow purpose of ensuring abnormal weight gain or loss was not a potential confound.

Elevated Plus Maze (EPM). Juveniles (PND 25) and adults, were assessed for anxiety-like behavior during a five min test session using one of four EPMs, as previously described (Ferguson & Berry, 2010). Briefly, each apparatus consisted of four connected black Plexiglas arms, each 10 cm wide and 50 cm long, elevated 50 cm above the floor. Two arms were enclosed within 40 cm walls (closed arms) and two arms had a short (8 mm) ledge around the edge (open arms). Each subject was gently placed on the central area facing the closed arm closest to the room wall, and the home cage and rolling cart were moved outside the test room.

Open Field (OF). Juveniles (PNDs 26-27) and adults were assessed for locomotor activity during two 30 min test sessions using one of eight OF apparatuses as previously described (Ferguson et al., 2012; Zhang et al., 2013). The clear Plexiglas arenas (each 40 x 40 x 40 cm) had a 16 x 16 photo beam detector around the outside floor perimeter for detection of horizontal movements and an elevated photo beam detector to measure vertical activity. The test room was maintained under normal fluorescent lighting. Each subject was

placed into the front corner of the same apparatus on each of two consecutive days. Opaque boards between adjacent apparatuses prevented visual contact.

For each of the two test days, activity was collected in five min intervals, and summed over the entire 30 min testing session (total activity). Behaviors assessed were total distance traveled (cm), average speed (cm/s), resting time (total time with no activity for > 2 s), and time and entries into the center area (defined as the central 20 x 20 cm). An "entry" was defined as consecutive breaking of two beams. PAS-Reporter (San Diego Instruments, San Diego, CA) was used to convert the raw x,y beam break data into the distance, speed, resting, and zone data for statistical analyses.

Zero Maze (ZM). Adults were assessed for anxiety-like behavior during a five min session using one of two ZM apparatuses, as previously described (Patisaul & Bateman, 2008; Patisaul et al., 2012; Shepherd et al., 1994). Each maze consisted of two open arms (9.5 cm wide) and two closed arms (29.5 cm high walls), was 123 cm in diameter and elevated 75.5 cm above the floor. Each of the two open arm areas had a 10 mm ledge around the edge (similar to the EPM). The subject was gently placed onto an open arm facing a closed arm and left undisturbed for 5 min.

Summary of primary endpoints in the behavioral tasks. The strongest indices of anxiety in these tasks are open arm activity in the EPM and ZM (less = heightened anxiety) and center activity in the OF (less = heightened anxiety)(Bailey & Crawley, 2009; Gould, Dao, & Kovacsics, 2009). The most robust indices of activity are closed arm exploration on the EPM and ZM and total distance traveled in the OF (over the full 30 min task). Habituation was assessed by comparing behavior across two successive days in the OF

(activity declines with experience) (Bailey & Crawley, 2009; Gould et al., 2009). Results from all assessed endpoints are presented in the tables and the most commonly assessed, salient endpoints for each testing apparatus depicted graphically.

Data decoding

All behavioral testing was completed and scored blind to exposure group. The blinded raw data were submitted to the National Toxicology Program Chemical Effects in Biological Systems (CEBS) database. It was then independently verified to account for all expected datasets and data points, and "locked" such that data could not be altered. After those events and prior to data analysis, the researchers were provided with the exposure code.

Statistical analysis of body weight and behavioral data

The statistical analyses were designed using published guidelines for low dose EDC studies with sample sizes in this range (Haseman et al., 2001) and to be consistent with prior work (examples in Supplemental Tables 4.1 and 4.2). Main effects and their interactions were examined using analysis of variance (ANOVA). A Fisher's protected Least Significant Difference (LSD) was used as the post-hoc test (when main effects or interactions were identified). While the Fisher's protected LSD does not provide the strong family-wise error control of alternative post-hoc procedures, it was selected over a more conservative approach to minimize risk of type-2 error (rejecting a meaningful effect). Because very few BPA effects (versus vehicle control) were identified, controlling for false-positives was not considered of high concern, as doing so would not impact data interpretation. All statistical analyses were implemented in R (R CoreTeam, 2014) and adults and juveniles were analyzed separately. For all endpoints, the nominal significance level reported is $p \leq 0.05$.

Body weight was analyzed by two-way ANOVA, with exposure and sex as factors. A main effect of sex was observed so all subsequent analyses were made within sex. Significant main effects of exposure were followed up with a protected Fisher's least significant differences (LSD) post-hoc test.

EPM data from two juvenile subjects (one 0.5 EE male and one BPA 2.5 female) and one adult vehicle control female were excluded from analyses because they fell from the apparatus. One adult 0.5 EE male was excluded from the ZM analyses because it was an extreme outlier (greater than twice the number of open arm entries as the next highest data point for that sex and exposure group). This exclusion did not affect the statistical significance of any exposure effects. Unfortunately, four adult females (three 0.5 EE and one BPA 2.5) could not be included in the analysis for the second OF day, because the data collection software was not properly started. Because of the reduced sample size for the adult female EE group on the second OF test day, data from the second OF day were only used to assess the impact of test day on the outcomes. Only data from the first OF day were analyzed in detail, graphed, and included in the figures and tables. For consistency, the juvenile OF data were approached the same way.

For EPM and ZM data sets, ANOVA models assessed effects of sex, exposure, and exposure by sex interactions. Because aspects of EPM and ZM behavior are sexually dimorphic, if a main effect of sex was found for any endpoint on that maze, all subsequent analyses were made within sex. Confirmation of known sex differences was considered to be an indication that the test was robust, powered sufficiently, and properly conducted. Significant main effects were followed up with a Fisher's protected LSD post-hoc test. As

commonly seen with a sample size of 12/sex/exposure group, achieving normality in all residual distributions within a given endpoint ANOVA model was rare (Cohen, Cohen, Stephen, & Aiken, 2002). Because violations of this assumption tend to produce false positives and there were no consistent treatment-associated effects (see Results), we did not differentially perform non-parametric tests in cases where deviation from normality may have been present. Rather, we applied a consistent modeling approach to all endpoints across each maze type (Cohen et al., 2002).

OF data sets were analyzed in two ways: the endpoint summed over the entire 30 min session and in 5 min intervals (i.e., a separate ANOVA for each 5 min interval). In both cases, for all endpoints a three-way ANOVA was conducted to test for main effects of sex, exposure, and test day (across the two days), and their interactions. Analysis of day 1 data (summed over the session and in 5 min intervals) was then conducted using two-way ANOVAs with sex and exposure as factors. Breaking the 30 min session down into 5 min intervals allows exploratory behavior to be assessed at different points across the session as behavior changes with experience (Bailey & Crawley, 2009; Goma & Tobena, 1978; Gould et al., 2009). The first 5 min of the test are thought to give the most informative general measures of anxiety (because novelty is highest). As the test progresses, activity declines as the animal becomes familiar with the arena; thus, differences in overall activity or center area behavior during the final intervals could be reflective of anxiety and/or exploratory behavior. Activity towards the end of the 30 min task is thought to reach a steady state so behavior in the final 5 min interval is considered to be the best indicator of general (not driven by novelty stress) locomotion (Gould et al., 2009). Significant main effects and interactions were

followed up with a Fisher's protected LSD post-hoc test. All tables (including Supplementary Tables 4.3 and 4.4) report p-values for the F-test associated with each endpoint across all factors tested.

We also performed a power analysis for a range of treatment effect sizes to evaluate possible risk of a type-2 error (rejecting the null hypothesis when an effect is present). To parameterize these calculations, we used the experimental data (treatment group-wise means and variances) from the adult male EPM measure of time spent in the open arms (see Results). The power calculations were implemented using the G*Power software (Faul, Erdfelder, Buchner, & Lang, 2009), then plotted using R.

Pairwise correlations between anxiety-related endpoints were conducted to assess data concordance across the OF, EPM, and ZM for the adult testing. The results (points colored by treatment and shaped by sex) are presented in Supplemental Figure 4.1, with the overall linear correlation between each pairwise outcome presented in the upper half of the figures and plotted in the lower half. The axis units are counts for "Entry" outcomes and seconds for "Time" outcomes.

Results

Terminal body weight

As expected, a significant effect of sex on body weight was observed at both ages ($p \leq 0.001$ for both ages; data not shown) with juvenile and adult males weighing more than same-age females.

Juveniles

In the EPM (Figure 4.2), no significant main effect of exposure group was found for any endpoint. Main effects of sex were identified for 4 of 11 endpoints, females spent less time in the central area ($p \leq 0.015$), exhibited more stretch attend postures ($p \leq 0.001$), had a shorter latency to enter the open arms ($p \leq 0.034$), and traveled more distance in the closed arms ($p \leq 0.005$). No significant interaction of sex and exposure was found for any endpoint.

OF data were first analyzed by assessing total behavior over the entire 30 min session of the first testing day (Figure 4.3). No significant main effects of exposure were found for any of the 4 endpoints. By contrast, significant main effects of sex were identified for all endpoints: females traveled less ($p \leq 0.006$), rested more ($p \leq 0.009$), made fewer center entries ($p \leq 0.014$), and spent less time in the center ($p \leq 0.011$). No significant interaction of sex and exposure was identified for any endpoint. Additionally, there was no significant effect of test day on any endpoint.

To obtain greater detail about possible impacts on behavior within the 30 min session, OF data from the first testing day were independently analyzed in five min intervals (Supplemental Table 4.3). Only the first day was analyzed to be consistent with the approach used for the adults (reported below), and because there were no significant effects of test day. Briefly, main effects of sex were found in 11 of 24 interval analyses but no significant interaction of sex and exposure was identified. Main effects of exposure were identified in only 3 of 24 interval analyses (Supplementary Table 4.3). In the second 5 min interval of the first day, the 2.5 and 25 BPA rats spent more time resting than the vehicle controls ($p \leq 0.003$ and $p \leq 0.001$, respectively). In the fourth interval, the BPA 25 group made fewer center

entries ($p \leq 0.005$) and spent less time in the center ($p \leq 0.003$) than vehicle controls. Significant main effects of test day were detected in only 2 of 24 instances.

Adults

In the EPM, significant main effects of exposure group were found for 5 of 11 endpoints, but post-hoc testing did not indicate that any BPA group was significantly different from the vehicle group. Instead, BPA and/or vehicle groups were significantly different from the EE group (Figure 4.4). EE and BPA 25 rats spent less time on the closed arms than BPA 2500 rats ($p \leq 0.001$ and $p \leq 0.006$, respectively). EE animals spent significantly more time on the open arms than the vehicle control, BPA 2.5, and BPA 2500 groups ($p \leq 0.05$, $p \leq 0.05$, and $p \leq 0.01$, respectively). The vehicle control and BPA 2.5 groups traveled more distance in the closed arms than the EE group ($p \leq 0.006$ and $p \leq 0.037$, respectively). EE and BPA 2500 groups traveled less distance in the center than the BPA 25 group ($p \leq 0.004$ and $p \leq 0.008$, respectively). The EE group entered the closed arms fewer times than the BPA 25 group ($p \leq 0.008$). Main effects of sex were identified in 6 of 11 endpoints, females spent more time in the open arms ($p \leq 0.001$), less time in the center ($p \leq 0.001$), traveled more distance (closed arms ($p \leq 0.001$), open arms ($p \leq 0.001$), and overall ($p \leq 0.001$)), and made more open arm entries ($p \leq 0.007$); effects consistent with known sex differences in rat EPM performance. No significant interactions of sex and exposure were identified for any endpoint.

No significant main effects of exposure were found for any OF endpoint when endpoints (day 1 only) were summed over the entire 30 min session (Figure 4.5). Main effects of sex were found for all endpoints, females traveled less distance overall ($p \leq 0.001$),

spent more time resting ($p \leq 0.009$), made fewer center entries ($p \leq 0.003$)), and spent less time in the center area ($p \leq 0.001$). No significant interaction of sex and exposure was found for any of those four day 1 endpoints. A significant main effect of test day was also identified for every overall endpoint; demonstrating that all groups habituated to the task. On the first test day, rats (regardless of sex or exposure group) traveled further ($p \leq 0.001$), rested less ($p \leq 0.001$), made more center entries ($p \leq 0.001$), and spent more time in the center area ($p \leq 0.001$).

Data from day 1 were then analyzed using separate ANOVAs for each 5 min interval. No significant main effects of exposure were identified in any interval (Supplementary Table 4.4). Main effects of sex were found in 18 of 24 interval analyses (shown in Supplementary Table 4.4) confirming that OF behavior was sexually dimorphic. No significant interactions of sex and exposure were found. Comparing behavior in each interval across days 1 and 2, significant main effects of test day were found in 17 of 24 interval analyses (shown in Supplementary Table 4.4) confirming across-session habituation regardless of sex or exposure.

In the ZM (Figure 4.6), no significant main effect of exposure was found for any endpoint. A main effect of sex was identified for 1 of the 7 endpoints and indicated that females performed fewer stretch attends ($p \leq 0.041$). No significant interactions between exposure and sex were identified for any ZM endpoint.

Estimating observed effect size of BPA and detection power

A subset of the experimental data was used to estimate the observed effect size of treatment and the associated detection power. The effect size, f, was defined as: $f = \sigma m / \sigma$,

where σm is the standard deviation of the group means and σ is the standard deviation within each group. Figure 4.7 shows the estimated power (the probability of rejecting a null hypothesis given that it is truly false) for a range of effect sizes. The effect size, f, was solved for plotting as η2, which is interpreted here as "proportion of variance explained by exposure group." Thus, the range of effect sizes plotted in Figure 4.7 represents effect sizes, η2, of 1% (f = 0.1) to 50% (f = 1). The data used to generate these curves were based upon observed data from the time in the open arms for adult males (vehicle and BPA 2500 groups) in the EPM. This data set was chosen for this analysis because main effects of exposure and sex were found for some of the endpoints on the EPM, including time in the open arms, and the variability was reasonably consistent across all exposure groups (regardless of sex). For this behavioral measure, our effect size was estimated as f = 0.37, which corresponds to an estimated power of 58% using 60 total animals (12 rats per each of 5 groups). Under this effect size (considered "moderate"), 95 total animals (19 rats per each of 5 groups) would be required to achieve 80% power. Note that these estimates do not account for any expected "ordering" of the treatment groups. If notions of non-monotonicity in complex behavioral responses were discarded, then alternative models might achieve slightly higher power estimates—given that other assumptions were held constant.

Correlation between outcome measures

Linear correlation patterns between anxiety-endpoints were explored for the three adult testing arenas. As expected, high empirical correlations (r) were found between related outcome measures within each apparatus (e.g., measures of speed, time, and number of entries into a specific area or arm). This is reflected in the groupings of significant results for

individual outcomes reported in all tables. Lower than expected correlations were found between related measures across the different testing arenas (Supplementary Figure 4.1), with the majority of across-assessment correlations $r < 0.15$. For example, concordance between time in the open ZM and EPM arms was a reasonable $r < 0.38$, but number of entries into the respective arms was poorly correlated at $r < 0.066$. Low concordance was observed in all exposure groups (depicted in Supplementary Figure 4.1) so the overall effect was not impacted by exposure group. The lack of robust across-apparatus correlations reinforces the weak or absent exposure-related effects observed in adults: if the sex or exposure-related effects observed in the EPM were robust, those effects should have been recapitulated in the ZM, which is specifically designed to evaluate similar phenomena.

Discussion

No systematic effects of BPA were observed on any endpoint assessed in adults and juveniles of both sexes, although there was nominal evidence of anxiety-related exploratory behavioral changes in the 2.5 and 25 μg/kg BPA exposed juveniles in the OF interval analysis. Overall evidence for BPA-related effects was minimal, weak, and inconsistent in both age groups and thus not interpreted to be indicative of a biologically meaningful effect on either anxiety or activity. Expected sex differences in EPM performance (summarized in (Simpson, Ryan, Curley, Mulcaire, & Kelly, 2012)) were statistically significant or suggested for most endpoints, and adult OF activity indicated a robust effect of test day (a classical indication of habituation), demonstrating that the design was sufficient to detect these large and well-established differences. Sex differences on the ZM, however, were not observed. Although it may be parsimonious to conclude from these results that perinatal exposure to

BPA doses below the NOAEL does not appreciably alter anxiety-related behavior and exploratory activity, confidence in such a conclusion is weakened by several factors. Low concordance between related behavioral tasks, particularly between the ZM and EPM, raises concerns regarding inter-maze reliability. Additionally, female adults were less active in the OF; the reverse of what is has historically been reported in rats (Gould et al., 2009). However, minimal to no sex differences in OF activity is within the normal range for NCTR Sprague Dawley rats (e.g. (Ferguson & Berry, 2010; Ferguson & Boctor, 2010)). Finally, power estimates of one of the most robust endpoint in the battery indicated that the effect size for BPA was smaller than predicted suggesting greater numbers would be needed to reject the null hypothesis with confidence. For all endpoints, inter-individual variation obscured potential exposure effects, but this variation is not out of the historical range for this strain in prior studies conducted at NCTR.

In the juveniles, no evidence of BPA-related effects was observed on EPM performance, but some sex differences were identified. Sex differences in the EPM are typically robust in adulthood (females in behavioral estrus are more active and explore the open arms more than males) but not in juveniles, thus the reported observations are consistent with that developmental trajectory. Similarly, no effects of BPA were detected on any OF endpoint when data from the 30 minute sessions were assessed as a whole, although when the 5 minute intervals were analyzed separately, the fourth interval (minutes 15-20 of the session) on day 1 identified effects suggestive of heightened anxiety in the BPA 25 group about midway through the task. Mid-trial activity changes are more difficult to interpret than those at the start or end of the task (Bailey & Crawley, 2009; Gould et al., 2009), and the

single, sporadic instance of altered center activity is likely due to chance and not indicative of a meaningful impact of BPA on anxiety or activity. Elevated overall time resting (in the BPA 2.5 and 25 groups; Supplementary Table 4.3) could potentially be interpreted as suggestive of heightened anxiety and reduced activity, but this spurious effect did not reach statistical significance even with a liberal statistical approach that does not robustly account for multiple comparisons. EE had sporadic effects on some measures, but overall outcomes were not predictive of BPA effects.

Interestingly, there was no effect of test day on OF behavior in juveniles, indicating no across-session habituation. Juveniles have a propensity to explore and are generally more active than adults so this result is not entirely inconsistent with typical rodent behavior. Comparable lack of across session habituation effects have been described in juvenile rats aged PND 40 or younger (Bronstein, 1972; Livesey & Egger, 1970). Similarly, OF data obtained from a prior study using a similar experimental design and BPA exposure, the same rat strain (tested over PNDs 40-42), and using the same OF apparatus and data collection software (Ferguson et al., 2012) also found no effect of sex or a difference between the first two sessions (activity declined by the third test day, however). Exposed (2.5 and 25 µg/kg bw BPA and 5 and 10 µg/kg bw EE) males displayed greater total activity indicating hyperactivity (Ferguson et al., 2012). Across the available literature (data published around and after the 2011 report jointly published by WHO and FAO (FAO/WHO, 2011) and up through December, 2014 are summarized in Supplemental Tables 4.1 and 4.2) BPA-related outcomes on juvenile anxiety and activity vary. While there is little consistent information regarding activity/exploration, the data are generally indicative of heightened anxiety.

Adult behavioral assessments indicated main effects of exposure for several EPM endpoints (Figure 4.4). Posthoc comparisons, however, revealed that these effects were attributable to differences between the BPA and EE groups (i.e., there were no significant differences between the BPA group and the vehicle control and very few between the EE group and the vehicle control). We have previously reported differences between BPA and EE exposures (Patisaul et al., 2012) and the present results reinforce our prior conclusion that BPA does not act strictly like an "estrogen" on the brain and behavior. While appropriate as a reference estrogen when exploring potential mechanisms of BPA, EE may not necessarily be an appropriate "positive control" for BPA effects on non-reproductive behavior. Adult rat EPM and ZM exploration is typically sexually dimorphic, with females in behavioral estrus more active and exhibiting increased exploration of "high anxiety" areas (i.e., open arms of the EPM and ZM) than females in other estrous phases or males. Although expected sex differences were detected in the EPM (most importantly, for open arm entries and time on the open arms), no significant sex differences in open arm behavior were detected in the ZM (Figure 4.6); thus, BPA-related impacts on sexually dimorphic behavior could not be ascertained on the ZM. Some prior studies have also not found sex differences on ZM behavior (A. A. Braun et al., 2011; Vorhees, Johnson, Burns, & Williams, 2009). Additionally, although OF sex differences on locomotor activity were the opposite of what is sometimes reported for rats (Gould et al., 2009), they are fully in line with the historical record of this strain assessed in this lab.

Of potential concern, exposure-related effects observed in the EPM at adulthood were not observed in the ZM, and concordance between some EPM and ZM endpoints was

markedly low, suggesting that performance on one task was neither predictive nor reflective of performance on the other. Although the EPM remains the "gold standard" for assessing anxiety-related behaviors, to overcome well-characterized limitations (including the opportunity for animals to perseverate in the center), other, related tests such as ZM were developed (A. A. Braun et al., 2011; Haller & Alicki, 2012). Diagnostic behaviors, including open arm activity, are expected to be highly concordant across mazes (at a level where their correlation (r) reaches statistical significance) and thus equivalently predictive of behavioral states. Percentage of time spent in the open arms (Supplementary Figure 4.2) was consistently higher in the ZM than the EPM for both male and female adults in all exposure groups. This effect may indicate that the ZM is a less aversive test than the EPM. For example, A. A. Braun et al. (2011) described increased open arm exploration in the ZM relative to the EPM in adult male Sprague-Dawley rats. Additionally, a logical consequence of the uninterruptable exploration time afforded by the ZM layout (which lacks the problematic EPM center region where animals can spend upwards of 20-30% of their time) means that, compared to the EPM, animals spend more overall time in either the open or closed arms of the ZM (Shepherd et al., 1994).

Sequential testing may have contributed to low concordance between EPM and ZM data, as anxiety and exploration may decrease with experience and task novelty. Essentially, conducting a battery of tests may have reduced the overall, general "fear" of being subjected to a behavioral task, particularly one designed to capture similar behaviors. While repeated exposures to the same task are well documented to reduce the diagnostic utility of that task (Armario & Nadal, 2013; Bailey & Crawley, 2009; McIlwain, Merriweather, Yuva-Paylor, &

Paylor, 2001; Paylor, Spencer, Yuva-Paylor, & Pieke-Dahl, 2006), it is unclear if prior experience to a similar task is comparably confounding. The adult rats in the present study were assessed for Barnes Maze performance (Johnson et al., manuscript in preparation) conducted under normal fluorescent lighting before initiating the tasks described herein. Test order was carefully considered and within historical norms for investigating endpoints relevant to locomotor and anxiety activity, without losing individual assay novelty. Some studies indicate that previous exposure to a novel environment increases subsequent activity and open arm entries in the EPM; however, this effect is not consistent across studies (Pellow et al., 1985; Walf & Frye, 2007; Weiss, Wadsworth, Fletcher, & Dourish, 1998).

The presence of ledges on the open arms of the EPM and ZM may have also impacted behavior. Using the existing EPM apparatus at NCTR was considered advantageous because those have previously been used with rats of this strain (Ferguson & Berry, 2010) and consistency between the current CLARITY-BPA study and prior NCTR studies was considered desirable. Replicability is high (in the range of $r = 0.8$ or greater) across years in the same lab with the same equipment, but markedly lower if equipment or other aspects of the lab environment change (Wahlsten, Bachmanov, Finn, & Crabbe, 2006). Thus, the two ZM apparatus constructed for this study were designed with ledges equivalent to those on the EPMs. Ledges, while reducing inadvertent falls, provide a degree of "security" and thus reduce anxiety associated with open arm exploration and, consequently, a depreciated ability to reveal responses to anxiolytics including benzodiazepines and chlordiazepoxide (Fernandes & File, 1996; Hogg, 1996). EPM ledges also decrease the utility of closed arm behavior as an overall measure of activity and negates head dipping as an indicator of anxiety

(Fernandes & File, 1996). Inclusion of ledges typically makes the task less aversive, which may have blunted diagnostic utility (Hogg, 1996). The addition of ledges also hampers the ability to directly compare the present results to those where ledges were not present. Previous studies of BPA, outlined in Supplementary Table 4.1, indicate that 4 of the 13 studies describing statistically significant effects of BPA used ledges on open arms (Gioiosa, Parmigiani, Vom Saal, & Palanza, 2013; Luo, Wei, Niu, Wang, & Wang, 2013; Tian, Baek, Lee, & Jang, 2010; Yu et al., 2011). One study using ledges did not find significant effects of BPA (Viberg et al., 2011). For the present studies, the overall impact of ledges cannot be fully determined but may be one of several factors which may have contributed to a lack of significant findings.

Other aspects of study design may have also contributed to data variability and low inter-arena concordance. Estrous cycle stage at the time of testing was monitored as much as was feasible to minimize estrous cycle effects. Adult females were tested in the EPM and ZM while in proestrus or early to late estrus (as established by vaginal lavage), so it is possible that some were not in behavioral estrus at the time of testing. An additional potentially influential factor is use of gavage. It is well known that perinatal stress can remodel the stress axis; amplifying risk of abnormal stress-responsivity, including heightened anxiety, and depressive-like behavior in adulthood (Markham & Koenig, 2011; Russo, Murrough, Han, Charney, & Nestler, 2012). In a related study, we showed that prenatal gavage alters estrogen receptor (ER) expression in neonatal brain regions fundamental to stress and fear-learning, anxiety, and activity (Cao et al., 2013). Gavage effects eclipsed those of BPA, raising concerns that gavage itself may interact with BPA exposure to induce

molecular, cellular, neural and behavioral changes; However, studies of siblings of those subjects, found no differences between gavaged and naïve controls (same strain, same housing facility) on preweaning behavior, OF activity, Barnes maze and water maze performance, novelty preference, motor coordination, adolescent play, running wheel activity, flavored solution intake, female sex behavior, manually elicited lordosis or circulating corticosterone levels measured at weaning or adulthood (Ferguson et al., 2012, 2011; Ferguson et al., 2014).

Because the data were primarily negative for exposure effect, power calculations based on the available data were conducted to assess the potential for Type II error (false negatives). These analyses detected a moderate power deficit arising primarily from the high inter-individual variability observed on these behavioral measures. Although the reliability of the experiments is high, in terms of measures within subjects and across test days, the inter-individual variability may have obscured a potentially real exposure-related signal. Given this, we estimated that a sufficiently powered study using these specific outcomes and exposure groups would minimally require a sample size of 19/sex/exposure group or 7 additional subjects/sex/exposure group than the current number assessed here, and considerably more for behaviors with smaller effect sizes. While sample sizes were within range of or exceeded historical norms for this lab and others, lower than anticipated power may at least partially account for why the present results contrast with prior work reporting evidence that early life BPA exposure can heighten anxiety and activity in these and related behavioral tests (summarized in Supplemental Table 4.1). Sex differences were detected in some, but not all, anticipated cases, demonstrating that, for most endpoints, the design was

robust enough to pick up influential factors with larger effect sizes than BPA. Importantly, power calculations are specific to the experiments herein, and are no way indicative of probable power levels for other unrelated endpoints in the CLARITY-BPA program, particularly those for which effect size is anticipated to be greater and inter-individual variability is anticipated to be lower. Post-hoc power analyses have well-characterized limitations (Levine & Ensom, 2001; Wagenmakers et al., 2014) and the vast majority of prior studies, conducted at NCTR and elsewhere, assessing the behavioral impacts of EDCs have used equivalent or smaller sample sizes (see Supplemental Tables 4.1 and 4.2 and also (FAO/WHO, 2011; Ferguson & Berry, 2010; Ferguson et al., 2011; Ferguson et al., 2014; Patisaul et al., 2005; Rebuli et al., 2014)).

Conclusions

The present studies represent a portion of the data obtained under the CLARITY-BPA program (Birnbaum et al., 2012; Schug et al., 2013). The potential for BPA exposure to affect behavioral effects was assessed in male and female juvenile and adult NCTR Sprague-Dawley rats that were siblings of animals used in an ongoing two year chronic study. Three BPA exposure levels were assessed concurrently with a negative (vehicle) control and a reference estrogen (EE) control. No compelling evidence of BPA-related effects on anxiety or exploratory behavior was found in the adults and only spurious evidence was found for heightened anxiety and activity in the juveniles. Although it is perhaps tempting to conclude that perinatal exposure to BPA levels below the NOAEL has little to no impact on affective behaviors, limitations of this study include lower than anticipated statistical power and maze concordance, and potential stress-related effects of gavage (pre- and post-natal). Subsequent

studies from the CLARITY-BPA project as well as others will provide further resolution on the potential effects of BPA by providing data on other neuro-related endpoints both at the behavior and molecular level and a wealth of other organ systems and outcomes.

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Figures

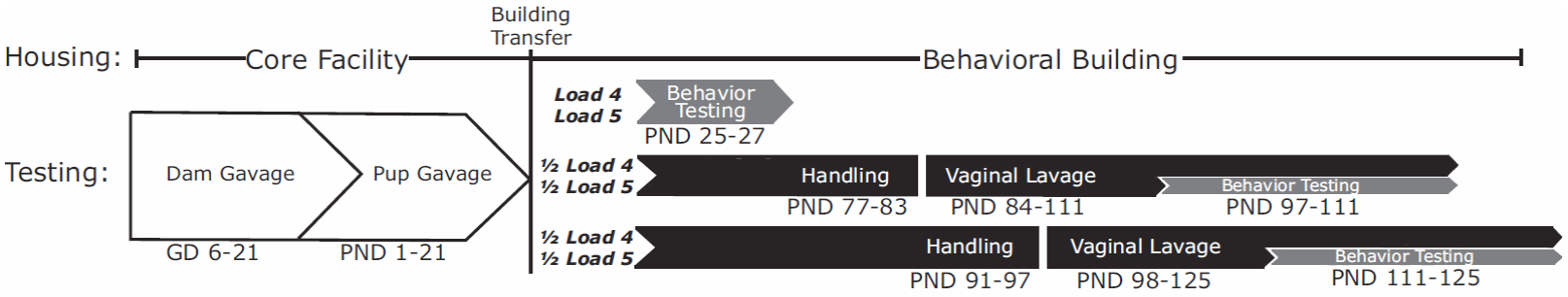


Figure 4.1: Methods Timeline. Visual depiction of the experimental methods timeline including dosing and housing. Dam and pup gavages occurred in the core animal facility (white arrows), and the experimental animals were transferred to a different animal facility on PND 21 and acclimated to the new facility from PND 21 to 25 for subsequent testing. Juveniles (gray arrow) were tested prior to puberty. Adults were tested in two groups (black arrows) and the time of behavioral testing for each group is indicated.

A

Juvenile Elevated Plus Maze ANOVA p-values

Endpoint	Effect of Exposure	Effect of Sex		Group by Sex Interaction
Time Spent - Closed Arms	0.509	0.381	F=M	0.362
Time Spent - Open Arms	0.282	0.728	F=M	0.713
Time Spent - Center	0.763	**0.015**	F<M	0.618
Stretch Attends	0.232	**0.001**	F>M	0.288
Latency to Enter Open Arms	0.777	**0.034**	F<M	0.485
Distance Traveled - Closed Arms	0.324	**0.005**	F>M	0.518
Distance Traveled - Open Arms	0.120	0.562	F=M	0.905
Distance Traveled - Center	0.552	0.175	F=M	0.922
Overall Distance Traveled	0.352	0.059	F=M	0.863
Open Arm Entries	0.336	0.507	F=M	0.820
Closed Arm Entries	0.948	0.127	F=M	0.507

F=M, no statistically significant sex difference

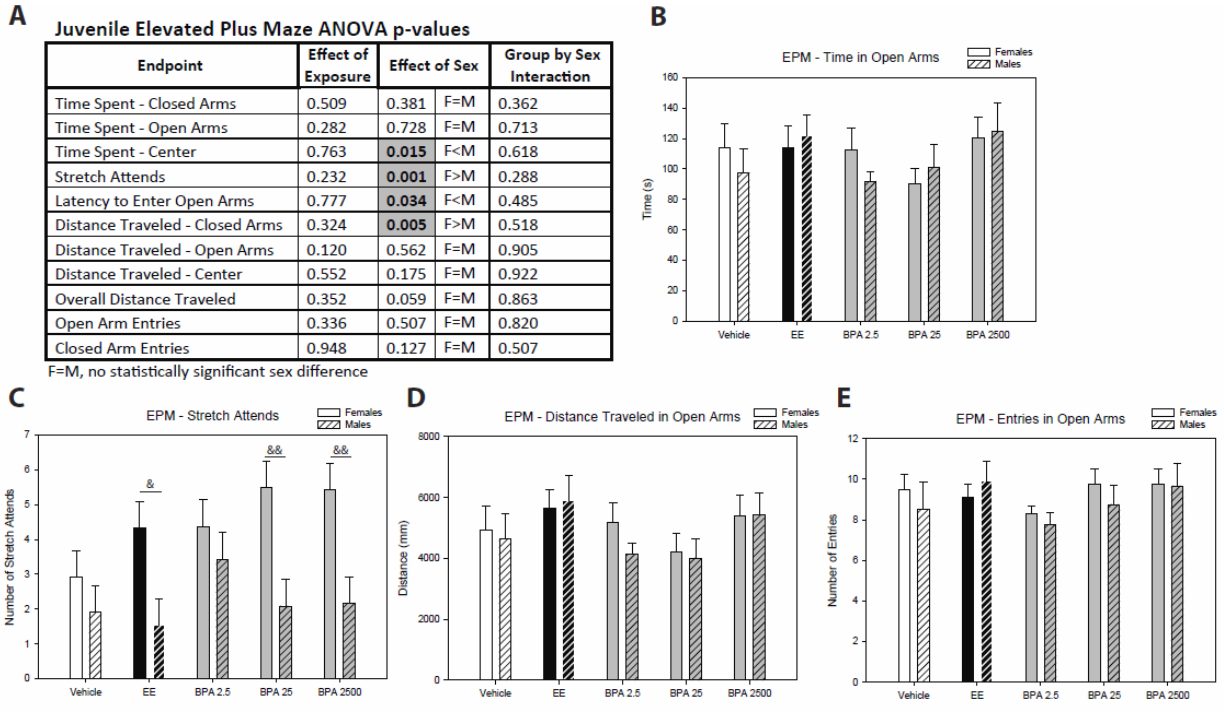


Figure 4.2: Juvenile EPM. (A) ANOVA p-values for main effects of exposure, sex, and their interaction for each endpoint. Significant effects are bolded and highlighted in gray and the direction of significant sex differences is indicated (M = male; F = female). (B) Time in the open arms did not differ by sex or across exposure groups. (C) Number of stretch attends was sexually dimorphic, with females performing more stretch attends than males. This sex difference was not statistically significant in the vehicle controls or the BPA 2.5 groups. No effects of EE or BPA were observed versus vehicle control. Distance traveled on the open arms (D) and number of open arm entries (E) were not impacted by sex or exposure. Graphs depict mean ± SEM. For all graphs, females are depicted in open bars and males in striped bars. Sex differences within exposure group are indicated by & $p \leq 0.05$ and && $p \leq 0.01$.

A

Juvenile Open Field ANOVA p-values

Endpoint	Effect of Exposure	Effect of Sex		Group by Sex Interaction	Effect of Test Day	
Overall Distance Traveled	0.264	**0.006**	F<M	0.906	0.661	1=2
Overall Time Resting	0.071	**0.009**	F>M	0.980	0.516	1=2
Total Entries – Center	0.373	**0.014**	F<M	0.981	0.897	1=2
Overall Time – Center	0.373	**0.011**	F<M	0.990	0.401	1=2

1=2, no statistically significant difference

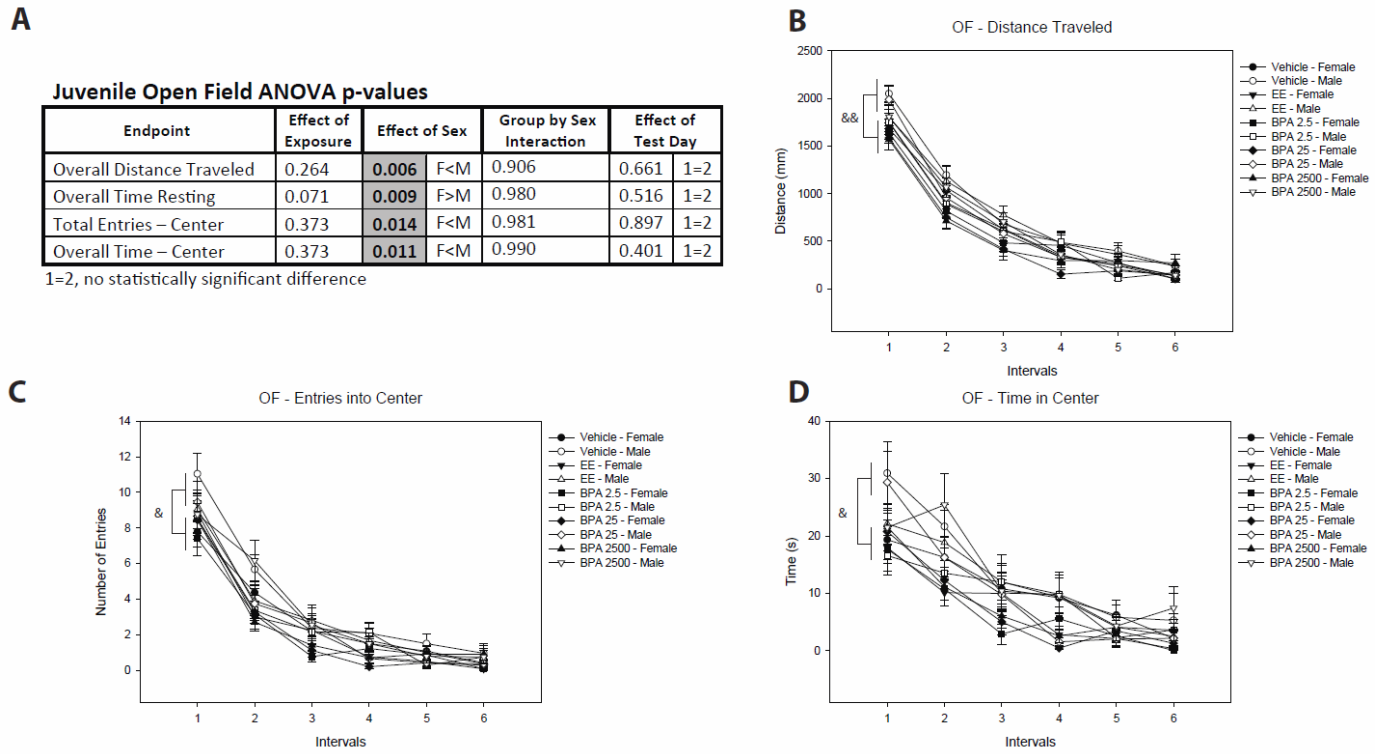


Figure 4.3: Juvenile OF. (A) ANOVA p-values for main effects of exposure, sex, their interaction, and test day for each endpoint. Significant effects are bolded and highlighted in gray. Sex and test day differences are indicated (M = male; F = female; 1 = first test day; 2 = second test day). Distance traveled (B), entries into the center (C), and time in the center (D) differed by sex, but did not differ across exposure groups. No effects of EE or BPA were observed versus vehicle control. Graphs depict mean ± SEM. For all graphs, females are represented by open shapes and males by black, filled shapes. Each interval was 5 min; all graphs show results from the first day of testing (data from the second day are not shown). Main effect of sex denoted by & $p \leq 0.05$ and && $p \leq 0.01$.

A

Adult Elevated Plus Maze ANOVA p-values

Endpoint	Effect of Exposure	Effect of Sex		Group by Sex Interaction	Group Differences
Time Spent - Closed Arms	**0.005**	0.351	F=M	0.393	EE < BPA 2500*** BPA 25 < BPA 2500**
Time Spent - Open Arms	**0.025**	**0.001**	F>M	0.380	Vehicle < EE* BPA 2.5 < EE* BPA 2500 < EE**
Time Spent - Center	0.508	**0.001**	F<M	0.365	
Stretch Attends	0.302	0.267	F=M	0.908	
Latency to Enter Open Arms	0.544	0.061	F=M	0.406	
Distance Traveled - Closed Arms	**0.029**	**0.001**	F>M	0.583	Vehicle > EE ** BPA 2.5 > EE*
Distance Traveled - Open Arms	0.271	**0.001**	F>M	0.658	
Distance Traveled - Center	**0.011**	0.445	F=M	0.956	BPA 25 > EE** BPA 25 > BPA 2500**
Overall Distance Traveled	0.132	**0.001**	F>M	0.568	
Open Arm Entries	0.319	**0.007**	F>M	0.850	
Closed Arm Entries	**0.031**	0.184	F=M	0.954	BPA 25 > EE**

*=$p \leq 0.05$, **=$p \leq 0.01$, ***=$p \leq 0.001$; F=M, no statistically significant sex difference

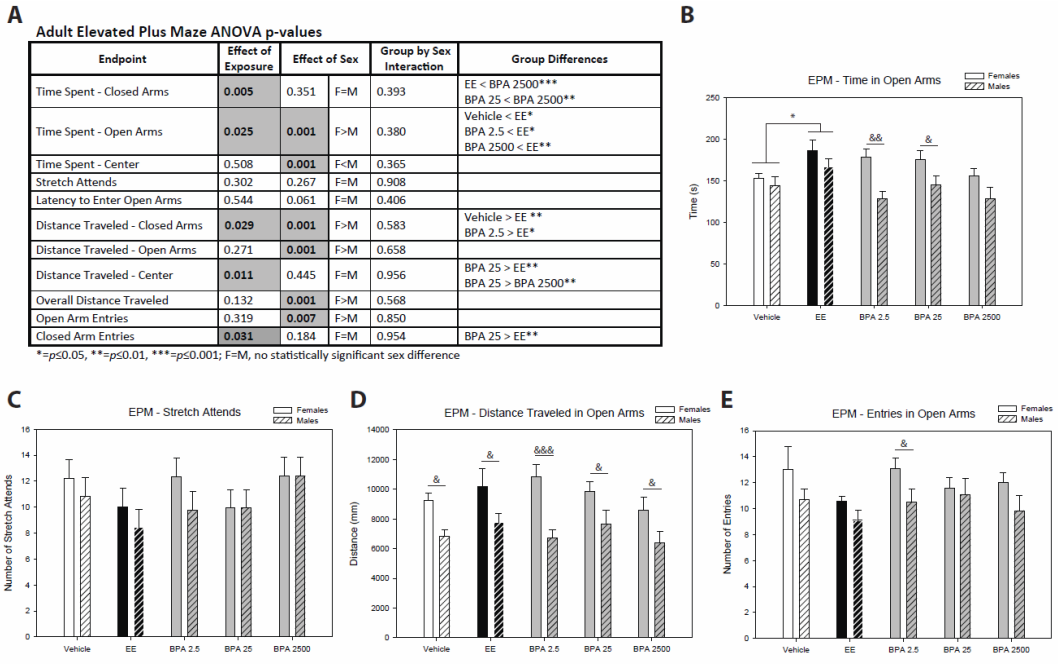


Figure 4.4: Adult EPM. (A) ANOVA p-values for main effects of exposure, sex, and their interaction for each endpoint. Significant effects are bolded and highlighted in gray. Sex and group differences are indicated (M = male; F = female). (B) Time in the open arms differed by exposure and sex. The EE group had a significantly longer time on the open arms than the vehicle group. BPA 2.5 and 2500 groups differed significantly from the EE group, but not the vehicle controls. Sex differences were identified in the BPA 2.5 and 25 groups, but not in the vehicle controls. (C) Number of stretch attends was not impacted by sex or exposure. (D) Distance traveled in the open arms was sexually dimorphic with females traveling farther. (E) Open arm entries were not impacted by exposure but were sexually dimorphic. This sex difference was only statistically significant in the BPA 2.5 group. Graphs depict mean ± SEM. For all graphs, females are depicted in open bars and males in striped bars. Group differences compared to the vehicle control group are indicated with *$p \leq 0.05$. Sex differences within exposure group are indicated by & $p \leq 0.05$; && $p \leq 0.01$; and &&& $p \leq 0.001$.

A

Adult Open Field ANOVA p-values

Endpoint	Effect of Exposure	Effect of Sex		Group by Sex Interaction	Effect of Test Day	
Overall Distance Traveled	0.227	**0.001**	F<M	0.793	**0.001**	1>2
Overall Time Resting	0.315	**0.009**	F>M	0.669	**0.001**	1<2
Total Entries – Center	0.300	**0.003**	F<M	0.813	**0.001**	1>2
Overall Time – Center	0.262	**0.001**	F<M	0.898	**0.001**	1>2

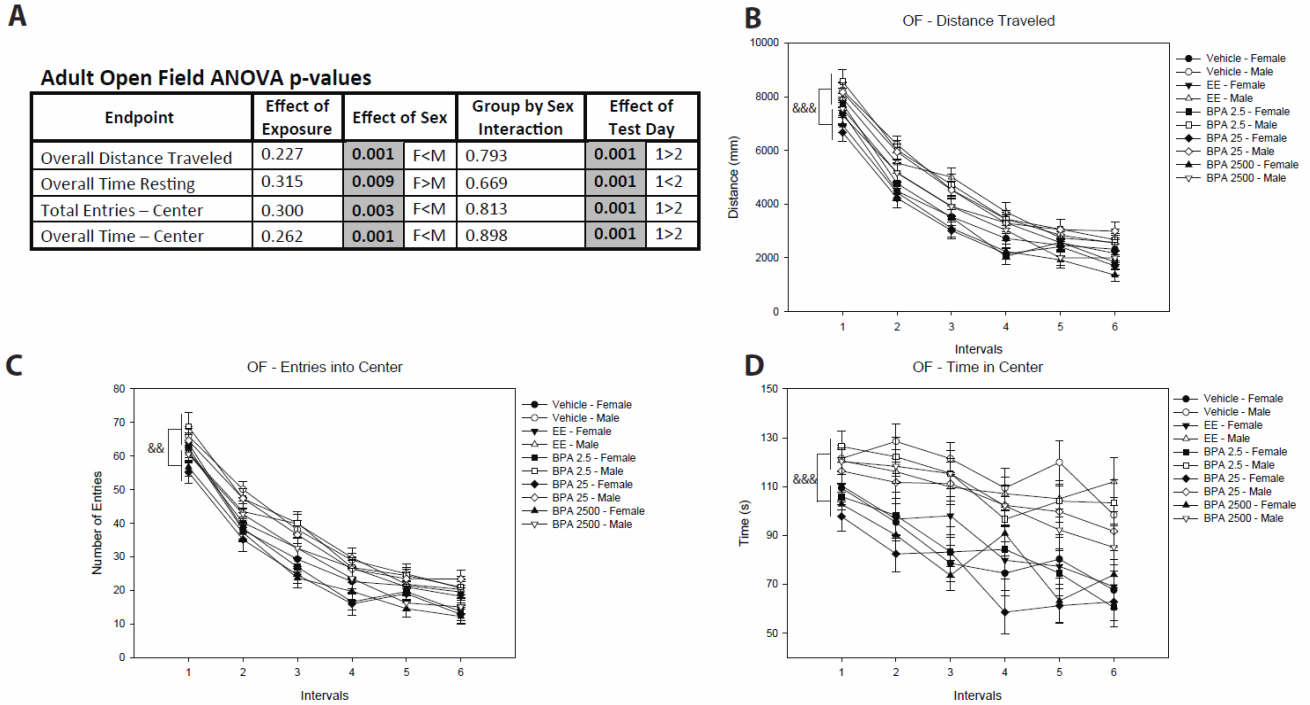


Figure 4.5: Adult OF. (A) ANOVA p-values for main effects of exposure, sex, their interaction, and test day for each endpoint. Significant effects are bolded and highlighted in gray. Sex and test day differences are indicated (M = male; F = female; 1 = first test day; 2 = second test day). Distance traveled (B), entries into the center (C), and time in the center (D) differed by sex, but did not differ across exposure groups. No effects of EE or BPA were observed. Graphs depict mean ± SEM. For all graphs, females are represented by open shapes and males by black, filled shapes. Each interval was 5 min; all graphs show results from the first day of testing (data from the second day are not shown). Main effect of sex denoted by && $p \leq 0.01$ and &&& $p \leq 0.001$.

A

Adult Zero Maze ANOVA p-values

Endpoint	Effect of Exposure	Effect of Sex		Group by Sex Interaction
Time Spent - Closed Arms	0.629	0.074	F=M	0.101
Time Spent - Open Arms	0.636	0.077	F=M	0.098
Stretch Attends	0.451	**0.041**	F<M	0.077
Latency to Enter Open Arm	0.495	0.582	F=M	0.837
Distance Traveled - Closed Arm	0.650	0.630	F=M	0.708
Distance Traveled - Open Arms	0.965	0.673	F=M	0.839
Overall Distance Traveled	0.708	0.602	F=M	0.908

F=M, no statistically significant sex difference

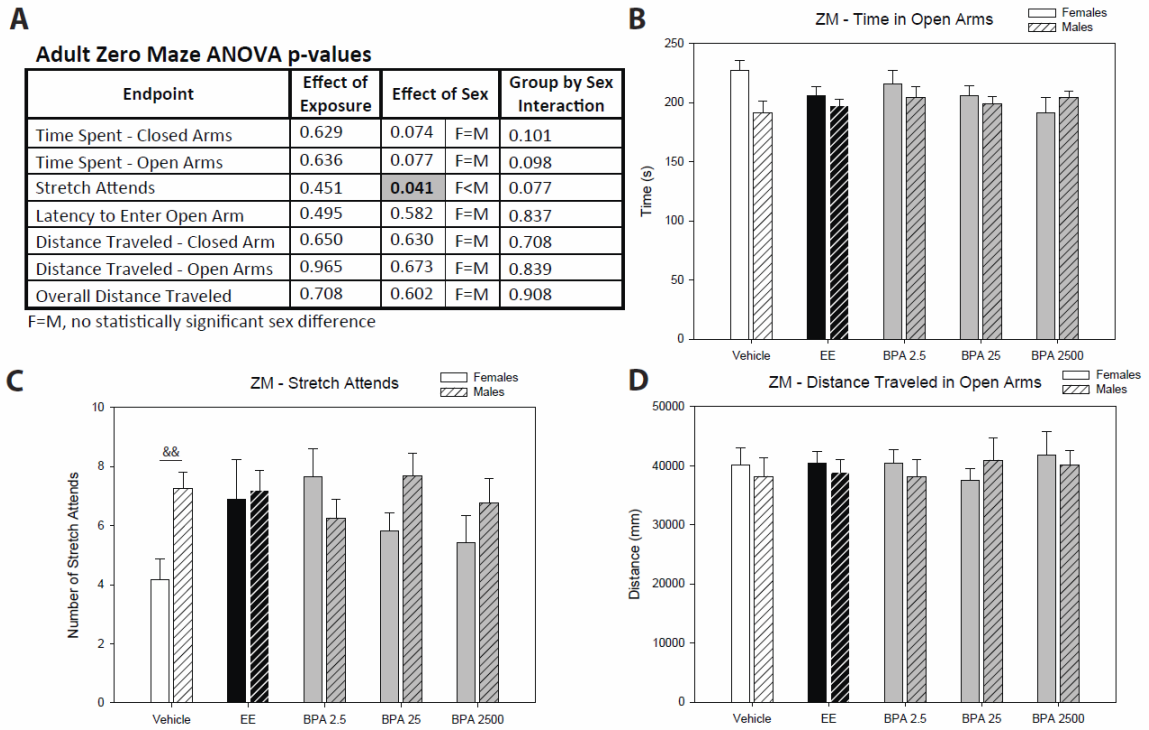


Figure 4.6: Adult ZM. (A) ANOVA p-values for main effects of exposure, sex, and their interaction for each endpoint. Significant effects are bolded and highlighted in gray. Sex and group differences are indicated (M = male; F = female). (B) Time in the open arms was not impacted by sex or exposure group. (C) Number of stretch attends was sexually dimorphic with females performing fewer stretch attends. No effects of EE or BPA were identified. (D) Distance traveled in the open arms was not impacted by sex or exposure group. Graphs depict mean ± SEM. For all graphs, females are depicted in open bars and males in striped bars. Sex differences within exposure group are indicated by && $p \leq 0.01$.

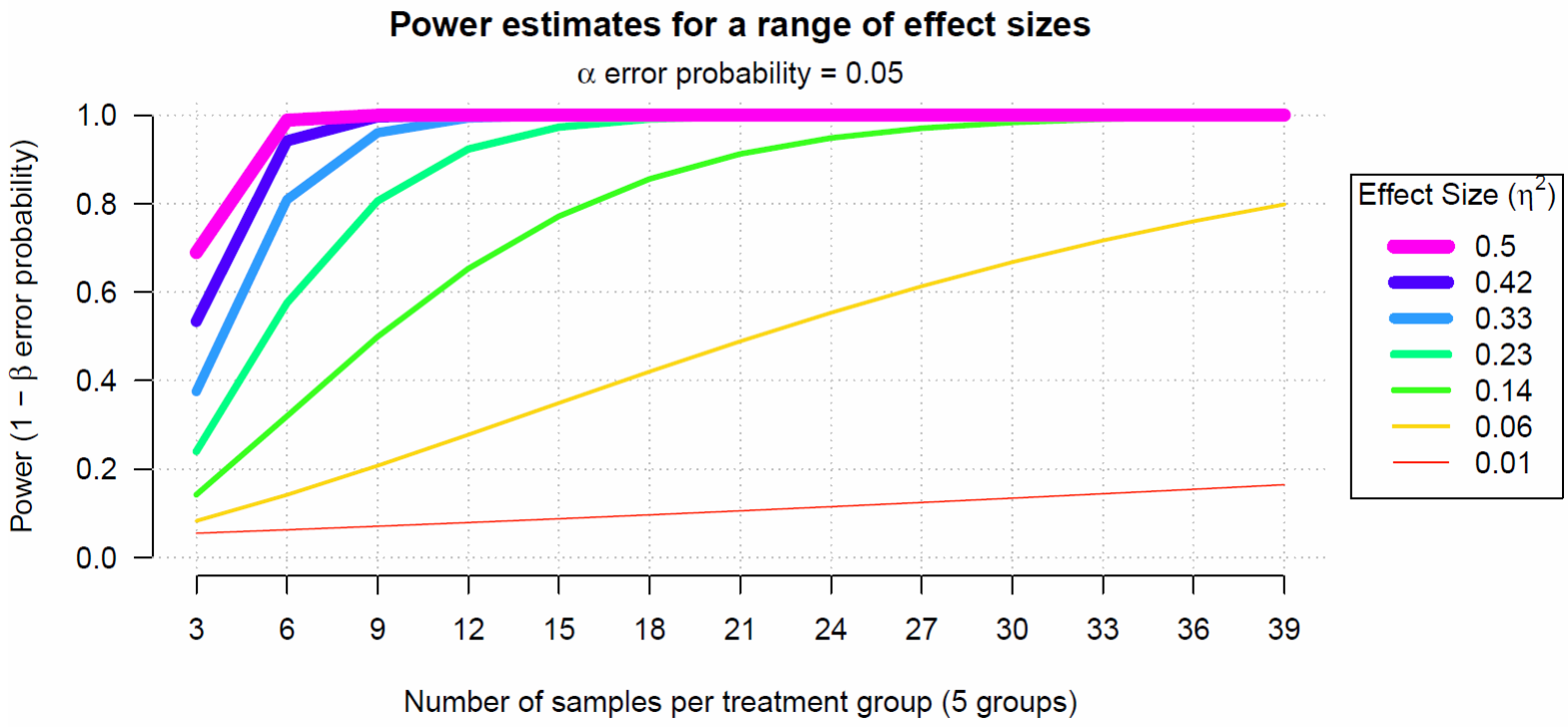


Figure 4.7: Theoretical power analysis. The estimated sample size (horizontal axis) required to achieve a given level of power (vertical axis) for various effect sizes (legend at right) via a fixed effects ANOVA model with Type I error probability $\alpha = 0.05$. For the present studies, effect size of male open arm behavior on the EPM was estimated to be approximately 0.4, which corresponds to an estimated power of 60% using 60 total animals (12 rats in 5 exposure groups). By these calculations group size in the present study (n = 12 per sex per group) was not sufficient to achieve the ideal power range of 80% or higher.

Supplementary Tables

Supplementary Table 4.1: Animal studies examining the relationship between BPA exposure and anxiety-related behaviors

Authors, Date	Animal Species, Strain	Sex	Exposure Route, Vehicle	Time of Exposure	BPA Dose(s), µg/kg BW/day	Endpoints Measured (Behavior Type)	Age at Testing	Results (indicates dose group with significant response)
(Cox, Gatewood, Howeth, & Rissman, 2010)	Mice, C57BL/6J	Both	Oral, diet (to the dam)	One week prior to breeding to parturition	50 mg/kg feed weight (~ 8,000 µg/kg bw)	EPM EPM	PND 20 Adult (age not specified)	Juvenile Males: Higher anxiety in distal ends of open arms. Adult Males: Higher anxiety in distal ends of open arms.
(Gioiosa et al., 2013)	Mice, CD-1	Both	Oral (to the dam for gestational exposure or to the pups for postnatal exposure)	GD11- birth (one group) or PND 0-8 (second group)	10	Novelty Preference OF EPM	PND 28-30 PND 70 PND 70	Females: Aversion to novelty Both sexes: Less inclined to explore. Females: More time in closed arms
(Goncalves, Cunha, Barros, & Martinez, 2010)	Rat, Wistar	Both	Oral, corn oil (to the dam)	GD 0 – birth PND 0 – PND 21 GD 0 – PND 21	40	OF	~PND 112	Both sexes: GD 0 – PND 21 and PND 0 - 21 groups decreased exploration
(Jasarevic et al., 2011)	Deer Mice	Both	Oral, diet (to the dam)	Two weeks prior to breeding to PND 21	50 mg BPA/kg feed weight (~ 5000 µg/kg bw)	EPM	~PND 74	Males: Spent less time in open arms and more time in closed arms.

Supplementary Table 4.1 continued

(Jasarevic et al., 2013)	Deer Mice	Both	Oral, diet (to the dam)	Two weeks prior to breeding to PND 21	50µg, 5mg, or 50 mg BPA/kg feed weight (~ 5, 500, 5000 µg/kg bw)	EPM	~PND 74	Males: Two higher dose groups spent less time in open arms and more time in closed arms.
(Jones & Watson, 2012)	Rat, Long Evans	Both	Oral, corn oil (to the dam)	GD 7 - PND 14	5, 50, 500 and 5,000	EPM Forced Swim Test	PND 90 – 150	Females: More fecal boli (500µg/kg bw). Females: More open arm entries (5µg/kg bw). Males: More general activity (5µg/kg bw). All sex differences, except defecation, eliminated in 5µg/kg bw. → Demasculinization of male anxiety-like behaviors. No effect.
(Kundakovic et al., 2013)	Mice, BALB/c	Both	Oral, corn oil (to the dam)	GD 0 - 19	2, 20 and 200	OF	PND 60	Females: Angiogenic Males: Anxiolytic Loss of sex differences

Supplementary Table 4.1 continued

(Luo et al., 2013)	Mice, CD1	Male	Oral, diet (to the test animal)	PND 35 - PND 70	50 mg/kg feed weight	EPM Light/Dark Box	Unspecified Unspecified	Males: Fewer open arm entries and less time on open arms Males: Fewer light side entries and longer latency to enter light side
(Luo et al., 2014)	Mice, CD-1	Female	Oral, diet (to the dam)	Two weeks prior to breeding to PND 21	50 mg/kg feed weight	OF ZM	PND 22 PND 24	Females: less time moving in center, fewer entries into center, shorter distance traveled in the center Females: Less time in open arms, fewer entries into open arms, traveled shorter distance in open arm.
(Matsuda et al., 2013)	Mice, C57BL/6J	Both	Injection (to the dam)	GD 10 – PND 20	0.25 (250ng)	Contextual fear conditioning	4 weeks 9 weeks	Females: higher freezing percentages in females BPA enhanced fear memory in juvenile females
(Matsuda et al., 2012)	Mice, C57BL/6J	Both	Injection (to the dam)	GD 10 – PND 20	0.25 (250ng)	OF	PND 28 and 56	Males: juveniles and adults spent less time in center.

Supplementary Table 4.1 continued

(Nakamura et al., 2012)	Mice, ICR/Jcl	Both	Injection, sesame oil (to the dam)	GD 0- PND 21	20	OF EPM	PND 21 PND 70 PND 24 PND 77	Females: More time in center Both sexes: Decreased total distance traveled Both sexes: Decreased total distance traveled Females: Decreased total distance traveled
(Panagiotidou, Zerva, Mitsiou, Alexis, & Kitraki, 2014)	Rats, Wistar	Both	Oral, cornflakes (to the dam)	GD 0 – PND 21	40	Forced Swim	PND 46	Females: increased duration of time needed to escape, less time immobile
(Patisaul & Bateman, 2008)	Rats, Long Evans	Male	Injection, sesame oil (to the test animal)	PND 0- PND 3	50	EPM	PND 56-61	Reduced entries and time spent in open arms. Increased time spent in closed arms.
(Patisaul et al., 2012)	Rats, Wistar	Male	Oral, drinking water (to the dam and to the pups)	GD 6 – PND 40	1mg/L drinking water (~300 µg/kg bw to the dams and ~ 500 µg/kg bw to pups)	Light/Dark Box EPM EPM	PND 24-28 PND 24-28 PND 60-70	Reduced exploration of light side (Data collapsed across sex) Reduced open arm activity (Data collapsed across sex). Greater latency to enter an open arm. Sex differences eliminated Demasculinization of male anxiety-like behaviors.

Supplementary Table 4.1 continued

(Tian et al., 2010)	Mice, ICR	Both	Oral, 1% DMSO (to dam or pups unspecified)	GD 7 - PND 21 and PND 22-36	100 and 500	OF EPM	Adult (age not specified)	Higher distance traveled in center (100µg/kg bw). Sex differences not accounted for. More time in open arms (500µg/kg bw). Sex differences not accounted for.
(Viberg et al., 2011)	Mice, NMRI	Male	Oral, egg lecithin and peanut oil (1:1) (to the test animal)	PND 10	320, 3,200, and 4,800	EPM	PND 90	No effect.
(Williams et al., 2013)	California Mice	Both	Oral, diet (to the dam)	Two weeks prior to breeding to PND 30	50 mg BPA/kg feed weight (~5000 µg/kg bw)	EPM	~PND 90	Males: No effect. Females: Reduced time in open arms. Loss of sex differences.
(Wolstenholme, Taylor, et al., 2011)	Mice, C57BL/6J	Both	Oral, diet (to the dam)	One week prior to breeding to GD 21	1.25 mg BPA/kg feed weight (~ 170 µg/kg bw)	EPM	PND 22	No effect in either sex.
(Wolstenholme et al., 2013)	Mice, C57BL/6J	Both	Oral, diet	7 to 10 days prior to breeding-GD 21 (birth) -birth	5 mg BPA/kg feed weight (~670 µg/kg bw)	Juvenile OF	PND 23-24	No effect on anxiety in either sex.

Supplementary Table 4.1 continued

(X. Xu, Tian, Hong, Chen, & Xie, 2011)	Mice, ICR	Both	Oral, (to the test animal)	PND 32-87	40, 400	OF EPM	PND 91 PND 92	Sex difference in rearing and grooming abolished in both BPA groups Males: Less open arm entries and less time spent in open arms Females: More open arm entries and more time spent in open arms Reversed sex difference
(X. Xu, Liu, et al., 2013)	Mice, ICR	Both	Oral, (to the test animal)	10-22 weeks of age	400, 4000, 40000	OF	~PND 160	Males: More rearings and time in central area.
(Yu et al., 2011)	Mice, C57BL/6J	Both	Injection, sesame oil (to the test animal)	PND 23 - 30	50	OF EPM Novel Cage Test	PND 60-70 PND60-70 PND 60-70	Males: More time in the center Females: Less time in the center Males: No effect Females: Less time in the open arm Males: Lower frequency of digging Females: No effect

Supplementary Table 4.2: Animal studies examining the relationship between BPA exposure and spontaneous motor activity

Authors, Date	**Animal Species, Strain**	**Sex**	**Exposure Route, Vehicle**	**Time of Exposure**	**BPA Dose(s), µg/kg BW/day**	**Endpoints Measured (Behavior Type)**	**Age at Testing**	**Results (indicates dose group with significant response)**
(O. S. Anderson et al., 2013)	Mice *a/a* C57Bl6J (derived from *a/a* X A^{vy}/a breedings)	Both	Oral, diet, (to the dam)	Two weeks prior to breeding to PND 22	50 ng, 50 µg or 50 mg/kg feed weight (~ 10 ng, 10 µg, 10 mg/kg bw)	Spontaneous Activity	3, 6 and 9 months	Females: Overall BPA exposure increased hyperactivity
(Ferguson et al., 2012)	Rats, Sprague Dawley	Both	Oral, carboxymethylcellulose (to the dam)	GD 6 - PND 21	2.5 and 25	OF	PND 40-42	Males: significantly more active
(Ishido, Masuo, Terasaki, & Morita, 2011)	Rats, Wistar	Male	Intracisternal injection	PND 5	20 µg (a priori)	Spontaneous Activity	4-5 weeks	Hyperactivity

Supplementary Table 4.2 continued

(Kiguchi et al., 2008)	Rats, Wistar	Male	Intracisternal, ethanol and olive oil	PND 5	20 and 40	Motor Activity Rearing, sniffing, and grooming behavior. Motor activity, rearing, sniffing, and grooming after methylphenidate administration.	PND 28	Higher motor activity during light phase (40 µg/kg bw). Higher counts of rearing, sniffing and grooming (40 µg/kg bw). Exposure did not affect response to methylphenidate.
(Kundakovic et al., 2013)	Mice, BALB/c	Both	Oral, corn oil (to the dam)	GD 0 - 19	2, 20 and 200	OF	PND 60	Males: Hyperactivity Females: Hypoactivity
(Kuwahara, Kawaguchi, Kohara, Cui, & Yamashita, 2013)	Rats, Sprague Dawley	Male	Oral, corn oil (to the dam)	GD 10 – PND 14	50 and 500	OF	7 weeks	Males: no effect on maze activity.
(Matsuda et al., 2012)	Mice, C57BL/6J	Both	Subcutaneous injection, phosphate buffered saline (to the dam)	GD 10 - PND 20	250 ng/kg bw	OF	4 weeks and 8 weeks	No effect on total distance traveled
(Ryan et al., 2010)	Rats, Long Evans	Female	Oral, corn oil (to the dam)	GD 7- PND 18	2, 20, and 200	Figure-8 Maze	Adult- not specified	Females: No effect on maze activity.
(Stump et al., 2010)	Rats, Sprague-Dawley	Both	Oral, diet	GD 0 - PND 21	10, 100, 5000, 50,000, and 150,000	Motor Activity	PND 13, 17, 21, and 61	No overall effect or in either sex.

Supplementary Table 4.2 continued

(van Esterik et al., 2014)	Mice, C57BL/6J X FVB	Both	Oral, diet (to the dam)	Two weeks prior to breeding to PND 21	3000	Spontaneous Activity	19-21 weeks	Males: Hypoactivity Females: trend to hyperactivity (couldn't statistically compare to just one control)
(Viberg et al., 2011)	Mice, NMRI	Male	Oral	PND 10	320, 3,200, and 4,800	Spontaneous Activity	PND 60 and 150	Increased locomotion, rearing, and total activity (3,200 and 4,800 µg/kg bw).
(C. Wang et al., 2014)	Rats, Sprague Dawley	Male	Oral, corn oil (to the dam)	GD 9-20	50, 500, 5,000, and 50,000	OF	PND 21	Lower motor activity (500 and 5,000 µg/kg bw) Lower rearing frequency (50, 500, 5,000, and 50,000 µg/kg bw) Lower grooming frequency (50, 500, and 50,000 µg/kg bw)
(Wolstenholme et al., 2013)	Mice, C57BL/6J	Both	Oral, diet	7 to 10 days prior to breeding-GD 21 (birth) -birth	5 mg BPA/kg feed weight (~670 µg/kg bw)	Juvenile OF	PND 23-24	Both sexes: Elevated activity in the F3 generation but not F1 or F2 generations.

Supplementary Table 4.3: Juvenile open field ANOVA p-values

Juvenile Open Field ANOVA p-values

Endpoint	Effect of Exposure	Effect of Sex		Group by Sex Interaction	Group Differences	Effect of Test Day	
Distance Traveled – Interval 1	0.807	**0.024**	F<M	0.772		**0.001**	1<2
Distance Traveled – Interval 2	0.162	**0.012**	F<M	0.860		0.103	1=2
Distance Traveled – Interval 3	0.252	**0.014**	F<M	0.808		0.429	1=2
Distance Traveled – Interval 4	0.222	0.090	F=M	0.957		0.258	1=2
Distance Traveled – Interval 5	0.436	0.854	F=M	0.321		0.972	1=2
Distance Traveled – Interval 6	0.824	0.416	F=M	0.273		0.382	1=2
Overall Distance Traveled	0.264	**0.006**	F<M	0.906		0.661	1=2
Time Resting – Interval 1	0.725	0.179	F=M	0.850		0.082	1=2
Time Resting – Interval 2	**0.033**	**0.014**	F>M	0.914	Vehicle < BPA 2.5** Vehicle < BPA 25***	**0.016**	1<2
Time Resting – Interval 3	0.152	**0.027**	F>M	0.869		0.147	1=2
Time Resting – Interval 4	0.283	**0.024**	F>M	0.978		0.284	1=2
Time Resting – Interval 5	0.183	0.666	F=M	0.351		0.923	1=2
Time Resting – Interval 6	0.816	0.263	F=M	0.409		0.363	1=2
Overall Time Resting	0.071	**0.009**	F>M	0.980		0.516	1=2
Entries – Center – Interval 1	0.876	0.171	F=M	0.791		0.223	1=2
Entries – Center – Interval 2	0.153	**0.015**	F<M	0.301		0.126	1=2
Entries – Center – Interval 3	0.670	**0.036**	F<M	0.857		0.517	1=2
Entries – Center – Interval 4	**0.048**	**0.020**	F<M	0.986	Vehicle > BPA 25**	0.243	1=2
Entries – Center – Interval 5	0.235	0.858	F=M	0.678		0.672	1=2
Entries – Center – Interval 6	0.432	0.237	F=M	0.795		0.831	1=2
Total Entries – Center	0.373	**0.014**	F<M	0.981		0.897	1=2
Time in Center – Interval 1	0.342	0.106	F=M	0.559		0.360	1=2
Time in Center – Interval 2	0.445	**0.009**	F<M	0.658		0.891	1=2
Time in Center – Interval 3	0.776	0.083	F=M	0.709		0.863	1=2
Time in Center – Interval 4	**0.047**	**0.034**	F<M	0.703	Vehicle > BPA 25** BPA 25 < EE*	0.183	1=2
Time in Center – Interval 5	0.304	0.999	F=M	0.927		0.531	1=2
Time in Center – Interval 6	0.394	0.079	F=M	0.972		0.873	1=2
Total Time – Center	0.373	**0.011**	F<M	0.990		0.401	1=2

*=$p \leq 0.05$, **=$p \leq 0.01$, ***=$p \leq 0.001$; F=M and 1=2, no statistically significant difference

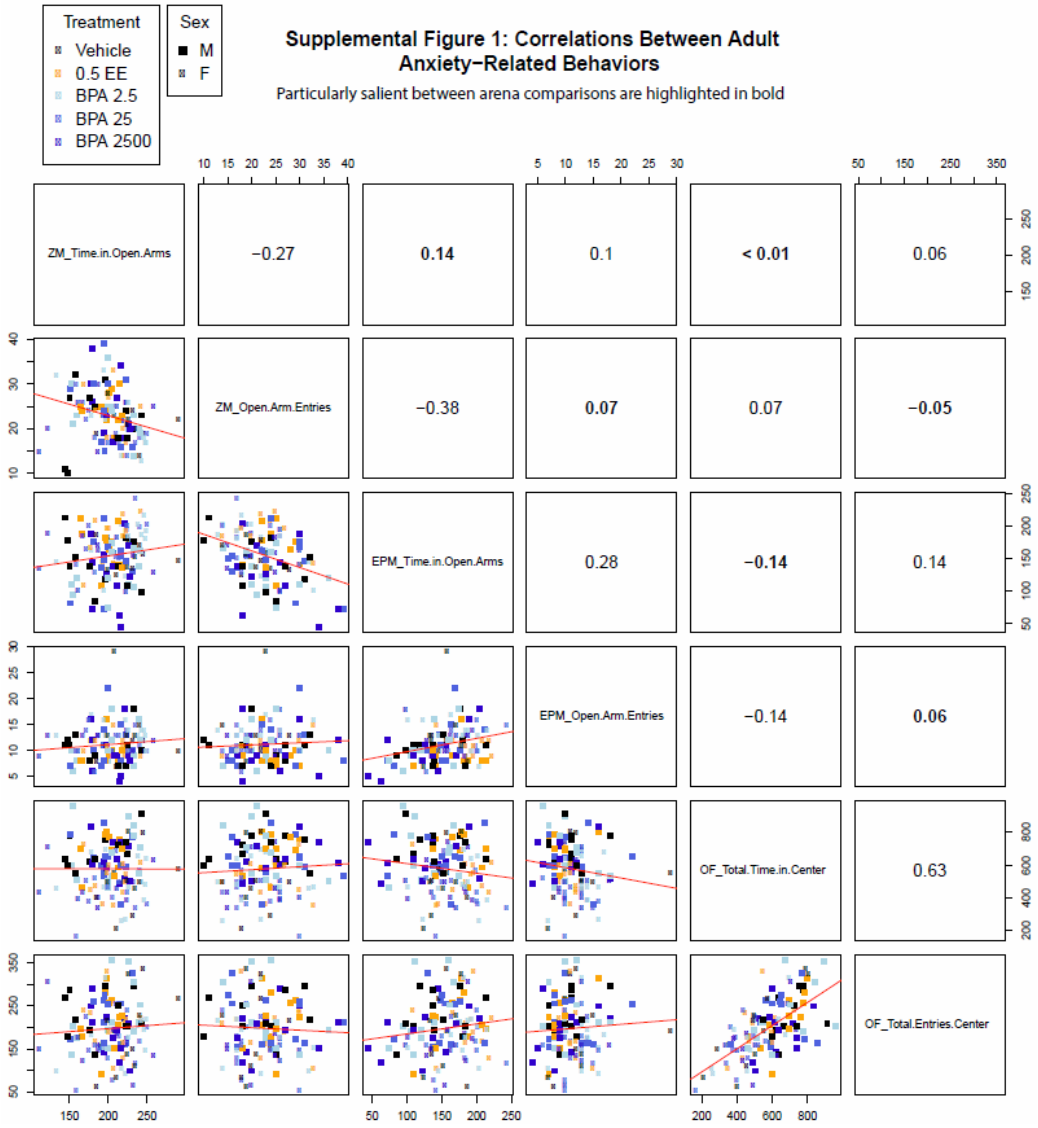
Supplementary Table 4.4: Adult open field ANOVA p-values

Adult Open Field ANOVA p-values

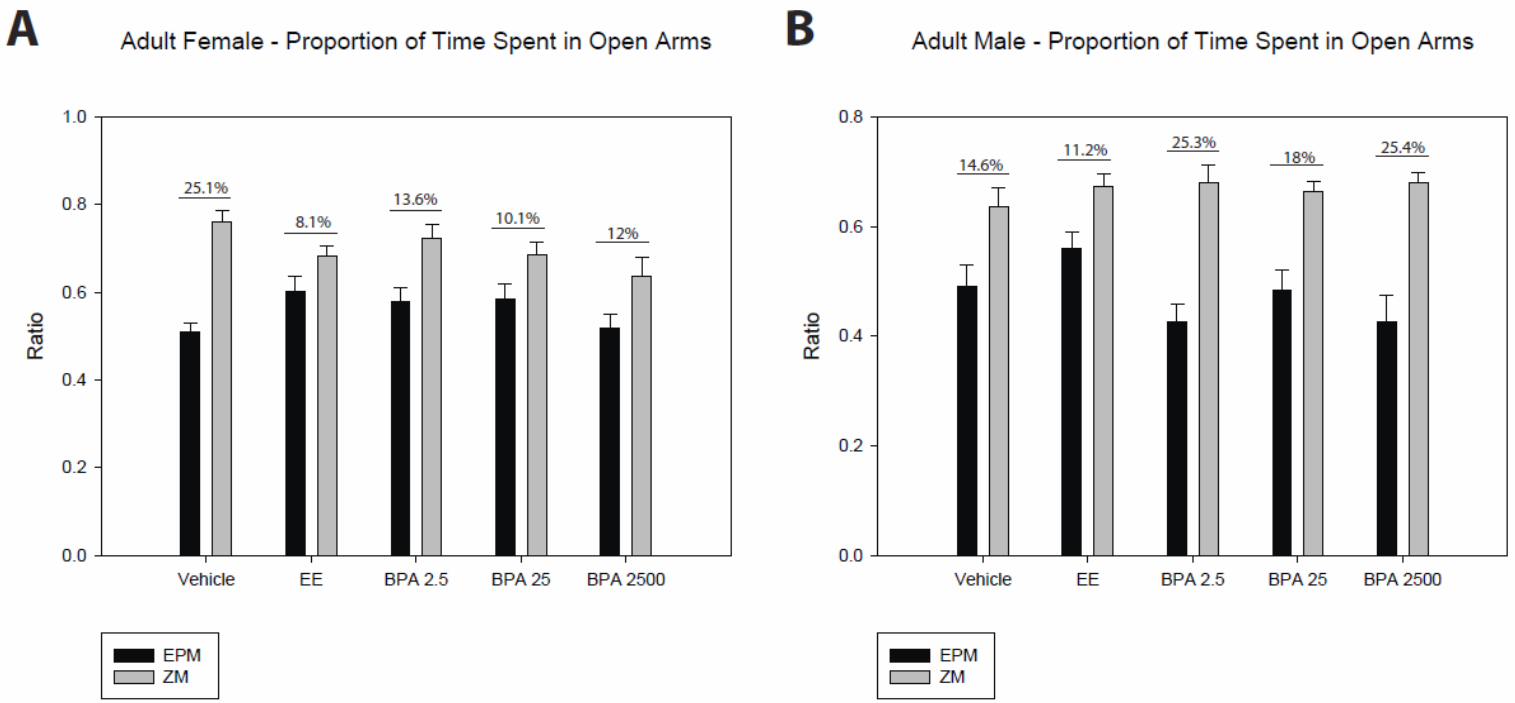
Endpoint	Exposure Group	Effect of Sex		Group by Sex Interaction	Effect of Test Day	
Distance Traveled – Interval 1	0.266	**0.004**	F<M	0.847	**0.013**	1>2
Distance Traveled – Interval 2	0.436	**0.001**	F<M	0.320	**0.001**	1>2
Distance Traveled – Interval 3	0.123	**0.001**	F<M	0.937	**0.001**	1>2
Distance Traveled – Interval 4	0.209	**0.001**	F<M	0.801	**0.009**	1>2
Distance Traveled – Interval 5	0.186	0.150	F=M	0.973	**0.003**	1>2
Distance Traveled – Interval 6	0.290	**0.008**	F<M	0.694	0.083	1=2
Overall Distance Traveled	0.227	**0.001**	F<M	0.793	**0.001**	1>2
Time Resting – Interval 1	0.564	0.098	F=M	0.928	0.052	1=2
Time Resting – Interval 2	0.842	**0.010**	F>M	0.194	**0.001**	1<2
Time Resting – Interval 3	0.131	**0.003**	F>M	0.852	**0.001**	1<2
Time Resting – Interval 4	0.235	**0.007**	F>M	0.503	**0.028**	1<2
Time Resting – Interval 5	0.207	0.373	F=M	0.924	0.077	1=2
Time Resting – Interval 6	0.522	0.053	F=M	0.858	0.198	1=2
Overall Time Resting	0.315	**0.009**	F>M	0.669	**0.000**	1<2
Entries – Center – Interval 1	0.286	0.140	F=M	0.821	**0.016**	1>2
Entries – Center – Interval 2	0.820	**0.001**	F<M	0.650	**0.001**	1>2
Entries – Center – Interval 3	0.119	**0.001**	F<M	0.936	**0.001**	1>2
Entries – Center – Interval 4	0.231	**0.005**	F<M	0.771	**0.014**	1>2
Entries – Center – Interval 5	0.197	0.158	F=M	0.990	**0.004**	1>2
Entries – Center – Interval 6	0.353	**0.029**	F<M	0.601	**0.040**	1>2
Total Entries – Center	0.300	**0.003**	F<M	0.813	**0.001**	1>2
Time in Center – Interval 1	0.520	**0.001**	F<M	0.887	**0.009**	1>2
Time in Center – Interval 2	0.340	**0.001**	F<M	0.900	0.179	1=2
Time in Center – Interval 3	0.660	**0.001**	F<M	0.420	**0.031**	1>2
Time in Center – Interval 4	0.459	**0.001**	F<M	0.344	0.605	1=2
Time in Center – Interval 5	0.130	**0.001**	F<M	0.878	**0.003**	1>2
Time in Center – Interval 6	0.630	**0.001**	F<M	0.547	0.066	1=2
Total Time – Center	0.262	**0.001**	F<M	0.898	**0.001**	1>2

*=$p<0.05$; F=M and 1=2, no statistically significant difference

Supplementary Figures



Supplementary Figure 4.1: Correlations matrices between adult anxiety-related behaviors. Correlation matrices for anxiety-related behaviors on the three testing arenas used for the adult rats in this study. Between-apparatus measures were not as strongly correlated as within-apparatus measures. The endpoints being compared are listed diagonally down the middle of the matrix. Behaviors of interest can be compared by reading down and across the corresponding rows creating a visual quadrant. The matrix is depicted at the bottom left of that quadrant and the and correlation coefficient (r) for that comparison is depicted in the upper right. All animals are plotted within each matrix cell and the exposure groups are delineated by color. Best fit lines for the data set are depicted in red.



Supplementary Figure 4.2: Proportion of time spent adult animals spent in open arms of the EPM and ZM. (A) Proportion of time that adult females spent in the open arms of the EPM and ZM. (B) Proportion of time that adult males spent in the open arms of the EPM and ZM. For both graphs, black bars represent the EPM and gray bars represent the ZM. Lines and percentages above the bars show the percentage difference between the two assays for the same exposure group.

CHAPTER 5—Conclusions

EDCs are ubiquitous and abundant in our environment, exposing humans to a myriad of chemicals on a daily basis. EDC exposure can be particularly detrimental to the developing fetus or early in adolescence during neurodevelopment. Although the EDC literature provides evidence of altered neurodevelopment as early as fetal life, with sex specific effects observed throughout the brain even before puberty, the specific mechanisms by which this occurs and the time frame over which the developing brain is sensitive are not clearly defined. This dissertation is significant because it provides data revealing the time course of ER expression in hormone-sensitive regions in the neonatal rodent brain and how developmental exposure to BPA alters this time course.

The described studies focused on Sprague-Dawley rats which is a standard rodent model used to investigate EDC exposure. Across the literature, numerous examples of significant effects of multiple developmental EDCs on neural endpoints abound, demonstrating that the potential for EDCs to act on the developmental brain are not strain or compound specific. Compared to the variety of compounds that humans are exposed to on a regular basis there were only a limited number of compounds represented in the neurodevelopmental literature, thus it would be beneficial to expand research on compounds such as organobromine and perfluorinated compounds into gestational and perinatal periods. Humans as well as rodents can be affected by EDCs, the human literature has found evidence of increased anxiety and activity in children when exposed to BPA, other compounds like PCBs have been shown to alter thyroid function, while still others, PBDEs and dioxin, have the potential to alter reproductive function and IQ. Rodent models have been found to be

similarly susceptible to the effects of EDCs and are a means to look more closely at the mechanism behind their action, as well as transgenerational effects within a reasonable amount of time.

The EDC that is the focus of this work is BPA. It was found to alter estrogen receptor expression in the neonatal, juvenile, and adult rat brain. In the neonatal rat brain, BPA, at exposure levels as low as 2.5 μg/kg bw/day, was found to alter the mediobasal hypothalamus and the amygdala, these effects were exposure level and sex specific. Importantly, effects of gavage, were found between the naïve and vehicle animals, indicating that method of exposure can also play an important role in outcomes. In this case, the findings may reflect an interaction of the prenatal BPA exposure and stress. In relation to functional significance, it is hypothesized that the alteration of estrogen receptor expression may play a role in hormone-sensitive behavioral changes that have previously been attributed to early life BPA exposure, though this conclusion is limited by the confound of the stress of gavage (Patisaul et al., 2012; Wolstenholme et al., 2012; Wolstenholme, Rissman, et al., 2011). In addition to the effects of BPA, this work shows unexpected findings of the stress of gavage. Stress related to the use of gavage had the ability to significantly alter brain estrogen receptor expression and sexual differentiation.

While certainly not a new concept, the reduction of unnecessary stress on the animals used in scientific research is and should always be taken into consideration in study design and reevaluated when needed. Based on the findings of our neonatal estrogen receptor expression study (Chapter 2), the use of gavage as a compound delivery mechanism may need reconsideration. The rationale behind its use is reasonable: to ensure precisely equal

amounts of the compound are delivered to each animal in the study. It is also widely believed that with continued use of this administration route, animals habituate to the technique, and stress is not a significant confounding issue. My studies show, however, that after dams were gavaged from GD 6-21, there was still striking evidence in the PND 1 pups of gavage-related effects. Estrogen receptor expression was significantly reduced in the vehicle (gavaged with vehicle) animals when compared to the naïve (non-gavaged) animals in the ARC and amygdala; two regions associated with stress. Thus the effect is hypothesized to be related to stress induced by the gavage technique. To confirm this hypothesis, additional evidence such as blood cortisol levels and other key markers of stress, would need to be evaluated. That was beyond the scope of the present studies and not available. Also, to definitively conclude that the effects observed here are attributable to gavage, and not the vehicle alone, further study is needed. An experiment to definitely determine whether gavage, and not the vehicle, is indeed capable of altering ER expression in the neonatal brain would include the naïve and vehicle groups from the neonatal study, as well as a group that is gavaged, but does not receive vehicle, and a group that is given the vehicle, but not by gavage. My data provide seminal evidence to warrant serious reconsideration of the use of the gavage technique, particularly for studies with neuroendocrine endpoints that might be sensitive to prenatal stress. There are many other ways to administer compounds orally to animals that are less invasive, such as dissolving the substance in ethanol or oil and administering it on food treats (such as sucrose tablets or cookies), or incorporating it in food or water.

In the juvenile and adult, estrogen receptor expression was altered by BPA at doses lower than the current oral reference dose (50 μg/kg bw/day), but not at the highest exposure level tested (2700 μg/kg bw/day). Effects were primarily seen in the preoptic area of the hypothalamus. Interestingly, Esr1 expression was altered in the juveniles, while Esr2 expression was altered in the adults, indicating that estrogen receptors may be variably sensitive to BPA based on hormonal state, whether the animal has undergone puberty (adults) or not (juveniles). In all three ages, neonatal, juvenile, and adult, effects of BPA were seen on ER expression at doses lower than what is currently considered "safe." The EPA has defined the LOAEL for BPA as 50 mg/kg bw/day, the lowest dose that was tested initially where adverse effects were observed, and the NOAEL as 5 mg/kg bw/day, or the exposure level at which no adverse effects will be seen for BPA. However, in the described studies, doses that were lower than the current LOAEL and NOAEL showed significant alteration in ER expression. The highest exposure level tested (2700 μg/kg bw/day) which was the closest to the current NOAEL showed no effects, but lower doses, as low as 2.5 μg/kg bw/day, showed significant effects of BPA on ER expression. These low dose effects demonstrate that the current standard for toxicological testing, beginning at very high doses and progressively decreasing dose until no significant effects are found, may not capture the range of exposures at which adverse effects are seen for all EDCs. EDCs, like BPA, may instead show adverse effects at very high doses and very low doses, while showing minimal, or no effects at median exposures.

Although numerous studies have attempted to characterize the impact of BPA on behavior, the results are inconsistent across the literature and not anchored to a definitive

mechanism (Patisaul et al., 2012; Wolstenholme et al., 2012; Wolstenholme, Rissman, et al., 2011). The behavioral studies in this dissertation were thus conducted to comprehensively address the relationship between BPA exposure, transcriptional estrogen receptor expression changes in sexually dimorphic brain regions, and behavioral effects Similar experimental design and dosing was used to allow for more direct comparison between experiments. However, there was no compelling evidence to indicate that BPA altered anxiety or activity in these animals. Sporadic evidence for altered behavior that was found in the juvenile animals, but not concluded to be convincing as biologically relevant. Study limitations including lower than anticipated statistical power and maze concordance, potential stress-related effects of gavage, the use of ledges on behavioral apparatuses, and females not all being in behavioral estrus at the time of testing, reduce confidence in the general conclusion that developmental BPA exposure does not impact behavior. Additionally, our behavioral battery was not exhaustive and thus does not rule out other behavioral outcomes including effects on memory or learning.

Additionally, the strain of rat that was used for these studies may also present a limitation. There are variations in physiology and behavior in all strains and stocks of rodents, each with pros and cons (Bonthuis et al., 2010). Sprague-Dawley rats were chosen based on their benefits for studying sex differences, female behavior can be masculinized with prenatal exposure estradiol and testosterone, as well as the presence of *Esr2* early in development, unlike other rat strains (Bonthuis et al., 2010). However, previous studies have indicated that Sprague-Dawley rats may be less sensitive to estrogen-like compounds, like BPA, than other strains of rat (Spearow & Barkley, 2001). We may have limited our ability

to find significant effects of BPA exposure by using this strain, however significant effects were found, especially in the developing brain. These significant findings in the developing brain may have been more frequent and substantial and contributed biologically significant alterations in anxiety and activity behavior as well, if we had used a strain that was more sensitive to xenoestrogens, like the Fisher-344 rat (Spearow & Barkley, 2001). Selection of the rat strain was beyond our control but considered important because these studies were conducted in collaboration with the FDA to help guide risk assessment. Using their animals in their facility ensures that the data will be included when policy makers decide how and where BPA should be used in products like food containers and water bottles.

In conclusion, there is sufficient evidence to suggest that EDCs can alter neurodevelopment, particularly pertaining to the alteration of hormone receptors, protein, and gene expression. EDCs can act in sex, region, and time specific manners and have the potential to impart organizational effects on the developing brain. Particularly, BPA has been shown in this dissertation to alter estrogen receptor expression in a time, sex, and region specific manner in neonatal, juvenile, and adult Sprague-Dawley rats, though no behavioral effects were evident as a consequence of estrogen receptor expression alteration. The major limitation of this study that must be taken into consideration along with BPA effects, include the effect of stress of gavage. This work will be combined with the larger CLARITY consortium body of work and will likely be used in the FDA's risk assessment of BPA. Based on a large body of literature currently available, as well as this large collaborative study, the CLARITY consortium will likely mark the conclusion of work on the compound BPA.

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