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Effects of Perinatal Bisphenol A Exposure on the Volume of Sexually-dimorphic Nuclei of Juvenile Rats: A CLARITY-BPA Consortium Study

Sheryl E. Arambula^{a,b}, Joelle Fuchs^a, Jinyan Cao^a, and Heather B. Patisaul^{a,b,c}

^aDepartment of Biological Sciences, North Carolina State University, Raleigh, NC 27695, USA

^bWM Keck Center for Behavioral Biology, North Carolina State University, Raleigh, North Carolina 27695, USA

^cCenter for Human Health and the Environment, North Carolina State University, Raleigh, NC 27695, USA

Abstract

Bisphenol A (BPA) is a high volume endocrine disrupting chemical found in a wide variety of products including plastics and epoxy resins. Human exposure is nearly ubiquitous, and higher in children than adults. Because BPA has been reported to interfere with sex steroid hormone signaling, there is concern that developmental exposure, even at levels below the current FDA No Observed Adverse Effect Level (NOAEL) of 5 mg/kg body weight (bw)/day, can disrupt brain sexual differentiation. The current studies were conducted as part of the CLARITY-BPA (Consortium Linking Academic and Regulatory Insights on BPA Toxicity) program and tested the hypothesis that perinatal BPA exposure would induce morphological changes in hormone sensitive, sexually dimorphic brain regions. Sprague-Dawley rats were randomly assigned to 5 groups: BPA (2.5, 25, or 2500 µg/kg bw/day), a reference estrogen (0.5 µg ethinylestradiol (EE₂)/kg bw/day), or vehicle. Exposure occurred by gavage to the dam from gestational day 6 until parturition, and then to the offspring from birth through weaning. Unbiased stereology was used to quantify the volume of the sexually dimorphic nucleus (SDN), the anteroventral periventricular nucleus (AVPV), the posterodorsal portion of the medial amygdala (MePD), and the locus coeruleus (LC) at postnatal day 28. No appreciable effects of BPA were observed on the volume of the SDN or LC. However, AVPV volume was enlarged in both sexes, even at levels below the FDA NOAEL. Collectively, these data suggest the developing brain is vulnerable to endocrine disruption by BPA at exposure levels below previous estimates by regulatory agencies.

Corresponding author and person to whom reprint requests should be addressed: Heather B Patisaul, Ph.D. Department of Biological Sciences, North Carolina State University, Raleigh, NC 27695, USA, Phone: 919-513-7567, hbpatisa@ncsu.edu.

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Keywords

Bisphenol A; brain; hypothalamus; amygdala; locus coeruleus; sexually dimorphic

1. INTRODUCTION

Perhaps one of the best-known and most intensely studied endocrine disrupting chemicals (EDCs) is bisphenol A (BPA). A high production volume chemical, BPA is used as a monomer in the production of polyvinyl chloride and polycarbonate plastics, epoxy resins, and a multitude of other commercial and consumer products (FAO/WHO, 2011). Human exposure to BPA is virtually unavoidable and occurs primarily from contaminated food and beverages. In industrialized countries, well over 90% of individuals are estimated to have detectable amounts of BPA in their bodies, albeit in small amounts (serum levels are typically in the range of 4 ng/ml or lower) (Bushnik et al., 2010; Calafat et al., 2005; Calafat et al., 2008; Casas et al., 2013; LaKind and Naiman, 2015). The most significant route of human exposure is thought to be ingestion, with dietary intake estimated to range from 0.1 – 1.4 µg/kg body weight (bw)/day, but exposure can also occur from other sources (FAO/WHO, 2011). BPA can cross the placenta and there is some evidence that it may accumulate in the fetus after repeated exposures (Ikezuki et al., 2002; Schonfelder et al., 2002; Taylor et al., 2008). In fetal rodents, BPA has been shown to preferentially accumulate in brain, in some cases to a greater degree in males than females (Negri-Cesi, 2015). In its 2014 updated safety assessment of Bisphenol A (BPA) for use in food contact applications, the US Food and Drug Administration defined the No Observed Adverse Effect Level (NOAEL) as 5 mg/kg bw/day based largely on two multigenerational rodent studies (documents available for download here: <https://www.fda.gov/NewsEvents/PublicHealthFocus/ucm064437.htm>).

BPA has been reported to interfere with the metabolism and signaling of endogenous steroid hormones, particularly estrogen, and numerous studies, including our own, have repeatedly shown in multiple species that developmental exposure to BPA can perturb sexually dimorphic brain development and behavior, even at exposures below the current FDA NOAEL (representative examples include (Braun et al., 2011; Jasarevic et al., 2013; Kinch et al., 2015; Patisaul et al., 2012b; Rebuli and Patisaul, 2015; Sullivan et al., 2014; Wolstenholme et al., 2011)). Although this compounding evidence is compelling, because few published studies are evaluated to be of high utility for human risk assessment, there remains a lack of consensus on the potential risks BPA pose to the developing brain in humans. The studies herein were specifically designed and conducted in response to that informational limitation under the CLARITY-BPA research program (Consortium Linking Academic and Regulatory Insights on BPA Toxicity) (Birnbaum et al., 2012; Heindel et al., 2015; Johnson et al., 2015; Schug et al., 2013), a multi-investigator effort coordinated and supported by the National Toxicology Program (NTP), National Institute of Environmental Health Sciences (NIEHS), and U.S. Food and Drug Administration (FDA) to help provide clarifying evidence. The present study tested the hypothesis that early-life BPA exposure can alter the volume of sexually dimorphic structures in the brain, and serves as a follow-up to our prior CLARITY-BPA paper describing non-reproductive behavioral outcomes in the same animals (Rebuli et al., 2015).

Designed to draw upon the strengths of academic and guideline-compliant studies in order to address research gaps and confirm prior findings, this and all other CLARITY-BPA studies are uniquely powerful because they incorporate research recommendations published by the WHO and others (Beronius et al., 2010; Beronius et al., 2009; Chapin et al., 2008; FAO/WHO, 2011; FDA, 2012; NTP, 2008) for enhancing robustness and reproducibility, including complete data blinding, use of only one animal per sex per litter (with the litter as the experimental unit), oral dosing, inclusion of a reference estrogen, and evaluation of multiple BPA doses, particularly levels at or below the FDA NOAEL. Additionally, as in our prior, published CLARITY-BPA studies (Arambula et al., 2016; Rebuli et al., 2015), sex was considered as a biological variable. Exposure levels and endpoints examined were established via consortium consensus and selected to maximize utility in risk assessment. The animals used for the present study were tested as juveniles for effects on sexually dimorphic, non-reproductive behaviors prior to sacrifice (Rebuli et al., 2015). Only limited and inconsistent evidence for heightened anxiety and exploratory behavior were observed, leading us to conclude that there were no systematic effects of BPA on the behavioral endpoints tested. Here we focused on exposure-related effects on the volume of sexually dimorphic brain nuclei in these animals with the hypothesis that perinatal exposure would abrogate volumetric sex differences. Sprague-Dawley rats from an existing colony at the National Center for Toxicological Research (NCTR-SD) were perinatally exposed to vehicle, BPA (2.5, 25, or 2500 µg/kg bw/day), or a reference estrogen (0.5 µg/kg bw/day 17α-ethinylestradiol (EE₂)). To ensure precise oral dosing, dams were gavaged from gestational day 6 (GD 6) until parturition and offspring were directly gavaged from postnatal day 1 (PND 1) to weaning (PND 21). PND 28 brains were coronally sectioned, thionin-stained for Nissl substance, and unbiased stereology was used to quantify the volume of sexually dimorphic brain regions.

Throughout the mammalian brain, several morphological and functional brain sex differences arise during the fetal and postnatal period in response to the organizational effects of steroid hormones (De Vries, 2004; McCarthy, 2008; Simerly, 2002). The two regions most classically associated with morphological sex differences in rodents are the aptly named sexually dimorphic nucleus (SDN) of the preoptic area and the anteroventral periventricular nucleus (AVPV). Both of these morphometric sex differences are mediated by estradiol but effects on apoptosis are opposite, resulting in the SDN being 5–7 times larger in males (Gorski, 1978) and the AVPV being nearly 1.6 times larger in females (Davis et al., 1996; Simerly et al., 1997). Volumetric sex differences of the SDN and AVPV emerge perinatally and during adolescence, respectively, and increase in magnitude until adulthood (Ahmed et al., 2008; Davis et al., 1996; Gorski, 1978; Simerly et al., 1997). The mechanisms by which the SDN and AVPV are sexually differentiated are well described, require estrogen receptor alpha (ERα), and can be predictably manipulated by exogenous hormones (Lenz and McCarthy, 2010; Schwarz and McCarthy, 2008; Simerly, 2002). Thus, they are considered particularly useful targets for assessing the endocrine disrupting properties of chemicals such as BPA.

The posterodorsal portion of the medial amygdala (MePD) was also selected for assessment because ERs are known to play a role in the sexual differentiation process (Cooke et al., 2003), and we have previously demonstrated that prenatal BPA exposure can alter sex-

specific patterns of MePD ER β expression (Cao et al., 2013). Although anatomical sex differences in the prepubescent rodent amygdala are not as great as in the preoptic area of the hypothalamus, the volume of the rat MePD is roughly 15 – 20% larger in prepubertal males than females (Cooke et al., 2007; Cooke and Woolley, 2005). Circulating levels of gonadal steroids maintain and enhance the MePD volumetric sex difference throughout puberty (Ahmed et al., 2008) and adulthood (Cooke et al., 2003), resulting in the adult male MePD being approximately 2 times larger than females (Cooke et al., 1999; Hines et al., 1992).

Lastly, we explored the effects of BPA on the volume of the locus coeruleus (LC), a nucleus located in the pons and selected because one laboratory has generated data suggesting perinatal BPA exposure can have sex-specific effects on LC volume in Wistar rats (Kubo et al., 2001; Kubo et al., 2003). A female biased sexual dimorphism in rodent LC volume has been reported, however, this appears to be strain- and species- dependent (Babstock et al., 1997; Garcia-Falgueras et al., 2005). Thus, whether or not a sex difference exists, and might be vulnerable to BPA, was of interest in our animal model. As the principal site of norepinephrine synthesis in the central nervous system, the LC plays a critical role in modulating behavioral, autonomic, and endocrine responses to stress.

2. MATERIALS AND METHODS

The study is a component of the CLARITY-BPA program and used the same animals for which behavioral data are already published (Rebuli et al., 2015). Because the comprehensive study design details are described in that prior publication, only the most directly relevant methods are summarized here.

2.1 Animal Husbandry

Sprague-Dawley rats from the National Center for Toxicological Research colony (NCTR-SD rats) were housed in an Association for Assessment and Accreditation of Laboratory Animal Care-(AALAC) accredited facility at NCTR (23 $\pm$ 3 °C, 50 $\pm$ 20% relative humidity, and 12:12 h light dark cycle, lights off at 0600 h). All aspects of this study were approved by the NCTR Institutional Animal Care and Use Committee (IACUC). Rats were housed in conditions designed to minimize unintentional exposure to BPA and other EDCs (use of glass water bottles with filtered water, thoroughly washed polysulfone caging and woodchip bedding) and a soy- and alfalfa- free diet (5K96 verified casein diet 10 IF, round pellets, γ -irradiated; Cat. 1810069, Purina Mills, Richmond IN) and Millipore-filtered water were provided ad libitum. Extracts of diet and other study materials were monitored for BPA and myco/phytoestrogens by liquid chromatography/mass spectrometry (Delclos et al., 2014) and all had levels below the average analytical method blanks (Heindel et al., 2015). Because these rats were bred for behavioral testing and thus required special housing, they were generated from the same colony as the CLARITY-BPA studies but not obtained from the mainline study and housed separately (in a different building) after weaning (Rebuli et al., 2015). The study was designed and executed with the litter as the statistical unit.

2.2 Reagents and Dosing

The BPA (CAS # 80-05-7, catalog # B0494, TCI America, Portland, OR) and ethinylestradiol (EE₂; CAS # 57-63-6, catalog #E4876, Sigma-Aldrich, St. Louis, MO) were more than 99% pure and administered in 0.3% aqueous carboxymethyl cellulose (CMC; catalog # C5013, Sigma- Aldrich, St. Louis, MO) by gavage daily at a volume of 5 ml/kg bw using a modified Hamilton Microlab ML511C programmable 115 V pump (Hamilton Co., Reno, NV) (Lewis et al., 2010).

Two weeks prior to mating, female NCTR-SD rats were randomly assigned to exposure groups stratified by body weight to ensure body weights were equivalent across all groups. Male breeders were assigned such that no sibling or first cousin mating occurred and mating was conducted as previously described (Delclos et al., 2014). To model the exposure route used to establish the FDA NOAEL, dams were orally gavaged daily with vehicle (0.3% CMC), 2.5, 25, or 2500 µg BPA/kg bw/day, or 0.5 µg EE₂/kg bw/day from GD 6 until the onset of labor (note: the full CLARITY-BPA study has additional exposure groups, see (Heindel et al., 2015). Neither the dams nor the pups were dosed on the day of birth (PND 0). On PND 1, litters were randomly culled to a maximum litter size of 10 (minimum size of 6) to achieve equal numbers of males and females. After the litter was culled, the pups were directly gavaged daily through weaning (PND 21). For pups younger than PND 5, the gavage needle was not inserted past the pharynx.

The 3 doses of BPA used in this study cover the lower range of exposures at which BPA-induced effects have been reported in scientific literature and include levels of BPA below the current FDA NOAEL of 5 mg/kg bw/day. Because many of the reported effects of BPA are hypothesized to be due to an estrogenic mode of action a reference estrogen group (0.5 µg EE₂/kg bw/day) was included.

2.3 Weaning and Tissue Collection

Offspring were weaned on PND 21 after their last daily gavage and identified by a tail tattoo with a unique identification number. As described previously, only offspring from litters with at least 9 pups and a reasonably balanced sex ratio at birth (no litter had more than a 4 pup sex difference except for 2 litters in load 5, which had a 5 pup sex difference: 9 males and 4 females) were used in this study (Rebuli et al., 2015). Animals were transferred to new rooms and housed in groups of 2–3 (same-exposure group, same-sex, same-age, non-siblings) under conditions identical to the preweaning rooms described above, apart from the light cycle (23:00 –11:00), which was adjusted to accommodate behavior testing. To ensure no test animals were housed alone, a same sex and age treatment-naïve “companion” animal was provided when needed. Twelve animals per sex per group were assigned to the current study (1/sex/litter). Behavioral testing occurred before puberty on PNDs 25 – 27 using a battery of behavioral tests predictive of anxiety (open-field and elevated plus maze), the outcomes of which are published (Rebuli et al., 2015). The animals (n = 120) were then sacrificed on PND 28 by CO₂ asphyxiation followed by rapid decapitation. Brains were collected, flash frozen on crushed dry ice and shipped from NCTR to North Carolina State University (NCSU) where they were stored at –80 °C.

2.4 Tissue Processing and Nissl Staining

The brain of each animal was cryosectioned (Leica CM1900, Nußloch, Germany) into three serial sets of 20 µm coronal sections, mounted onto Superfrost plus slides (Fisher Scientific, Pittsburgh, PA) and stored at -80 °C. On the day prior to staining, one set of sections was thawed and dried at room temperature overnight. The sections were then defatted in 100% xylene, rehydrated in a series of descending ethanols and Milli-Q water (Merck Group, Darmstadt, Germany), and stained for Nissl substance with thionin (0.2%) to visualize anatomical structures. The slides were then dehydrated in ascending ethanols, cleared in 100% xylene, and cover-slipped with DPX mounting medium (VWR International Inc., Poole, England).

2.5 Stereological Quantification

Unbiased stereology was performed using the Stereologer™ software (Stereology Resource Center, Inc., MD) on a Leica DM2500P microscope (Leica Microsystems, Wetzlar, Germany) equipped with a motorized stage (Applied Scientific Instrumentation, Eugene, OR) and a video camera (IMI Technology Co., Seoul, Korea). Procedures for volumetric assessment were similar to those we have used for prior studies (McCaffrey et al., 2013; Patisaul et al., 2007).

Unilateral contours of the AVPV, SDN, and LC and bilateral contours of the MePD were drawn at low magnification (5x) from the live image with the assistance of a rat and mouse brain atlas (Paxinos, 1991, 2007). Bilateral measurements of the MePD were taken because there are subtle volumetric differences between the left and the right MePD as well as between the sexes (Cooke et al., 2007; Johnson et al., 2008). A uniform grid of points with an area per point of 2000 µm² was randomly superimposed over each section and all of the points lying within the region of interest were selected. Based on these counts, volume was estimated using the Cavalieri method (Gundersen and Jensen, 1987) and coefficient of error for individual volume estimates was less than 10% (CE < 0.10).

The volume of the AVPV and SDN were independently defined and measured by two blinded investigators to confirm that the measurement methodology was reproducible. For the analysis, the data from both investigators was averaged. A single investigator, blinded to exposure groups, then quantified the volume of the LC and MePD. Only animals for which every section within regions of interest were perfectly intact were examined. Thus, because of tissue damage or uneven staining, some material could not be analyzed: 2 brains were excluded for the SDN, 3 for the AVPV, 24 for the left MePD, 20 for the right MePD, and 13 for the LC. The number of animals excluded was higher for the MePD because this region is volumetrically larger than the others and thus requires more sections to fully measure. If even a single section was damaged or missing, then the animal was excluded to prevent measurement error. Sample sizes for all endpoints are indicated in the figures.

2.6 Data Decoding

To ensure that all investigators remained blinded during data collection, all tissue samples provided by NCTR were given a unique identifier and designated with a letter (A, B, C, etc.) to denote each experimental group (grouped by exposure and sex) but blind the NCSU team

to exposure. Coded raw data were submitted to the NTP Chemical Effects in Biological Systems (CEBS) repository after all volumetric measurements were complete. After a CEBS administrator performed a quality control analysis, the raw data was archived, and the NCSU investigators were then unblinded and began the statistical analysis. This code was not the same as the one used for these animals while they underwent behavioral testing. Thus, all information obtained from these animals was collected under blinded conditions.

2.7 Statistical Analysis

Statistical analysis for all of the data was performed and graphed using Prism version 7 (GraphPad Software, Inc., La Jolla, CA). The statistical approach was designed to be consistent with published guidelines for low dose EDC studies (Haseman et al., 2001) and previously published stereological studies of similar scale (equivalent or smaller sample sizes) in the rat brain (Adewale et al., 2011; McCaffrey et al., 2013). Within each exposure group, no same-sex littermates were included, so potential litter effects did not need to be statistically accounted for. Because it can differ, the volume of the left and right MePDs were first analyzed individually. Significant differences in size were not observed, thus a combined data set was generated by calculating an average (of left and right) MePD size (for animals in which both could be measured) or by using the single available value (from the left or the right). This produced a single, representative MePD volume for each animal.

Prior to all statistical analysis, data were assessed within each region using the Shapiro-Wilk normality test ($\alpha = 0.05$) and violations were only found for residual groups within the MePD and SDN. In some cases this was due to the presence of statistical outliers, which we ultimately chose not to remove in order to ensure a full accounting of all data. Violations of normality may increase the chance of type I error, but are not uncommon with sample sizes of 9–12/sex/group (Cohen et al., 2002). Because occurrences of non-normality or outliers did not meaningfully impact the statistical outcome or interpretation of the data, rather than differentially perform non-parametric tests in cases where deviation from normality occurred, we applied a constant modeling approach to all endpoints in each region of interest.

For each region of interest, all data were first analyzed by a two-way analysis of variance (ANOVA) with sex and exposure as factors to identify significant main effects and their interactions. To maximize resolution regarding potential sex-specific effects, the data were then analyzed within sex by a one-way ANOVA and the Dunnett's Multiple Comparison post hoc test was used to compare each exposure group to the same-sex vehicle control group. Lastly, t-tests were used to identify sex differences and, most importantly, to ensure known sex differences were detected in the unexposed control groups. Confirmation of known sex differences in the vehicle controls was considered to be an indication that the measurements were robust, sufficiently powered to detect a difference in the range of that effect size, and properly conducted. All analyses were two-tailed and the level of significance for all data was set at $p = 0.05$.

3. RESULTS

All results are summarized in Table 1. As expected, the volumes of the SDN, AVPV, and MePD were sexually dimorphic in the vehicle control groups. Overall, BPA and EE₂ had minimal effects on these volumetric sex differences.

3.1 SDN Volume

There was a main effect of sex [$F(1, 108) = 279.60, p = 0.001$] on the volume of the SDN but no effect of exposure and no significant interaction. In all exposure groups SDN volume was significantly larger in males than females (Figure 1B and Table 1). When exposure was examined within sex, there were no significant effects of BPA or EE₂ observed.

3.2 AVPV Volume

Two-way ANOVA revealed a main effect of sex [$F(1, 107) = 115.40, p = 0.001$] and exposure group [$F(4, 107) = 23.79, p = 0.001$], plus a significant interaction [$F(4, 107) = 6.97, p = 0.001$] on AVPV volume. Within females, perinatal exposure to 2.5, 25, and 2500 $\mu\text{g BPA/kg bw/day}$ increased the volume of the AVPV ($p = 0.008, p = 0.031$, and $p = 0.001$, respectively; Figure 2B). In males, perinatal exposure to 25 and 2500 $\mu\text{g BPA/kg bw/day}$ increased AVPV volume when compared to the same sex vehicle control group ($p = 0.001$ for both). In all exposure groups, AVPV volume was found to be significantly larger in females than males (Figure 2B and Table 1). Perinatal exposure to 0.5 $\mu\text{g EE}_2/\text{kg bw/day}$ did not significantly affect AVPV volume in either sex.

3.3 MePD Volume

In the left MePD, two-way ANOVA revealed a main effect of sex [$F(1, 90) = 80.98, p = 0.001$] and exposure [$F(4, 90) = 2.98, p = 0.02$], plus a significant interaction [$F(4, 90) = 3.34, p = 0.01$]. When exposure was examined within sex, however, no significant effects of BPA or EE₂ on left MePD volume were identified in either sex. As expected, left MePD volume was significantly larger in males than females in all exposure groups (Figure 3 and Table 1).

In the right MePD, there were significant main effects of sex [$F(1, 86) = 91.31, p = 0.001$] and exposure [$F(4, 86) = 4.91, p = 0.001$] on volume. Compared to the same-sex vehicle control, right MePD volume was significantly increased in females perinatally exposed to 2500 $\mu\text{g BPA/kg bw/day}$ ($p = 0.001$). Right MePD volume was significantly larger in males than females for all exposure groups (Figure 3 and Table 1).

Using the data set in which the right and left MePD measurements were combined, there was a main effect of sex [$F(1, 99) = 140.30, p = 0.001$] and a significant interaction of sex and exposure [$F(4, 99) = 3.06, p = 0.02$] on MePD volume. Within sex, however, perinatal exposure to BPA or EE₂ had no significant effects on MePD volume in either males or females (Figure 4B). Male MePD volume was significantly larger than females in all exposure groups (Figure 4B and Table 1).

3.4 LC Volume

Two-way ANOVA revealed a significant main effect of exposure [$F(4, 97) = 2.73, p = 0.034$] but not sex on the LC volume. Compared to the same sex vehicle control animals, LC volume was significantly increased in males perinatally exposed to $0.5 \mu\text{g EE}_2/\text{kg bw/day}$ ($p = 0.02$) (Figure 5B). In all exposure groups, LC volume was not found to be significantly different between males and females (Figure 5B and Table 1). No significant effects of BPA on LC volume were identified in either sex.

4. DISCUSSION

BPA effects were mainly confined to the AVPV, which was increased by perinatal exposure in both sexes at multiple dose levels. Perinatal exposure to $2.5 \mu\text{g/kg bw/day}$ BPA increased AVPV volume in females and exposure to 25 and $2500 \mu\text{g BPA /kg bw/day}$ increased volume in both males and females. Because endogenous estradiol, acting through $\text{ER}\alpha$ and $\text{ER}\beta$, is masculinizing in the AVPV (Bodo et al., 2005; Patchev et al., 2004), these effects suggest that BPA is functionally acting as an estrogen antagonist (McCarthy, 2008). Within the MePD, an effect of BPA on volume was detected only in the right MePD in males exposed to the $2500 \mu\text{g BPA/kg bw/day}$ exposure group. Because the left and right MePD nucleus have numerous structural and functional differences, an effect on only one side is biologically plausible. LC volume was not found to be sexually dimorphic in this animal model, and slightly but statistically increased by EE_2 but not BPA. EE_2 did not, however, masculinize any of the sexually dimorphic nuclei, demonstrating that it was not effective as a positive control. These data show that perinatal BPA exposure, at an oral exposure range well below the current FDA NOAEL, is capable of disrupting brain sexual differentiation in rats.

Well-characterized as sexually dimorphic, the rat AVPV contains several robust sex differences critical to the sex-specific neuroendocrine and reproductive functions of this region, including the capacity to coordinate steroid positive feedback in females (Simerly, 2002). Identification of the AVPV as a sensitive target for BPA is a consistent finding across the literature, with a couple of prior studies reporting volumetric effects. Some evidence for disruption of AVPV volume was observed in prepubertal female offspring of CD-1 mice exposed to $250 \text{ ng BPA kg/day}$ delivered by osmotic pump from GD 8 to lactational day 16. An important distinction, however, is the direction of the effect. While total AVPV volume was not actually quantified, perinatal BPA exposure led to a significant decline in the number of tissue sections spanning the rostral-caudal extent of the AVPV in prepubertal females (Rubin et al., 2006). In contrast, a prior experiment from our lab group found neonatal exposure to $250 \mu\text{g}$ of BPA by subcutaneous injection every 12 hours during the first two days of life (approximately $42 \text{ mg BPA/kg bw/day}$) had no effect on the AVPV volume of adult male Sprague-Dawley rats (females not examined) (Patisaul et al., 2007). The directional disparity in volumetric outcomes may at least partially result from experimental design differences (dose, route of administration, timing of exposure) and variation in the neural structure of rats and mice (Bonthuis et al., 2010).

BPA-related disruption of sexually dimorphic endpoints within the AVPV has also been reported. The AVPV contains a sexually dimorphic population of dopaminergic neurons that

are more abundant in females than males and differentiate under the influence of ER α and ER β (Bodo et al., 2005; Simerly et al., 1985; Simerly et al., 1997). Of the three studies examining the effect of developmental BPA exposure on this population in the prepubertal rodent AVPV, two found sex- and dose-specific effects. Neonatal exposure to 250 μ g of BPA by subcutaneous injection every 12 hours during the first two days of life increased the number of dopaminergic neurons in the male rat prepubertal AVPV but had no effect in females (Patisaul et al., 2006). In CD-1 mice, transplacental and lactational exposure, spanning GD 8 to PND 16 to much lower BPA exposures (25 and 250 ng/kg bw/day) decreased the number of dopaminergic neurons in the prepubertal female AVPV but had no effect on males (Rubin et al., 2006). Conversely, the third experiment, which used the same route and timing of exposure as the present study, found no effects of perinatal BPA exposure to 2.5 or 25 μ g/kg bw/day on AVPV dopaminergic cell numbers in PND 21 male and female NCTR-SD rats (Ferguson et al., 2015). Notably, while a female-biased sexual dimorphism in dopaminergic cells was reported, the expected sex difference in total AVPV volume was not found. Although we were able to detect a volumetric sex difference in the present study, it was smaller than has been reported in prior studies, suggesting that AVPV sex differences may be smaller in the NCTR-SD strain. Alternatively, this discrepancy may simply be a result of age. While this sex difference emerges pre-pubertally, the differential cell death that enhances the sex difference is not complete until the late perinatal period (Ahmed et al., 2008; Davis et al., 1996; Gorski, 1978; Simerly et al., 1997). Notably, the sensitive window for AVPV differentiation occurs earlier, just after birth, suggesting that any action of BPA or EE₂ on AVPV volume most likely occurred perinatally.

Kisspeptin neurons in the AVPV are also sexually dimorphic, with adult females possessing many more kisspeptin neurons than males (Kauffman et al., 2007) and the ontogeny of this sex difference is primarily mediated by estrogen activity on ER α (Patisaul et al., 2012a; Patisaul et al., 2009). Moreover, kisspeptin is the principal regulator of gonadotropin secretion and crucial for the timing of pubertal onset and ovulation (Oakley et al., 2009; Pineda et al., 2010; Pinilla et al., 2012). Prior rodent studies provide compelling evidence that developmental BPA exposure can alter levels of kisspeptin mRNA expression (*Kiss1*) and the number of kisspeptin immunoreactive (kisspeptin-ir) neurons in the AVPV in a sex- and age- specific manner. For instance, in the rat, perinatal exposure to 2 μ g BPA/kg bw/day increased the number of kisspeptin-ir neurons at PND 30, 50, and 90 in males (Bai et al., 2011) and neonatal exposure to 50mg BPA/kg bw/day was found to downregulate *Kiss1* expression in adult females (Patisaul et al., 2009). Due to the nature of the study design and other logistical constraints, we were not able to examine kisspeptin or other AVPV-related endpoints other than volume.

BPA-related effects in the AVPV appear complex, as reported outcomes vary by dose, sex, age, and species/strain of rodent model. Nevertheless, results from prior experiments, together with the observations reported herein, indicate that BPA disrupts sexual differentiation of the AVPV, most likely by interfering with endogenous estrogen. Previous studies demonstrate that developmental BPA exposure can alter the expression of ER α and ER β in the AVPV (Cao et al., 2014; Monje et al., 2009; Rebuli et al., 2014). This disruption may occur via an epigenetic mechanism, as BPA has been shown to induce lasting changes in DNA methylation of genes encoding estrogen receptors (Kundakovic et al., 2013; Nugent

et al., 2010). Thus, BPA may be interfering with sexual differentiation by acting directly on ERs, or indirectly by altering ER levels. The observed impacts on AVPV sexual differentiation contribute to, and are consistent with a large body of literature reporting BPA-related effects on reproductive behavior and physiology (Peretz et al., 2014; Ziv-Gal and Flaws, 2016).

In contrast to the AVPV, no effects of perinatal BPA exposure on SDN volume were observed in either sex. Available literature regarding BPA-related effects on SDN volume has yielded contradictory results that have been attributed to inconsistencies in key study design elements, including methodological differences for SDN-volume measurements and defining the borders of the SDN. Generally, studies using levels of BPA above the FDA NOAEL have found SDN volume to be unaffected by prenatal and/or postnatal exposure (Kwon et al., 2000; Nagao et al., 1999; Patisaul et al., 2007; Takagi et al., 2004). The one exception to this found perinatal BPA exposure ranging from 10,000 to 10,000,000 µg/kg bw/day orally administered to dams through cookie treats, diminished SDN volume in adult male Long Evans rats (McCaffrey et al., 2013). Notably, calbindin-ir was used to delineate the anatomical boundaries of the SDN. Calbindin is a calcium-binding protein expressed in a subdivision of sexually dimorphic SDN neurons (Sickel and McCarthy, 2000). It is possible that calbindin-ir reveals the border of the SDN more precisely than classical histological staining methods, including Nissl. This may contribute to the contradictory results reported for doses of BPA equivalent to or below the FDA NOAEL. For example, SDN volume evaluated by cresyl violet stain was unaltered in adult Wistar rats perinatally exposed to 30, 300 and 1500 µg BPA/kg bw/day (Kubo et al., 2001; Kubo et al., 2003). In contrast, a study that delineated the SDN with calbindin-ir, and employed the same animal model and exposure paradigm as the present study, found SDN volume was increased in males perinatally exposed to 2.5 and 25.0 µg BPA/kg bw/day (He et al., 2012). That outcome supports our general conclusion that disruption of sexually dimorphic brain volumetrics can occur at doses well below the FDA NOAEL.

To our knowledge, this is the only study to examine the effects of BPA exposure on the volume of the MePD. Right MePD volume in males exposed to the 2500 µg BPA/kg bw/day was increased, but it is difficult to interpret the significance of this singular finding. Prior studies from our lab group have found that developmental BPA exposure, at levels below the FDA NOAEL, can alter ER β expression in the amygdala (Patisaul et al., 2012b), and the MePD expression of ERs α and β in a sex- and dose-dependent manner (Cao et al., 2014; Cao et al., 2013). ER β expression appears to be particularly sensitive to disruption by BPA in this region. Because both androgen and estrogen receptors mediate MePD sexual differentiation (Johansen et al., 2004), it is at least conceivable that BPA-induced alterations in ER expression underlie the morphometric change reported here but subsequent work will be needed to comprehensively explore that possibility. The sex-biased outcome may also reflect sex-biased exposure as at least one prior study has found that BPA accumulates to a higher degree in the fetal brain of males compared to females in Balb-C mice (Mita et al., 2012). Brain levels were not directly measured in the present study. Additionally, because the endocrine system contains multiple feedback loops, it is possible that brain effects could result from BPA-related actions extramural to the brain.

One lab has previously explored the effects BPA on LC volume and both studies from that group used Wistar rats (Kubo et al., 2001; Kubo et al., 2003). Perinatal exposure to levels of BPA below the FDA NOAEL increased male LC volume and decreased female LC volume, thereby reversing the sex difference. In Wistar rats the female LC contains more neurons and has a greater overall volume than the male LC. This, however, appears to be strain-specific, as sex differences have not been observed in Long Evans or Sprague-Dawley rats (Babstock et al., 1997; Garcia-Falgueras et al., 2005; Garcia-Falgueras et al., 2006; Guillamon et al., 1988; Pinos et al., 2001). Here we found no sex difference in LC and no effect of BPA exposure at any of the exposure levels examined. Considered collectively, these results highlight the importance of strain when designing and interpreting rodent EDC studies.

0.5 µg/kg bw/day EE2 was not masculinizing for any of the sexually dimorphic endpoints examined and thus not an effective positive control. Notably, our two prior CLARITY-BPA studies on the brain and behavior also failed to consistently find any effects of EE₂ on sexually dimorphic endpoints (Arambula et al., 2016; Rebuli et al., 2015). Historically, the vast majority of studies establishing the mechanisms by which brain nuclei are sexually differentiated by steroid hormones have used 17β-estradiol itself or estradiol benzoate via injection at doses typically ranging from 2 to 50 µg (estimating that a female rat pup weighs ~ 6 g this dose range would be comparable to 333 – 8,333 µg /kg bw) (Arnold and Gorski, 1984; Gorski, 1978; Gorski and Wagner, 1965; Gurney and Konishi, 1980). For the CLARITY-BPA program and other toxicological studies, oral dosing is preferable because it models the typical human exposure route, thus EE₂ was used as the reference estrogen. Failure to produce measurable effects on SDN or AVPV volume could indicate that the dose was insufficient, dosing was improperly conducted, EE₂ does not reach the brain, EE₂ is not masculinizing in brain, or the this strain of rat is resistant to estrogen. Of these, the first possibility is considered most likely. While there is considerable historical toxicological data on EE₂ and uterine weight, only a paucity of studies have examined the impact of EE₂ on sexually dimorphic neuroendocrine or behavioral endpoints. One, using exposures ranging from 0.05 to 50 µg/kg bw/day demonstrated doses above 5 µg/kg bw/day were most effective at masculinizing sexually dimorphic behaviors related to reproduction in the Wistar rat (Ryan et al., 2010). Most relevant to the present study, work from a different lab group that used the same SD-NCTR strain found perinatal oral exposure to 5.0 and 10.0 µg/kg/day successfully masculinized the SDN volume in females (He et al., 2012). These data support the conclusion that the dose of EE₂ used here was likely insufficient, and also emphasize the need to establish a dose-response relationship for EE₂ and hypothalamic masculinization across this and other toxicologically valued strains.

Finally, the data reported herein are consistent with the other studies from the CLARITY-BPA consortium published to date. Collectively all have shown sporadic and generally modest effects of BPA on the brain and behavior, cardiac endpoints, and female ovarian follicle numbers and sex steroid levels (Arambula et al., 2016; Gear et al., 2017; Johnson et al., 2015; Patel et al., 2017; Rebuli et al., 2015). Significantly, the observed increase in female AVPV volume occurred at the same doses reported to cause effects in other CLARITY-BPA studies. We previously reported, for example, that prenatal exposure to 2.5 µg BPA/kg bw/day upregulated hypothalamic expression of ERα in neonate females (Arambula et al., 2016). Continuous exposure to 2.5 µg/kg bw/day from GD 6 to PND 21

also reduced the numbers of primordial, primary, preantral, and total healthy follicles in the ovary of PND 21 females (Patel et al., 2017). These consistencies support concerns that BPA can produce effects below the FDA NOAEL in multiple systems.

5. CONCLUSIONS

This study provides evidence that developmental BPA exposure, at levels below the FDA NOAEL, can impact sexual differentiation of the AVPV. Neither BPA nor EE₂ impacted the volume of the SDN or the MePD, and EE₂ had a minimal effect on LC volume. Limitations of this study include potential stress-related effects of gavage (Cao et al., 2013) and the failure of the reference estrogen to masculinize any of the sexually dimorphic endpoints, an outcome which likely reflects insufficient dose. Emerging data from ongoing CLARITY-BPA projects will establish the degree to which BPA impacts the development of other tissue types and organ systems. Comprehensive approaches that simultaneously assess molecular and functional phenotypes, as well the size of specific brain regions, are needed to fully characterize the neurodevelopmental effects of BPA exposure, but collectively, these data support prior conclusions that prenatal BPA exposure, even at exposure levels below the current FDA NOAEL, alters sexual differentiation of the brain.

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HIGHLIGHTS

- Some hypothalamic brain nuclei have morphometric sex differences resulting from perinatal sex steroid hormone action.
- We hypothesized that perinatal exposure to BPA, an endocrine disruptor, would alter morphometric sex differences in the juvenile rat brain.
- The volume of sexually dimorphic rat brain nuclei were quantified using unbiased stereology.
- BPA increased the volume of the anteroventral periventricular nucleus in both males and females.
- Our data suggest BPA, even at doses below the FDA NOAEL, can interfere with brain sexual differentiation.

Figure 1.

(A) Representative thionin stained coronal sections showing the sexually dimorphic nucleus (SDN). Sections are arranged from rostral to caudal and the dotted line indicates the boundaries of the area measured. Perinatal exposure to BPA or EE_2 had no significant effects on SDN volume in either sex (B). As expected, SDN volume was significantly larger in males than females in all exposure groups. Significant sex differences in volume are represented by $^{\dagger\dagger\dagger}p < 0.001$. Error bars represent the 95% confidence interval and sample size is provided at the bottom.

Figure 2.

(A) Representative thionin-stained coronal sections showing the anteroventral periventricular nucleus (AVPV). Sections are arranged from rostral to caudal and the dotted line indicates the boundaries of the area measured. In females, perinatal exposure to 2.5, 25, and 2500 μg BPA/kg bw/day increased AVPV volume (B). In males, perinatal exposure to 25 and 2500 μg BPA/kg bw/day increased AVPV volume. As expected, AVPV volume was found to be significantly larger in females than males in all exposure groups. Exposure to 0.5 μg EE₂/kg bw/day had no significant effects on AVPV volume. Significant differences in volume compared to the same-sex vehicle group are represented by *** $p < 0.001$, ** $p < 0.01$, and

* $p < 0.05$. Significant sex differences in volume are represented by $^{\dagger\dagger}p < 0.01$, and $^{\dagger}p < 0.05$. Error bars represent the 95% confidence interval and sample size is provided at the bottom.

Figure 3.

In the right medial posterodorsal amygdala (MePD), perinatal exposure to 2500 μg BPA/kg bw/day increased MePD volume compared to the same sex vehicle controls. However, overall evidence for BPA- and EE_2 - related effects were minimal and inconsistent on both the volume of the left and right MePD. In both the left and right MePD, volume was found to be significantly larger in males than females in all exposure groups. Significant differences in volume compared the same-sex vehicle group are represented by **p < 0.01. Significant sex differences in volume are represented by †††p < 0.001, ††p < 0.01, and †p < 0.05. Error bars represent the 95% confidence interval and sample size is provided at the bottom.

Figure 4.

(A) Representative thionin-stained coronal sections showing the posterodorsal subnucleus of the medial amygdala (MePD). Sections are arranged from rostral to caudal. The dotted line indicates the boundaries of the area measured. Perinatal exposure to BPA or EE₂ had no significant effects on MePD volume (B). MePD volume was larger in males than females in all exposure groups. Significant sex differences in volume are represented by ††† $p < 0.001$, and †† $p < 0.01$. Error bars represent the 95% confidence interval and sample size is provided at the bottom.

Figure 5.

(A) Representative thionin-stained coronal sections showing the locus coeruleus (LC). Sections are arranged from rostral to caudal. The dotted line indicates the boundaries of the area measured. LC volume was significantly increased in males perinatally exposed to 0.5 μg EE₂/kg bw/day (B). BPA had no effects on LC volume. Significant differences in volume compared the same-sex vehicle group are represented by * $p < 0.05$. Error bars represent the 95% confidence interval and sample size is provided at the bottom.

Table 1Effect of Sex and Perinatal BPA or EE₂ Exposure on the Volume of Juvenile Rat Brain Nuclei.

Endpoint	Group	Effect of Sex	<i>p</i>
SDN Volume	Vehicle	F < M	<i>p</i> 0.001
	2.5 BPA	F < M	<i>p</i> 0.001
	25 BPA	F < M	<i>p</i> 0.001
	2500 BPA	F < M	<i>p</i> 0.001
	0.5 EE ₂	F < M	<i>p</i> 0.001
AVPV Volume	Vehicle	F > M	<i>p</i> 0.003
	2.5 BPA	↑F > M	<i>p</i> 0.001
	25 BPA	↑F > M↑	<i>p</i> 0.023
	2500 BPA	↑F > M↑	<i>p</i> 0.001
	0.5 EE ₂	F > M	<i>p</i> 0.001
Left MePD Volume	Vehicle	F < M	<i>p</i> 0.001
	2.5 BPA	F < M	<i>p</i> 0.001
	25 BPA	F < M	<i>p</i> 0.008
	2500 BPA	F < M	<i>p</i> 0.001
	0.5 EE ₂	F < M	<i>p</i> 0.010
Right MePD Volume	Vehicle	F < M	<i>p</i> 0.001
	2.5 BPA	F < M	<i>p</i> 0.001
	25 BPA	F < M	<i>p</i> 0.001
	2500 BPA	↑F < M	<i>p</i> 0.001
	0.5 EE ₂	F < M	<i>p</i> 0.006
Averaged MePD Volume	Vehicle	M > F	<i>p</i> 0.001
	2.5 BPA	M > F	<i>p</i> 0.001
	25 BPA	M > F	<i>p</i> 0.001
	2500 BPA	M > F	<i>p</i> 0.001
	0.5 EE ₂	M > F	<i>p</i> 0.001
LC Volume	Vehicle	M = F	ns
	2.5 BPA	M = F	ns
	25 BPA	M = F	ns
	2500 BPA	M = F	ns
	0.5 EE ₂	↑M = F●	ns

Notes: All brain nuclei except the LC were sexually dimorphic in size and there was no instance where exposure eliminated that difference. “↑” represents a significant increase in volume compared with the same-sex vehicle control. “ns” represents a p-value which was not significant.