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The impact of neonatal bisphenol-A exposure on sexually dimorphic hypothalamic nuclei in the female rat

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Abstract

Now under intense scrutiny, due to its endocrine disrupting properties, the potential threat the plastics component bisphenol-a (BPA) poses to human health remains unclear. Found in a multitude of polycarbonate plastics, food and beverage containers, and medical equipment, BPA is thought to bind to estrogen receptors (ERs), thereby interfering with estrogen-dependent processes. Our lab has previously shown that exposure to BPA (50mg/kg bw or 50µg/kg bw) during the neonatal critical period is associated with advancement of puberty, early reproductive senescence and ovarian malformations in female Long-Evans rats. Here, using neural tissue obtained from the same animals, we explored the impact of neonatal BPA exposure on the development of sexually dimorphic hypothalamic regions critical for female reproductive physiology and behavior. Endpoints included quantification of oxytocin-immunoreactive neurons (OT-ir) in the paraventricular nucleus (PVN), serotonin (5-HT-ir) fiber density in the ventrolateral subdivision of the ventromedial nucleus (VMNvl) as well as ERα-ir neuron number in the medial preoptic area (MPOA), the VMNvl, and the arcuate nucleus (ARC). Both doses of BPA increased the number of OT-ir neurons within the PVN, but no significant effects were seen on 5-HT-ir fiber density or ERα-ir neuron number in any of the areas analyzed. In addition to hypothalamic development, we also assessed female sex behavior and body weight. No effect of BPA on sexual receptivity or proceptive behavior in females was observed. Females treated with BPA, however, weighed significantly more than control females by postnatal day 99. This effect of BPA on weight is critical because alterations in metabolism, are frequently associated with reproductive dysfunction. Collectively, the results of this and our prior study indicate that the impact of neonatal BPA exposure within the female rat hypothalamus is region specific and support the hypothesis that developmental BPA exposure may adversely affect reproductive development in females.

Keywords

xenoestrogen; endocrine disruption; brain; sexual differentiation; development; estrogen receptor; neuroendocrine

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Conflicts of Interest None

1. Introduction

It is well established that mammalian neuroendocrine development is sensitive to changes in steroid hormone levels (Simerly, 2002; Wallen, 2005b). This sex specific organization of endocrine sensitive tissues and circuits is thought to be vulnerable to endocrine disruption by compounds that act as 'hormone mimics.' Such compounds can mimic or interfere with the natural organizational effects of hormones by interacting with hormone receptors or disrupting hormone-dependent signaling pathways (Gore, 2008; Welshons et al., 2006). Collectively referred to as endocrine disrupting compounds (EDCs), they are now ubiquitous in the human environment and their potential health effects are only beginning to be elucidated. The present study tested the hypothesis that exposure to the EDC bisphenol A (BPA) during the neonatal critical period interferes with the sex specific organization of the female rat hypothalamus. Each of the hypothalamic areas selected for the present study is well known to be densely populated with estrogen receptor (ER) containing neurons and critical for some aspect of female reproduction, including regulation of the estrous cycle, mating or maternal behaviors. We also assessed the impact of neonatal BPA exposure on sexual proceptivity and body weight, two endpoints of emerging concern (Monje et al., 2009; Ryan et al., 2010b).

Apprehension over the long-term health effects of neonatal and childhood exposure to EDCs arises from animal data indicating that the critical periods of fetal, infant and pubertal development are more sensitive to low doses of hormones than adult tissues, and thus more vulnerable to endocrine disruption (Selevan et al., 2000; Vom Saal and Moyer, 1985). Research focused on neonatal critical windows of development in rodents (prior to and immediately after birth) has shown that perinatal exposure to BPA can alter reproductive development and influence reproductive potential later in life, a concept referred to as the 'Fetal Basis of Adult Disease' (FEBAD) hypothesis (Heindel, 2005; Heindel and Levin, 2005; Patisaul and Adewale, 2009; Vandenberg et al., 2009). In rodents, perinatal exposure to low, environmentally relevant doses of BPA (ranging between 2µg/kg and 250µg/kg body weight) has been shown to advance puberty and reproductive senescence, alter estrous cyclicity, disrupt ovarian and mammary gland development and has been correlated with an increased incidence of mammary tumors (Adewale et al., 2009; Durando et al., 2007; Honma et al., 2002; Markey et al., 2001; Markey et al., 2003; Susiarjo et al., 2007). All of these reproductive and neuroendocrine endpoints are regulated by the hypothalamic-pituitary-gonadal (HPG) axis. Thus, disruption of hypothalamic sexual differentiation could be a unifying mechanism underlying this suite of effects induced by neonatal BPA exposure.

BPA entered commercial production in the 1950's after initially undergoing development as a possible synthetic estrogen (Dodds et al., 1938), and is now primarily used as a component of polycarbonate plastics, epoxy resins and thermal paper receipts (Biedermann et al., 2010). It can be found in a wide variety of food and beverage containers, medical equipment and plastic tubing, among other products (Calafat et al., 2009; Vandenberg et al., 2007; Vandenberg et al., 2009; Welshons et al., 2006). The Centers for Disease Control (CDC) has estimated that an estimated 93% of the US population has detectable levels of BPA in their bodies (Calafat et al., 2005). Urine analysis has detected BPA in men and women across all age groups, but children and hospitalized infants have significantly higher circulating levels of BPA than adults, prompting concern about the long term health effects that might result from developmental exposure (Calafat et al., 2009; Lakind and Naiman, 2010; Meerts et al., 2001; Welshons et al., 2006). Exposure can occur throughout life, including during fetal development because BPA fails to bind to α -fetoprotein and can thus jeopardize the developing fetus through placental transfer. Once the infant is born, further susceptibility can occur through lactational transfer from the mother, or by interacting with BPA-containing toys and products, thus exposing the child during multiple critical developmental

periods (Ikezuki et al., 2002; Nagel et al., 1999; Tsutsumi, 2005). The lowest observed adverse effect level (LOAEL) for BPA is currently set in the US at 50 mg/kg body weight (bw) per day and the “safe” or reference dose is defined as 50µg/kg bw per day.

Although evidence that BPA exposure to doses equivalent to or lower than the LOAEL can adversely affect the development of the female reproductive tract is relatively robust, whether or not it can affect neuroendocrine development remains unclear. We have previously shown, for example, that neonatal exposure to BPA can affect adversely affect ovarian development, pubertal timing, and the ability to maintain a regular estrous cycle in female rats (Adewale et al., 2009; Patisaul et al., 2009b). The goal of the present study was to determine, within this same group of animals, if sexually dimorphic hypothalamic development had also been disrupted. In rodents, estrogen is well recognized to masculinize hypothalamic regions which govern sex specific physiology and behavior, including ovarian cyclicity and sexual receptivity (Bachman, 1995; Baum, 1979; McCarthy, 2008; Simerly, 2002). In males, testosterone from the testis enters the bloodstream and is carried to the brain where it is aromatized to estrogen. It is this estrogen which then masculinizes the brain (Simerly, 1998; Simerly, 2002). The female brain, on the other hand, develops largely in the absence of estradiol and is considered to be feminized. Thus, exposure to EDCs that mimic estrogen could potentially masculinize the female hypothalamus. In primates, the relative role steroid hormones play in hypothalamic differentiation are less well understood but androgens are now recognize to be important for masculinization (Wallen, 2005b). To further elucidate potential mechanisms through which BPA might be acting within the female rat brain, we employed the ER α -selective agonist PPT as an additional positive control group to compare the effects of ER α agonism with BPA exposure. Estradiol benzoate (EB) and vehicle were also included as additional control groups (positive and negative respectively).

BPA is hypothesized to work through an ER mediated pathway and although the RBA of BPA for both ER α and ER β is similarly low (Kuiper et al., 1998; Welshons et al., 2003), at least one study has shown it to be a more effective ligand for ER β than ER α (Routledge et al., 2000). We therefore hypothesized that low dose exposure to BPA, during the neonatal critical window of postnatal days (PND) 0–4, would disrupt the organization of sexually dimorphic regions of the hypothalamus important in regulating female reproductive behavior and physiology, particularly those known to contain ER β . It was further hypothesized that this disruption would result in a male-like pattern of development in neonatally exposed females. The neonatal window was selected as the exposure period because it is a well recognized critical window of sensitivity in the rat (Bachman, 1995; Baum, 1979; Simerly, 2002), approximately akin to the 2nd trimester in humans (Bayer et al., 1993) (www.endocrinedisruption.com).

We focused on four ER-rich hypothalamic regions involved in female reproduction and which exhibit sexually dimorphic differences in volume (size or cell number) or hormone receptor expression between males and females. The first area of interest, the paraventricular nucleus (PVN), is the primary site of oxytocin (OT) synthesis. OT is crucial for many aspects of maternal, social, sexual and cognitive behaviors and in many species its release is sensitive to levels of estradiol (Amico et al., 1997; Lee et al., 2009; Neumann, 2008; Rosenblatt et al., 1988). Because OT neurons co-express ER β (also referred to as ESR2), but not the ER α (ESR1) subtype (Hrabovszky et al., 2004; Patisaul et al., 2003; Sharon and Allan, 1997), we hypothesized that any BPA-induced effects within this region are likely mediated via ER β and not through ER α . The second area of interest was the ventrolateral (vl) subdivision of the ventromedial nucleus (VMN) an area critical for regulation of female sex behavior (McCarthy, 2008; Pfaff and Sakuma, 1979). It receives numerous serotonergic (5-HT) projections and is sexually dimorphic, with males having a significantly greater

density of 5-HT fibers than females (Patisaul et al., 2008). The medial preoptic area (MPOA) was selected as the third area because it is another region critical for the regulation of the female reproductive cycle, as well as maternal and sexual behavior (Baskerville and Douglas, 2008; Charlton, 2008; Numan and Stolzenberg, 2009). The MPOA expresses both ER isoforms and exhibits sexually dimorphic expression of ER β but not ER α , with males showing greater expression of ER β than females (Kudwa et al., 2004; Weiser et al., 2008). The final area of interest was the arcuate nucleus (ARC), which, while involved in feedback regulation of the female reproductive cycle, is typically not considered to be sexually dimorphic in volume or cell number, thus we hypothesized that this area might be more resistant to endocrine disruption (Walsh et al., 1982). Body weight and sexual behavior were also assessed as part of this project. Elucidating the mechanisms by which BPA disrupts the ontogeny and function of the HPG axis will help establish whether or not this compound poses a potential threat to human health.

2. Materials and Methods

2.1. Animals and Neonatal Exposure

Animals used for this study were also used for a prior study and detailed methods concerning housing conditions, diet, behavioral testing and surgical procedures can be found in (Adewale et al., 2009; Patisaul et al., 2009b). Briefly, female pups were obtained from cross-fostered litters born to timed pregnant Long Evans rats (n = 10, Charles River, NC) and housed on a 12h light cycle at the Biological Resource Facility at North Carolina State University (NCSU). Throughout the study all animals were maintained on a semi-purified, phytoestrogen-free diet *ad libitum* (AIN-93G, Test Diet, Richmond, IN). Animal care, maintenance and surgery were conducted in accordance with the applicable portions of the Animal Welfare Act and the U.S. Department of Health and Human Services "Guide for the Care and use of Laboratory Animals" and approved by the NCSU Institutional Animal Care and Use Committee.

Beginning on the day of birth, the female pups were cross-fostered to minimize potential litter effects and subcutaneously (sc) injected with vehicle (0.05 ml sesame oil), estradiol benzoate (EB, 25 μ g, Sigma, St. Louis), 50 μ g/kg bw bisphenol-A (Low dose BPA, Sigma), 50 mg/kg bw bisphenol-A (High dose BPA), or the ER α agonist 4,4',4''-(4-Propyl-[1H]-pyrazole-1,3,5-triyl)trisphenol (PPT; 1 mg/kg bw, Tocris Biosciences, Ellisville, MS). PPT is a selective agonist for ER α , with a 400-fold preference for ER α and minimal binding to ER β (Stauffer et al., 2000). All compounds were dissolved in ethanol, and then sesame oil at a ratio of 10% EtOH and 90% oil as we have done previously (Adewale et al., 2009). The vehicle was also prepared with this ratio. We have found this vehicle to cause less skin irritation than the alternative vehicle, DMSO. The animals (n = 6–9 per group) received injections daily from the day of birth, defined as postnatal day zero (PND 0), through PND 3 (4 injections total).

2.2. Body Weight, Ovariectomy and Behavioral Testing

All pups were weaned into same-sex littermate pairs on PND 22, ear tagged, and maintained on a reverse light schedule (lights off from 10:00 to 22:00) for the remainder of the experiment. Body weight was recorded at PND 31 and PND 99 and females were ovariectomized (OVX) between PND 148 and PND 169 and allowed at least two weeks to recover before behavioral testing. To induce sexual receptivity to test for lordosis and proceptive behavior, the OVX females were sc injected with 10 μ g EB at 0900h, followed 48 hours later by a sc injection of 500 μ g progesterone (P) (same vehicle as above) and paired with vigorous males as described previously (Adewale et al., 2009; Bateman and Patisaul, 2008). Each test was 10 minutes in length.

We have previously reported no effect of BPA on lordosis quotient, used as a measurement of sexual receptivity (Adewale et al., 2009), but a more recent paper reported an effect of neonatal BPA on proceptive behavior (Monje et al., 2009). We therefore chose to revisit our data and quantify proceptive behavior as measured through the number of hops and darts performed by each female during the duration of the test. Hops and darts are solicitation or 'precopulatory' behaviors exhibited by sexually receptive female rats when in the presence of a potential mate and can be an additional indication of female sexual receptivity (Erskine, 1989). All behavior was scored from our archived video recordings of the behavioral tests by an observer blind to treatment groups.

2.3. Brain Collection, and Immunohistochemistry

Nineteen days after sex testing was completed, all animals were again sequentially administered EB and P and sacrificed by transcardial perfusion with 4% paraformaldehyde 6–8 hours after the progesterone injection as we have done previously (Bateman and Patisaul, 2008). This was done to simulate proestrous levels of estrogen and progesterone, an approach that is commonly used to standardize hormone levels and assess brain sexual dimorphisms (Becker et al., 2005). Brains were removed, post-fixed, cryoprotected, and stored at -80°C , then sliced into 35 μm coronal sections, and divided into four series of free-floating alternating sections, one of which was used for a previous study (Adewale et al., 2009). For each animal three sets of coronal sections were used. One set of coronal sections comprising the PVN, one containing both the VMNvl and the ARC and one containing the MPOA were immunolabeled using immunohistochemistry (IHC) methods described in detail elsewhere (Bateman and Patisaul, 2008; Patisaul et al., 2008).

2.4. Immunohistochemistry and Antibodies

Sections from the PVN were collected from a subset of animals (PPT group excluded due to an insufficient number of quality sections) and labeled for OT and FOS using a cocktail of primary antibodies directed at OT (raised in mouse, 1:20,000, Chemicon MAB5296) and FOS (raised in goat, 1:1000, SC-52-6, Santa Cruz Biotechnology). Secondary labeling was performed sequentially (using methods detailed in (Patisaul et al., 2006)) with the appropriate biotinylated secondary antibodies (1:200, Vector Laboratories, Burlingame, CA) and an avidin-biotin amplification step (ABC Elite, Vector Laboratories). OT was developed in 3,3'-diaminobenzidine (DAB) and FOS was developed in DAB with nickel enhancement (NiDAB). Sections comprising the VMNvl and the ARC were immunolabeled for 5-HT fibers and ER α using a cocktail of primary antibodies directed against 5-HT (raised in goat, 1:6000, Immunostar 20079) and ER α (raised in rabbit, 1:20,000, Upstate (now Millipore) 06-935) followed by secondary antibodies Alexa-Fluor donkey anti-rabbit 555 and Alexa-Fluor donkey anti goat 488 at 1:200 (Molecular Probes, A31572 and A11055 respectively). Sections comprising the MPOA were immunolabeled for ER α (1:20,000) followed by secondary antibody Alexa-Fluor donkey anti-rabbit 555 at 1:200. After secondary antibody incubation, sections were rinsed, mounted onto slides (Superfrost Plus, Fisher, Pittsburgh, PA), and coverslipped using a standard glycerol mountant.

2.5. Quantification of OT Cell Density and Activity

For each animal, numbers of OT-ir cells were quantified to assess cell density and the number of cells immunolabeled for both OT and FOS were counted to assess OT activity (Nishitani et al., 2004; Witt and Insel, 1994). Both were counted in three anatomically matched sections representing the rostral, middle and caudal regions of the PVN by unbiased stereology (Gundersen et al., 1988) using the stereology module of the MCID Core software package (InterFocus Imaging, Cambridge, England). Similar to what we have done previously (Patisaul et al., 2006), the unilateral contours of the PVN were drawn at low magnification (10 $\times$) from a live image and then analyzed and counted at higher

magnification (100×). The optical fractionator probe was then used to count the cells (25 × 25 μm² counting frame, 35 sampling sites per section). Once the cells were counted, the number of OT-ir cells and the percentage of double labeled cells were established for each animal, and the means for each treatment group subsequently calculated.

2.6. Quantification of 5-HT Fiber Density

5-HT fiber density within the VMNvl was visualized and quantified using a previously published protocol (Patisaul et al., 2009a; Patisaul et al., 2008). All sections were scanned using a Leica TCS SPE confocal microscope fitted with a 63× oil corrective objective lens. For each scan, a set of serial image planes (z-step distance = 1 μm) was collected through the entire thickness of each section. Image stacks were analyzed using the Image J software package (National Institutes of Health (NIH), Bethesda, MD) as previously described (Bateman and Patisaul, 2008). To control for variations in tissue thickness that would result in unequal numbers of image planes, substacks of consecutive image planes that excluded the rostral and caudal edges of the tissue sections were created for each set of scans. Substacks consisted of 31 sequential image planes. Anatomical identification was made using a brain atlas and only data from sections with consistent staining throughout the entire thickness were included in the analysis (Paxinos and Watson, 2004). Individual images contained within each substack were first binarized and depixelated to minimize the inclusion of background fluorescence. Fibers were then skeletonized to a thickness of one pixel to compensate for differences in individual fiber thickness and brightness. The number of resulting bright pixels in each image plane was then quantified using the Image J Voxel Counter plug-in (NIH). The voxel counts were averaged within the substack to obtain a single measure for each section that was then used as a quantitative representation of average 5-HT fiber density within the volume sampled (Bateman and Patisaul, 2008; Patisaul and Polston, 2008).

2.7. Quantification of ERα Cell Density

Coronal sections comprising the VMNvl, MPOA and ARC were imaged on a Leica TCS SPE confocal microscope fitted with 63× and 20× objectives. A set of serial image planes from the VMNvl and the ARC were collected at 63×, and from the MPOA at 20× throughout the entire thickness of the section. For the VMNvl and ARC, three anatomically matched sections per animal were scanned at a z-step distance of 1 μm and then standardized to substacks of 31 image planes. Within the MPOA two sections per animal were scanned at a z-step distance of 3 μm and standardized to substacks consisting of 10 planes for each section. All anatomical identification was made using a brain atlas and only data from sections with consistent staining throughout the entire thickness were included in the analysis (Paxinos and Watson, 2004). Individual images within each substack were thresholded and counted using the 3D object counter plugin for Image J. For images from the VMNvl and ARC the minimum object size detected was set at 200 voxels and for images within the MPOA minimum object size was set at 20 voxels. Maximum object size was left at the default value for all scans. All animals were counted by an observer blind to treatment groups and were screened for thresholding error; any cells missed by the 3D object counter were manually counted and added to the total number of cells. The two sections per animal collected for the MPOA were counted separately and then averaged together to determine ERα cell number.

2.8. Statistical Analysis

Statistical analysis of all data was done using SigmaPlot version 11.0. Proceptive behavior was analyzed by one-way ANOVA with exposure group as the factor. A one-way ANOVA followed up with a Fisher's Least Significant Differences (LSD) post hoc test was used to detect group differences in body weight and ERα cell numbers. For OT cell density we ran a

two-way ANOVA on mean percentage of double labeled OT/FOS-ir cells as well as the overall number of OT-ir cells. Based on the above results a region specific one-way ANOVA followed up with a Fisher's LSD post hoc test was run for each region. For 5-HT fiber density, based on previously published work, we hypothesized an increase in 5-HT fiber density to male-like levels, and therefore ran a one-way ANOVA followed up with a one-sided Dunnett's post hoc test against our OIL controls with the hypothesis that EB and PPT would increase 5-HT fiber density (Patisaul et al., 2009a; Patisaul et al., 2008).

3. Results

3.1. Neonatal BPA Increased Adult Body Weight

Body weight was assessed at PND 31 and PND 99. No significant differences in weight were observed at PND 31 but a significant effect of treatment emerged by PND 99 ($F(5,50) = 1.0$, $p < 0.427$ and $F(4,33) = 6.76$, $p < 0.001$ respectively). The high dose BPA treated females weighed significantly more than control females at PND 99 (Figure 1A; $p < 0.05$). Mean body weight for the control females was 290g, compared to 326g for the high dose BPA females and 296g for the low dose females. The mean body weight of the PPT treated females was 325g, a weight similar to the high dose BPA females, but not statistically significant from the control females due to high variability within this group ($p = 0.097$). As anticipated, the EB treated females weighed significantly more than the control females ($p < 0.001$, 374g).

3.2. Neonatal BPA Did Not Impact Sexual Proceptivity

Results concerning receptive behaviors were published previously (Adewale et al., 2009) so for the present study only proceptive behaviors were analyzed. One way ANOVA revealed no significant effect of exposure (Figure 1B). It was readily apparent, however, that although proceptive behaviors were not qualitatively impacted by neonatal exposure to either BPA or PPT, EB exposed females were not proceptive because none of these animals displayed any hopping or darting behaviors. These observations are consistent with our prior finding that sexual receptivity was unaffected in all but the EB exposed females (Adewale et al., 2009).

3.3 Numbers of OT Neurons, but not OT Activity, Within the Rostral PVN were Increased by Neonatal BPA

A two-way ANOVA on the overall number of OT neurons within the PVN indicated a significant main effect of region ($F(2,64) = 59.4$, $p < 0.001$), treatment ($F(3,64) = 2.78$, $p < 0.048$), and a significant interaction of region by treatment ($F(6,65) = 2.3$, $p < 0.045$). The number of OT neurons was greatest in the rostral portion of the PVN regardless of treatment. Within this subregion, neonatal exposure to EB ($p < 0.01$), high dose BPA ($p < 0.01$) or low dose BPA ($p < 0.05$) significantly increased the number of OT neurons (Figure 2). A two-way ANOVA for double labeled OT/FOS neurons found a main effect of region ($F(2,64) = 24.06$, $p < 0.0001$) but not treatment. The density of double labeled cells was greatest in the caudal sections ($p < 0.001$) compared to early and middle regions, but was unaffected by neonatal exposure.

3.4. 5-HT Fiber Density in the VMNvl was Unchanged by Neonatal BPA

There was a main effect of treatment on 5-HT fiber density within the VMNvl ($F(4,34) = 4.66$, $p < 0.004$) (Figure 3). As expected 5-HT-ir was significantly higher in females treated neonatally with EB ($p < 0.001$) compared to control females. Additionally, females treated with the ER α agonist PPT showed a significant increase in 5-HT fiber density compared to

controls ($p < 0.04$). There was no significant effect of BPA exposure at either the high (50mg/kg) or low (50µg/kg) dose ($p < 0.48$ and $p < 0.11$ respectively).

3.5. Numbers of ER α Neurons in the VMNvl, MPOA and ARC were Unchanged by Neonatal BPA

Quantification of ER α cell density within the VMNvl, MPOA and ARC revealed no significant effects of either dose of BPA, however, a main effect of treatment on ER α cell density within the MPOA ($F(4,35) = 5.74$, $p < 0.001$) was observed. Neonatal exposure to PPT or EB significantly reduced ER α -ir cell density within the adult female MPOA ($p < 0.001$ and $p < 0.028$ respectively) (Figure 4).

4. Discussion

Neonatal exposure of female rats to the plasticizer BPA impacted the sex specific organization of the hypothalamus in a region specific manner. Both doses of BPA significantly increased the number of OT neurons within the rostral PVN, an effect that, to our knowledge, has not yet been documented. The overall activity of these neurons in response to hormone priming, however, appeared to be unchanged as the density of OT and FOS colabeling did not differ among the treatment groups. BPA failed to alter any other hypothalamic endpoint evaluated, however, including 5-HT-ir innervation within the VMNvl or the number of neurons containing ER α within the VMNvl, MPOA or the ARC. Because the PVN contains primarily ER β , not ER α , and the regions where BPA had no significant effects densely express ER α , collectively the results suggest that BPA may be more active through ER β . Within the rodent hypothalamus, ER β is known to play a role in sexual differentiation during development and, while not as clearly understood, ER β is also known to be expressed in humans during gestation (Brandenberger et al., 1997; Enmark et al., 1997; Mosselman et al., 1996; Rissman et al., 1997; Shughrue et al., 1996; Weiser et al., 2008). It is therefore possible that interference with this receptor by BPA may adversely impact the development and organization of ER β mediated pathways. Additionally, we observed no impact of neonatal BPA exposure on female proceptive behaviors, a result which is in agreement with our previous data showing no affect of BPA on lordosis behavior (Adewale et al., 2009). We did observe, however, a dose dependent, significant increase in body weight. The 50mg/kg bw BPA females weighed an average of 36g (12.4%) more than controls causing concern that early exposure to BPA could contribute to adult weight gain.

4.1. BPA Increased OT Neuronal Number in the PVN

Here we have shown that neonatal BPA exposure can significantly increase the number of OT neurons within the anterior PVN, however, the physiological significance of this effect and the mechanism by which this occurs remains unclear. It is well established that ER β , but not ER α co-localizes with OT neurons throughout the PVN (Alves et al., 1998) an observation which leads us to hypothesize that BPA's effects within this nucleus are more likely via an ER β , rather than an ER α , mediated pathway. Recent evidence in support of the hypothesis that BPA is more influential on ER β mediated pathways within the brain and body emerged from a study revealing that BPA down regulates ER β mRNA expression in the hippocampus (Xu et al., 2010) and the observation that mice lacking ER β fail to display BPA-induced ovarian malformations seen in wild-type counterparts (Susiarjo et al., 2007). While such studies suggest that BPA works preferentially through ER β rather than ER α , the possibility that BPA also acts through non-classical estrogen mediated pathways cannot be ruled out. For example, BPA may also be working through one or more "non-classical" estrogen pathways, such as the membrane receptor GPR30 or other estrogen related receptors (ERRs) (Matsushima et al., 2008; Takayanagi et al., 2006; Takeda et al., 2009; Thomas and Dong, 2006).

Interestingly, the ability of PVN OT neurons to respond to estrogen does not appear to have been altered by neonatal exposure to BPA. Quantification of FOS induction in OT neurons following hormone priming (with estrogen and progesterone) revealed no significant group differences. Collectively our data indicate that although the total number of PVN OT neurons is increased by neonatal BPA exposure, their ability to respond to hormone priming is proportional to and not significantly different from control animals. Prior studies in knockout mice have shown that estradiol administration in adulthood increases OT mRNA expression in the PVN of wild-type, but not ER β knockout mice, indicating the importance of this receptor in estradiol mediated increases in OT (Nomura et al., 2002; Patisaul et al., 2003). Although we could not quantify ER β density because a suitable antibody is not currently available, the lack of group differences in OT activity following hormone priming suggest that ER β activity is, at least within this specific pathway, functionally normal in the BPA exposed animals.

In addition to OT and ER β , PVN neurons also express a myriad of other peptide hormones including vasopressin, galanin, growth-hormone releasing hormone, thyrotropin releasing hormone, vasoactive intestinal peptide and corticotropin releasing hormone among others (Kiss et al., 1991). As such, the PVN is recognized to play a pivotal role in mediating hypothalamic responses to stress, feeding and drinking behavior, lactation and parental care. The PVN is comprised of magnocellular and parvocellular regions each with distinct subdivisions that have well delineated distributions of neurotransmitter-specific neuronal populations (Armstrong et al., 1980; Swanson and Sawchenko, 1980). The anterior region is characterized by small to medium sized parvocellular neurons which send direct projections to the median eminence and other hypothalamic regions (Swanson and Kuypers, 1980; Swanson and Sawchenko, 1980) and a distinct population of OT-producing magnocellular neurons in a region now defined as the anterior commissural region but still considered part of the PVN (Armstrong et al., 1980; Kiss et al., 1991; Swanson et al., 1986). It is within these anterior subregions that BPA induced a significant increase in the number of OT-ir neurons. To reasonably predict the physiological and behavioral impacts of increased neuronal density, further work will be needed to better isolate specifically which other peptide hormones and neurotransmitters within these regions are impacted by developmental exposure to BPA. Our results, however, demonstrate that sexual behavior is not significantly impacted. OT is a key peptide hormone involved in a number of reproductive, social and maternal behaviors, including but not limited to: social recognition, nursing, maternal care, aggression, the facilitation of lordosis/reproductive behavior in non-lactating females and regulation of the female reproductive cycle (Baskerville and Douglas, 2008; Caldwell et al., 1994; Choleris et al., 2006; Ferguson et al., 2001; Insel et al., 1997; Lee et al., 2009; Neumann, 2008; Pedersen and Boccia, 2002; Schulze and Gorzalka, 1992). Though not specific to OT, BPA has been shown to affect maternal behavior of both the exposed dam and her pups. Exposure of pregnant rat dams to BPA during gestation and immediately after parturition reduced subsequent grooming of pups as well as time spent over the nest (Della Seta et al., 2005). In another study, female mice exposed to 10 μ g/kg BPA as pups or as pregnant adults, but not both, showed decreased time spent nursing and more time out of the nest than controls. Interestingly, females exposed both as pups and when pregnant as adults showed no difference from controls in maternal behavior (Palanza, 2002). Such studies indicate that our observed increase in OT neurons by BPA exposure could have behavioral consequences not assessed in this study.

Alterations in OT neuronal number could also have ramifications outside the PVN because OT has been shown to have important organizational effects throughout the brain. Thus the activity of these neurons in the BPA exposed animals may differ from controls during development rather than adulthood, a possibility we are now actively investigating. In neonatal voles, for example, OT expression is important for the regulation and induction of

ER α during development, and manipulation of OT can alter ER α expression in both males and females (Kramer et al., 2007; Pournajafi-Nazarloo et al., 2007). Although we found a significant effect of neonatal BPA on OT neuron number in the PVN, we did not find a significant effect on the number of ER α neurons in any region examined. It is plausible that ER α mRNA levels are impacted even though neuron number is not, a possibility that remains to be determined. OT release is not limited to the PVN thus effects may be more widespread. For example, the supraoptic nuclei (SON) also contains a distinct population of OT neurons (Gimpl and Fahrenholz, 2001) suggesting that it may also be vulnerable to endocrine disruption by BPA and other EDCs. Moreover, levels of OT and its receptor within the brain change as development progresses, making OT signaling pathways a potentially vulnerable target for EDC disruption across the lifespan, not just within the neonatal critical period (Tribollet et al., 1992). For example, in adult females of many species, OT expression increases concomitantly with estradiol and progesterone, especially during mating, pregnancy and lactation (Lee et al., 2009) (Amico et al., 1997; Broad et al., 1993). Thus, estrogen-mediated OT activity may be impacted by EDC exposure in adulthood. For instance, we have shown that ovariectomized, hormonally replaced female rats that consumed an over the counter soy supplement had lower levels of oxytocin receptor (OTR) expression in the VMNvl at proestrous, an effect which was accompanied by decreased sexual behavior (Patisaul et al., 2001). While OT and OTR regulation in adult and pubertal rodents is mediated largely by estradiol and progesterone, in neonates, OT release is also influenced by GABA, suggesting another possible route by which BPA could impact OT activity (Amico et al., 1997; Dreifuss et al., 1992; Hrabovszky et al., 2004; Ivell and Walther, 1999; McCarthy, 1995; Nomura et al., 2002; Patisaul et al., 2003; Shughrue et al., 2002; Viero and Dayanithi, 2008). The widespread distribution of OT within the neonatal rat brain and the sensitivity of OT release to estradiol indicate that the potential vulnerability of OT signaling pathways to EDC disruption might be an element of neonatal brain organization that has, so far, gone relatively unappreciated.

4.2. BPA did not Affect 5-HT Fiber Density in the VMNvl

The sexually dimorphic VMNvl is larger in males, who also possess more 5-HT fibers, and is a key area mediating gender specific sex behavior. It is also well known to be sensitive to changes in gonadal hormone levels, especially estrogen (Matsumoto and Arai, 1986; Patisaul et al., 2009a; Patisaul et al., 2008; Pozzo Miller and Aoki, 1991). We found no effect of BPA exposure on 5-HT-ir fiber density within the VMNvl however, consistent with our previous observations, selective agonism of ER α resulted in male-typical 5-HT-ir levels (Patisaul et al., 2009a; Patisaul et al., 2008). While serotonergic neuronal cell bodies are located in the dorsal raphe and nearly universally found colocalized with ER β , the density of 5-HT-ir fibers extending into the VMNvl appear to be sensitive to ER α , but not ER β agonism during neonatal life (Patisaul et al., 2009a). Serotonergic fibers make numerous contacts on ER α containing neurons within the VMNvl and although the density of these fibers is strongly sexually dimorphic, the functional role for this sex difference remains ambiguous. We previously hypothesized that 5-HT fibers contribute to the suppression of the lordosis response in males. This postulate is consistent with numerous studies showing that lesions within the VMNvl can restore lordosis behavior in male rats (Kakeyama and Yamanouchi, 1994; Moreines et al., 1988). In PPT exposed females, however, even though 5-HT levels appear to be elevated to male-typical levels, we found no concomitant decrease in female sexual behavior indicating that other neurotransmitters may be involved in abrogating this response. Finally, the ability of the ER α agonist PPT, but not BPA, to alter 5-HT-ir fiber density further supports the hypothesis that BPA influences hypothalamic organization through a non-ER α regulated mechanism.

4.3. BPA had No Effect on ER α Neuronal Number within the VMNvl, MPOA and ARC

We found no effect of BPA exposure on the number of ER α neurons in any of the regions examined. A significant reduction following neonatal EB or PPT exposure, however, was observed in the MPOA. The ability of EB and PPT to reduce ER α in the MPOA and not the VMNvl or ARC suggests that ER levels in the MPOA could be more sensitive to neonatal hormone exposure than in the other two regions. Unlike the VMNvl and ARC, which primarily express ER α , the MPOA expresses both ER α and ER β (Shughrue et al., 1998). The impact of neonatal PPT exposure on adult ER-ir indicates the potential sensitivity of this area to endocrine disruption, particularly to compounds with relatively potent activity on ER α . We found no impact of BPA on ER neuron number within the MPOA but this observation does not rule out the possibility that BPA may preferentially act through ER β , and thus alter ER β neuron number while not affecting ER α levels. Alternatively, under normal developmental circumstances, males typically express higher levels of ER β than females (Shughrue et al., 1997) thus the male MPOA may be more sensitive to BPA. Unfortunately we were not able to determine the impact of BPA on ER β cell density in the MPOA because a suitable antibody is not commercially available, but it would be intriguing to see if neonatal exposure to BPA differentially affected the expression of ER α and ER β within the male and female MPOA. Alternative experimental approaches could be used to address this possibility. Moreover, it is important to establish if EDCs acting primarily via ER α can elicit effects in this region similar to those of PPT.

It is important to note that the failure of BPA, PPT and EB to alter ER α expression in the VMNvl and the ARC could be due to the timing of exposure or the insensitivity of these particular endpoints to endocrine disruption. The ARC in particular is typically not considered to be sexually dimorphic in volume or cell number and is therefore thought to be more resistant to endocrine disruption during the neonatal period (Walsh et al., 1982). While not sexually dimorphic in adulthood, however, there is emerging research indicating the ARC may exhibit dimorphic organization during puberty (Kauffman, 2010) suggesting that sensitivity within the ARC could be age, hormone and/or receptor specific. Whether or not ER α or ER β agonism within any of these areas is sensitive to EDCs during other critical development periods remains to be determined.

4.5. Sexual Behavior

As each nucleus assessed within the hypothalamus is involved in some aspect of female reproductive behavior, we also looked at BPA's effect on female proceptivity. Consistent with our previous results indicating no effect of BPA or PPT on lordosis (Adewale et al., 2009), we found no effect on female proceptive behaviors. Although the EB exposed females displayed no signs of proceptivity this effect was not statistically significant. The observation is consistent, however, with the lack of sexual receptivity reported for these animals in our prior studies (Adewale et al., 2009). Intensity of behavior within each group, including our controls, was highly variable, which, although not atypical for complex behaviors, is likely why our EB females did not turn out to be significantly different from the control group. Collectively, these results suggest that agonism of both ER α and ER β , as seen by EB but not BPA or PPT, may be necessary to alter sexual behavior (Adewale et al., 2009; Patisaul et al., 2009a). A recent study reported decreased proceptive behavior in BPA treated females, but consistent with our observations, showed no difference in lordosis quotient (Monje et al., 2009). These differences could be due to the fact that different strains of rats were used in each study, along with a different dosing regimen. Additionally, our Long Evans rats were on a phytoestrogen free diet, while the Wistar rats used in the study by Monje and colleagues (2009) were not, thus effectively exposing the rats to multiple endocrine disrupting compounds simultaneously and introducing the potential for additive effects. Our data is also consistent with a number of other studies indicating the failure of

BPA exposure to elicit adverse effects on female lordosis or proceptivity in rodents (Farabollini et al., 1999; Kubo et al., 2003; Kwon et al., 2000; Ryan et al., 2010a). Additionally, we have previously shown that selective ER agonism and phytoestrogen exposure also fail to reduce lordosis behavior, suggesting that this pathway may be less sensitive to disruption by certain EDCs (Bateman and Patisaul, 2008).

4.6. Effects of BPA Exposure on Body Weight

We tracked the impact of BPA on total body weight because a relationship between developmental exposure and adult weight has recently become a point of considerable interest and forms the foundation for the Fetal Basis of Adult Disease (FEBAD) hypothesis (Heindel, 2005). Effects on body weight have been reported for a number of EDCs, including the pharmaceutical diethylstilbestrol (DES) and the phytoestrogens genistein and daidzein (Newbold et al., 2009). Our results add to a growing list of studies showing that perinatal BPA exposure can increase total body weight (Howdeshell et al., 1999; Nikaido et al., 2004; Patisaul and Bateman, 2008; Rubin and Soto, 2009). Here we demonstrate that this effect may be both dose and age dependent, with our high dose BPA (50mg/kg bw) females exhibiting a significant increase in body weight over controls by early adulthood (PND 99) but not puberty (PND 31). Importantly, human adipose tissue is known to express both ER isoforms (Hugo et al., 2008) and studies suggest that perinatal exposure to BPA may permanently alter the estrogenic sensitivity of peripheral tissues allowing the consequences of early exposure to persist into adulthood (Khurana et al., 2000; Markey et al., 2005; Rubin and Soto, 2009). This effect may predispose BPA exposed infants to obesity in adulthood, a condition associated with a number of medical and reproductive problems (reviewed in (Newbold et al., 2009). Consistent with this hypothesis, our previous study demonstrated that female rats from the 50mg/kg bw BPA group displayed early reproductive senescence and abnormal ovarian morphology compared to controls (Adewale et al., 2009).

Unfortunately definitive conclusions about the influence of BPA and other EDCs on body weight are hard to discern due to the variety of exposure methods and exposure windows seen in the literature (Newbold et al., 2009; Rubin and Soto, 2009). Certain trends, however, seem to be emerging and they support the theory that BPA, like many EDCs, works through a non-monotonic, or inverse “U” shaped, dose curve. Most of the low dose effects were seen when exposure of pups occurred during gestation and lactation as the mother was exposed orally, often via drinking water. For example, Rubin and colleagues exposed pregnant Sprague-Dawley dams to either 0.1mg/kg bw or 1.2 mg/kg bw via drinking water from gestational day (GD) 6 through the end of lactation (PND 21). Both groups of pups showed increases in body weight over controls in the first two weeks, however, as the pups aged the low dose group weighed significantly more than the high dose group from PND 11 to PND 110 (Rubin et al., 2001). Similarly, studies in mice have shown that dams exposed during gestation and lactation, via drinking water to doses 2–25µg/kg bw BPA, 1µg/ml and 10µg/ml, have pups with significantly higher body weight than controls at both pre and post pubertal time points (Miyawaki et al., 2007; Rubin and Soto, 2009). Miyawaki and colleagues found that while both the 1µg/ml and the 10µg/ml BPA doses increased body weight, the 1µg/ml dose increased adipose tissue weight significantly more than the 10µg/ml dose (Miyawaki et al., 2007; Rubin and Soto, 2009).

Not all gestational exposure studies have found lasting differences in body weight. In a recent study by Ryan and colleagues, mice exposed gestationally to low, environmentally relevant doses of BPA at 1 part per billion, showed an initial increase in body weight over controls, but this difference disappeared in adulthood (Ryan et al., 2010c). This group theorized that, because increased weight was accompanied by increased body length, this effect may represent a faster growth rate rather than a phenotype which predisposes the animal for obesity. Other studies utilizing direct, sc exposure of mouse pups during the first

week of life showed no effect of BPA on weight at doses of 10, 100 or 1000 μ g/kg bw (Newbold et al., 2007). These results contrast with ours but collectively, this body of research indicates that BPA's effects on metabolism and body weight are dose and time sensitive, and that exposure during gestation seems to have a greater impact on body weight than postnatal exposure. The variability in results is likely related to the non-monotonic dose response curve that has been reported for many actions of BPA, however the exact mechanisms through which BPA affects body weight are not fully understood and warrant further exploration (Rubin and Soto, 2009; Vandenberg et al., 2009; Welshons et al., 2006).

4.7. Summary and Conclusion

The question of whether or not the endocrine disruptor BPA poses a threat to human neuroendocrine and reproductive development remains under intense scrutiny. Recent media attention has raised public awareness of BPA, which can be found in a number of commercially available products and is estimated to be produced in volumes totaling over 6 billion pounds each year worldwide (Vandenberg et al., 2007; Vandenberg et al., 2009). Contamination of both human and wildlife populations occurs through numerous sources including the atmosphere, food and beverage containers, plastic pipes and plastic medical equipment, among others (Calafat et al., 2009; Lakind and Naiman, 2010; Le et al., 2008; Vandenberg et al., 2007). A recent review of the 2005–2006 National Health and Nutrition Examination Survey estimates that daily human exposure to BPA falls within the range of 25–50 ng/kg, with children under the age of 11 exhibiting higher levels of exposure than adults. This estimate, while below the EPA's reference dose of 50 μ g/kg bw/day, does not take into account non-dietary modes (Biedermann et al., 2010) or timing of exposure. The effects of BPA on development are still controversial, thus the method, source and timing of exposure are all very critical points that need to be taken into account when determining the potentially deleterious effects of BPA or any endocrine disrupting compound.

Although reproductive control in rodents and other mammals is governed by the hypothalamic–pituitary–gonadal (HPG) axis (McEwen et al., 1979; Simerly, 2002) there are key differences in how the human and rodent brain is sexually organized which must be taken into account when considering human risk. While sexual differentiation of the brain in rodents and many other species is known to be regulated by estradiol exposure, it is thought that androgens play a more influential role in the sexual differentiation of the primate (both human and non-human) brain (Herman et al., 2003; Muhlenstedt and Schneider, 1979; Wallen, 2005a). It is therefore possible that the actions of BPA and other EDCs on sexual differentiation of the brain in rodents are not predictive of concomitant effects in humans. However, it is important to recognize that the impact of BPA and other EDCs is not limited to the process of brain sexual differentiation, but also encompasses many other endpoints including: the timing of pubertal onset, regulation of the estrous or menstrual cycle, gonadal and reproductive tract development, body weight, hormone sensitive cancers as well as social and maternal behaviors (Benachour and Aris, 2009; Fujimoto et al., 2006; Gore, 2008; Hunt et al., 2003; Markey et al., 2001; Newbold et al., 2007; Palanza, 2002; Patisaul and Adewale, 2009; Rubin et al., 2001; Schönfelder et al., 2002; Susiarjo et al., 2007; Varayoud et al., 2008; Vivacqua et al., 2003; vom Saal et al., 2007). Additionally, while human brain differentiation may be more influenced by androgens rather than estrogens, the relative importance of each across fetal and neonatal life is far from certain. Moreover, the brain still expresses estrogen, and other hormone receptors, making it a potential target for EDCs.

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Figure 1.

(A) Body weight on PND99 was significantly impacted by neonatal exposure with females exposed to EB or the higher dose of BPA weighing significantly more than the vehicle (OIL) controls. (B) Effect of neonatal BPA exposure on female preceptive behaviors. There was no significant effect of exposure on preceptive hopping and darting behavior. Qualitatively, the EB exposed animals were not preceptive because none displayed this behavior. (* $p \leq 0.05$; ** $p \leq 0.001$)

Figure 2.

(A) Representative image depicting OT (white arrows) and OT/FOS double labeling (black arrows) within the PVN of a control (OIL) adult female rat. (B) Depiction of a co-labeled OT/FOS neuron observed at higher power. (C) Neonatal exposure to EB or BPA at either dose significantly increased the number of OT-ir neurons present within the adult PVN compared to OIL controls. The coexpression of OT-ir and FOS-ir was not significantly different between groups. (* $p \leq 0.05$; ** $p \leq 0.01$; scale bar = 50 μm)

Page 30 of 31

Figure 3.

Representative confocal images depicting 5-HT fibers within the VMNvl of a (A) vehicle treated (OIL) female and an (B) EB exposed female. (C) Neonatal exposure to EB or PPT significantly increased 5-HT-ir fiber density within the adult female VMNvl. Although there was no significant effect of either BPA dose, there was a trend for increased fiber density in the 50µg BPA group (* $p \leq 0.05$; ** $p \leq 0.001$; † $p = 0.11$; scale bar = 20µm).

Figure 4.

Representative confocal images depicting ERα-ir labeling within neuronal nuclei within the MPOA of a (A) vehicle treated (OIL) female and an (B) EB exposed female. (C) Average ERα-ir neuronal number within the MPOA. Neonatal exposure to either EB or PPT significantly decreased the number of ERα-ir cells within the adult female MPOA while BPA had no significant effect. (* $p \leq 0.03$, ** $p \leq 0.001$; scale bar = 40 μ m)