Published in final edited form as:

Neurotoxicology. 2012 January ; 33(1): 23–36. doi:10.1016/j.neuro.2011.11.002.

Neonatal Bisphenol A Exposure Alters Sexually Dimorphic Gene Expression in the Postnatal Rat Hypothalamus

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Abstract

Developmental exposure to Bisphenol A (BPA), a component of polycarbonate and epoxy resins, has been purported to adversely impact reproductive function in female rodents. Because neonatal life is a critical window for the sexual dimorphic organization of the hypothalamic-pituitary-gonadal (HPG) axis, interference with this process could underlie compromised adult reproductive physiology. The goal of the present study was to determine if neonatal BPA exposure interferes with sex specific gene expression of estrogen receptor alpha (ER α), ER beta (ER β) and kisspeptin (Kiss1) in the anterior and mediobasal hypothalamus. Long Evans (LE) neonatal rats were exposed to vehicle, 10 μ g estradiol benzoate (EB), 50 mg/kg BPA or 50 μ g/kg BPA by subcutaneous injection daily from postnatal day 0 (PND 0) to PND 2. Gene expression was assessed by in situ hybridization on PNDs 4 and 10. Within the anterior hypothalamus ER α expression was augmented by BPA in PND 4 females, then fell to male-typical levels by PND 10. ER β expression was not altered by BPA on PND 4, but significantly decreased or eliminated in both sexes by PND 10. Kiss1 expression was diminished by BPA in the anterior hypothalamus, especially in females. There were no significant impacts of BPA in the mediobasal hypothalamus. Collectively, BPA effects did not mirror those of EB. The results show that neonatal hypothalamic ER and Kiss1 expression is sensitive to BPA exposure. This disruption may alter sexually dimorphic hypothalamic organization and underlie adult reproductive deficiencies. Additionally, the discordant effects of EB and BPA indicate that BPA likely disrupts hypothalamic organization by a mechanism other than simply acting as an estrogen mimic.

Keywords

BPA; preoptic area (POA); arcuate nucleus (ARC); kisspeptin; estrogen receptor (ER); hypothalamus; development; sexual differentiation; brain; endocrine disruption

1. INTRODUCTION

BPA was initially developed as a possible synthetic estrogen (Dodds and Lawson, 1936), and is now a high volume production component of polycarbonate plastics, epoxy resins,

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dental sealants, thermal receipts and other products (Biedermann et al., 2010, Vandenberg et al., 2007). Human exposure is nearly ubiquitous, with urinary levels higher in children than adults (Calafat et al., 2008). The health impacts of BPA exposure remain controversial but growing evidence suggests that it has the potential to impose adverse outcomes on reproductive (Beronius et al., 2010, Cabaton et al., 2011, Howdeshell et al., 1999, Vandenberg et al., 2009) cardiovascular (Pant et al., 2011) and metabolic (Groff, 2010, Newbold, 2010) health. The hypothalamus is an integral part of each of these systems and thus conceivably a central target through which BPA could induce widespread effects across multiple organ systems. We and others have shown that BPA exposure can alter the sex specific organization of hypothalamic regions in the murine brain known to be important for coordinating gonadotropin release and sexual behavior, most notably the anteroventral periventricular nucleus of the hypothalamus (AVPV) (Patisaul et al., 2006, 2007, Rubin et al., 2006) and the arcuate nucleus (ARC) (Patisaul et al., 2009). Collectively these observations support the hypothesis that disruption of hypothalamic organization during critical windows of development could underlie a suite of neuroendocrine BPA effects.

Although long considered weakly estrogenic, the specific mechanisms by which BPA interacts with molecular and cellular targets within the hypothalamus and elsewhere are not clearly established (vom Saal et al., 2007). Classically, BPA is thought to disrupt genomic pathways mediated by the two primary forms of the nuclear estrogen receptor (ER); ER alpha (ER α) and ER beta (ER β). Compared to estradiol, BPA has a binding affinity approximately 10,000–100,000 fold weaker for both ER isoforms (Andersen et al., 1999, Barkhem et al., 1998, Blair et al., 2000, Gould et al., 1998) but appears to bind each with relatively equal affinity (Kuiper et al., 1998) indicating that it has the potential to interact with either.

It is well established that estrogen is masculinizing during perinatal development in the murine brain, and that exposure to even low levels of estrogen or xenoestrogens during this time can permanently alter neuroendocrine pathways critical for mediating steroid negative feedback, gonadotropin release, energy homeostasis and sexual behavior (Amateau et al., 2004, Bader et al., 2011, Faulds et al., 2011, Gore, 2008, Simerly, 2002). We have recently shown that the expression of ER α and ER β is sexually dimorphic within numerous subregions of the neonatal rat hypothalamus (Cao and Patisaul, 2011). For example, within the AVPV, ER β expression is higher in males on the day of birth while ER α expression is higher in females, suggesting that the two isoforms may play different functional roles in the sexual differentiation of this region. This sex difference is transient, and eliminated within 48 hours, suggesting that if BPA exposure during only the first two days of life alters expression levels, estrogen sensitivity during this critical period of sexual differentiation may be impacted. Here we tested the hypothesis that neonatal BPA exposure can affect the sex specific hypothalamic expression of ER α and ER β across postnatal development, focusing specifically on the AVPV, ARC and two other subregions (the medial preoptic area (MPOA) and the ventrolateral division of the ventromedial nucleus (VMNvl)) known to be essential for the neuroendocrine control of gonadotropin release and other aspects of female reproductive physiology, metabolic regulation and behavior (Nance, 1976, Pfaff and Keiner, 1973, Pfaff et al., 1994, Simerly, 2002). Although previous studies have explored similar hypotheses in a variety of species, they primarily looked at adult expression in only a single sex and either employed a different exposure window, (Ceccarelli et al., 2007), focused on only one hypothalamic area (Mahoney and Padmanabhan, 2010, Monje et al., 2007), or employed techniques with less anatomical resolution such as QT-PCR (Khurana et al., 2000, Monje, Varayoud, 2007). Thus, the present studies build on this prior work by (1) characterizing the specific hypothalamic subregions vulnerable to disruption in both sexes (2) identifying this disruption early in postnatal development and (3) more carefully identify the critical window of exposure for ER disruption by BPA. Collectively, these studies test

the hypothesis that perturbation of ER expression during the neonatal period of hypothalamic sexual differentiation could be a mechanism by which BPA induces a wide range of effects, some of which may not emerge until adulthood.

We also sought to determine if BPA exposure can disrupt the expression of *Kiss1*, a gene which codes for the kisspeptin family of peptides. Kisspeptin is now recognized to be the primary “gatekeeper” of gonadotropin release and essential for initiating pubertal onset (Oakley et al., 2009, Pineda et al., 2010). It also plays a role in energy homeostasis and is a putative effector of leptin actions on gonadotropin releasing (GnRH) neurons (Castellano et al., 2010). There are two populations of *Kiss1* neurons in the murine brain, an anterior group which extends through the AVPV into the MPOA (a region now collectively referred to as the rostral periventricular area of the third ventricle (RP3V)) and a mediobasal hypothalamic group confined to the ARC. In adults, the RP3V population is sexually dimorphic, with females having higher expression levels than males (Kauffman et al., 2007), while the ARC population is not (Losa et al., 2010). These observations suggest that the anterior population participates in steroid positive feedback on gonadotropin release, while the posterior population plays a role in steroid negative feedback (Oakley, Clifton, 2009, Pineda, Garcia-Galiano, 2010). Nearly all *Kiss1* neurons in the RP3V and ARC co-express ER α , and a subset of RP3V *Kiss1* neurons co-express ER β (Smith et al., 2005, Smith et al., 2006) suggesting that disruption of ER expression within this neuronal phenotype could affect its sex specific ontogeny and function. Gestational and/or postnatal BPA exposure has been shown to alter *Kiss1* mRNA expression within whole hypothalamic micropunches (Navarro et al., 2009, Xi et al., 2010), and RP3V *Kiss1*-immunoreactivity (Bai et al., 2011, Panzica et al., 2009) during peripuberty and adulthood. Although RP3V *Kiss1* expression is not apparent in either sex until approximately the second week of life (Cao and Patisaul, 2011, Clarkson et al., 2009) we have shown that neonatal exposure to estrogen or an ER α selective agonist (Bateman and Patisaul, 2008, Patisaul, Todd, 2009) can defeminize kisspeptin signaling pathways in the adult RP3V, suggesting that the first few days of life may be a critical window of BPA vulnerability, and that this disruption may be apparent prior to puberty. In contrast to the RP3V, *Kiss1* mRNA in the ARC is robustly expressed from PND 0 through PND 19, with higher levels in females than males within the first week of life (Cao and Patisaul, 2011). This indicates that *Kiss1* expression may be influenced by the neonatal estrogen and vulnerable to endocrine disruption by a chemical like BPA. Here we examined whether neonatal BPA exposure affects the sexual dimorphic ontogeny of *Kiss1* mRNA expression in the RP3V and ARC.

The present study is the first to precisely characterize when, where and how neonatal BPA exposure affects sexual dimorphic ER and *Kiss1* gene expression in the postnatal rat brain. Collectively, the results reveal the potential for BPA to dysregulate the differential expression of ER α , ER β and *Kiss1* during a critical window of hypothalamic organization, findings which will help elucidate the mechanisms by which BPA can induce persistent effects across neuroendocrine systems.

2. MATERIALS AND METHODS

2.1 Animal care, neonatal exposure and tissue collection

Pups were born to timed pregnant Long Evans rats ($n = 13$; Charles River, Raleigh, NC), housed at the Biological Resource Facility at North Carolina State University (NCSU) under a 14:10 h light: dark cycle (lights on at 0700 h) at 23°C and 50% average relative humidity. Animals were housed in thoroughly washed polysulfone caging with woodchip bedding, and fed a semi-purified, phytoestrogen-free diet ad libitum (AIN-93G, Test Diet, Richmond, IN) to minimize exposure to exogenous BPA, phytoestrogens, and other endocrine disrupting compounds (Brown and Setchell, 2001, Degen et al., 2002, Thigpen et al., 2007). All

animals were maintained 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 all studies were approved by the Institutional Animal Care and Use Committee of NCSU.

Beginning on the day of birth (defined as postnatal day zero (PND0)), female and male pups (n = 6–9 per sex per group) were subcutaneously (sc) injected with vehicle, estradiol benzoate (EB, 10 μ g, Sigma, St. Louis), low dose (50 μ g/kg bw) BPA (LBPA, Sigma), or high dose (50 mg/kg bw) BPA (HBPA). The high dose is the lowest observed adverse effect level (LOAEL) for oral exposure established by the U.S. Environmental Protection Agency (EPA), and the low dose is the FDA reference dose considered “safe” for human oral exposure. Although oral administration would have reflected the most typical human exposure route, injection was used to ensure consistent exposure across individual animals for this, primarily mechanistic, study. Injection likely results in a higher internal dose than oral exposure (Doerge et al., 2010) although at least one study has shown that this difference is not significant in neonatal mice (Taylor et al., 2008). EB was included as a positive control at a dose sufficient to induce complete masculinization of the hypothalamus and prevent the onset of regular estrous cycles (Aihara and Hayashi, 1989, Nagao et al., 1999). All compounds were first dissolved in 100% ethanol (EtOH, Pharmaco), and then sesame oil (Sigma) at a ratio of 10% EtOH and 90% oil (Patisaul, Fortino, 2006). The vehicle was a mixture of 10% EtOH and sesame oil. Injections (0.05 ml) were administered every 24 h as described previously (Patisaul, Todd, 2009) from PND 0 through PND 2. This neonatal critical period in rodents is approximately equated with the late second and third trimesters of human brain development (Grumbach, 2002).

Litter sizes ranged from 9 to 17 pups and were not standardized for size or sex ratio. All pups within the litter were administered the same compound to prevent cross-contamination (3 litters each for vehicle, EB, and LBPA, 4 litters for HBPA). Further measures taken to reduce cross-contamination included storing the vials in separate containers, changing gloves after each litter was handled, always handling the vehicle control litters first, and always handling the EB exposed litters last. Each injection was administered slowly enough to minimize seepage and the pups were not returned to the dam until the injection site had healed enough to be leak-free (about 15 minutes post-injection). Pups were sacrificed by rapid decapitation on PNDs 4 and 10, the heads rapidly frozen on powdered dry ice and stored at –80°C until cryosectioning. To minimize potential litter effects, each experimental group contained no more than 2 pups of each sex per litter, and each experimental group contained pups from at least 3 litters.

2.2 In situ hybridization histochemistry (ISHH)

All brains were cryosectioned (Leica CM1900, Nussloch, Germany) into three serial sets of 18 μ m coronal sections, mounted onto Superfrost plus slides (Fisher Scientific, Pittsburgh, PA), and stored at –80°C until ISHH processing. For each animal, one set of sections was used for ISHH of ER α , ER β and Kiss1. For each gene, all sections containing the anterior hypothalamus were processed simultaneously as a large batch, then all sections containing the mediobasal hypothalamus were processed as a subsequent batch using transcriptional templates for ER α , ER β and Kiss1 generated and used as described previously (Cao and Patisaul, 2011). The hybridized slides were apposed to Kodak Biomax MR X-ray film (Eastman Kodak, Rochester, NY, USA) for 14 to 40 days depending on the ROI (Cao and Patisaul, 2011). Autoradiographic 14C microscopes (Amersham Life Sciences, Arlington Heights, IL, USA) were included to generate the optical density curve needed for quantification.

Because Kiss1 expression is weak on PND 10 (Cao and Patisaul, 2011, Clarkson, Boon, 2009), and thus difficult to accurately quantify by optical density, the hybridized Kiss1 slides were dipped in NTB3 emulsion (Kodak, Rochester NY), developed, counterstained, and the resulting silver grains quantified as described previously (Cao and Patisaul, 2011).

2.3 Image analysis and quantification

Autoradiograms depicting ER α , ER β , and Kiss1 signal were imaged and quantified using the digital densitometry application of the MCID Core Image software program (InterFocus Imaging Ltd, Cambridge, England) following conventional procedures similar to what we and others have described elsewhere (Cao and Patisaul, 2011, Kuhar et al., 1986, Patisaul et al., 1999). In the anterior hypothalamus, labeling was quantified in the AVPV and MPOA. In the mediobasal hypothalamus, labeling was quantified in the VMNvl and ARC. Because the VMNvl and ARC are larger than the AVPV and MPOA, these regions were subdivided into rostral and caudal portions (designated the rostral VMNvl (rVMNvl) and rostral ARC (rARC), and the caudal VMN (cVMNvl) and caudal ARC (cARC) respectively. For both the VMNvl and the ARC, the beginning of the caudal subregion was defined as the caudal border of the lateral part of the retrochiasmatic area (Rchl).

Expression at each age (PNDs 4 and 10) was analyzed separately using a sampling template encompassing the region of interest (ROI) to standardize the area examined. Identification of each ROI was conducted using conventional landmarks and the aid of a standard rat brain atlas (Paxinos and Watson, 2007) as detailed previously (Cao and Patisaul, 2011). ROI and background levels were measured bilaterally from anatomically matched sections. For quantification of all three genes, 2 bilateral sections per animal were used for the AVPV and MPOA analysis, and also for each subregion of the VMNvl and ARC. For quantification of ARC Kiss1, 4 bilateral sections per animal were used. The resulting values for each brain section after background subtraction were then averaged to obtain a representative measurement (for that region) for each animal. Optical densities were converted to nCi/g tissue equivalents using a “best fit” curve (3rd degree polynomial) generated from the autoradiographic ¹⁴C microscales. In all cases, signal was within the limits of the curve.

The emulsion dipped and counterstained slides containing Kiss1 labeling, were imaged using a Retiga 2000R color camera (QImaging, Surry, British Colombia, Canada) under the 20 $\times$ plan apo objective for ARC and 40 $\times$ for the RP3V of our Leica 5000DM microscope. The presence of dense clusters confined within discreetly counterstained nuclei was considered to be confirmation of label specificity and identified as Kiss1 neurons.

2.4 Statistics

All datasets were first tested for homogeneity of variance using a Bartlett’s test then analyzed by two-way analysis of variance (ANOVA) with sex and exposure group as factors. A significant interaction was found in almost all cases, and one way ANOVA was then used to analyze the effect of exposure within each sex. Significant effects were followed up with the Dunnett’s Multiple Comparison post hoc test to compare each exposure group to the same sex vehicle control group. T-tests were used to identify sex differences in mRNA expression within each ROI. All analyses were two-tailed and results were considered significant when $P \leq 0.05$.

3. RESULTS

3.1 Summary of Results

A summary of the overall results is provided in Table 1. In the anterior hypothalamus, ER expression was higher in females than males by PND 10 in the MPOA but equivalent to

male expression in the AVPV. Neonatal exposure to EB eliminated the sex differences primarily by reducing ER expression in females. BPA effects were dose and age dependent. On PND 4, ER α expression was augmented in both sexes of the HBPA group, but, by PND 10, female expression was lower compared to the unexposed controls. LBPA exposure produced lower ER α expression in the female AVPV and male MPOA by PND 10 but no appreciable effects in either sex were detected on PND 4. ER β expression was significantly reduced in both BPA exposure groups by PND 10 with AVPV expression virtually eliminated in both sexes. In the posterior hypothalamus, ER expression was higher in females compared to males on both PND 4 and 10 and neonatal EB exposure eliminated these differences by abrogating expression in females. BPA had no effect on ER expression in females but the low dose resulted in elevated ER β expression in the male cVMNvl by PND 10. As expected (Cao and Patisaul, 2011, Clarkson, Boon, 2009, Clarkson and Herbison, 2006), Kiss1 was barely detectable in the PND10 RP3V, and EB exposure eliminated expression while BPA exposure appeared to reduce it in females. In the ARC, Kiss1 levels were higher females compared to males on PNDs 4 and 10 and EB exposure lowered female expression thereby eliminating the sex difference. Neonatal BPA exposure did not eliminate the sex difference in expression levels at either age examined.

3.2 ER α mRNA expression in the RP3V

As expected (Cao and Patisaul, 2011), in the vehicle control groups ER α expression was sexually dimorphic and readily detected in all subregions examined, with the density of ER α mRNA signal generally higher in the MPOA than the AVPV, and more robust on PND 4 than PND 10 (Figure 1).

3.2.1 AVPV—In the PND 4 animals, two way ANOVA revealed a significant effect of sex ($F(1, 36) = 15.23, P \leq 0.0004$) and exposure group ($F(3, 36) = 16.5, P \leq 0.0001$) plus a significant interaction ($F(3, 36) = 12.4, P \leq 0.0001$) on ER α expression. There was a significant effect of exposure in females ($F(3, 18) = 13.89, P \leq 0.0001$) and males ($F(3, 18) = 6.363, P \leq 0.004$) (Figure 1A,B), with ER α expression significantly higher in both sexes of the HBPA group compared to the same sex vehicle controls, but unaltered in either sex of the LBPA group. In contrast, EB exposure reversed the sexual dimorphic expression of ER α . By PND 10 (Figure 1D,E), ER α mRNA expression was appreciably lower and no longer sexually dimorphic in the EB and BPA exposed groups. Two way ANOVA revealed a significant effect of exposure group ($F(3, 36) = 13.29, P \leq 0.0001$) but not sex, and a significant interaction ($F(3, 36) = 3.623, P \leq 0.02$). ER α expression was only altered in females ($F(3, 20) = 25.34, P \leq 0.0001$), and significantly lower than controls in the EB ($P \leq 0.01$) and BPA groups ($P \leq 0.01$).

3.2.2 MPOA—On PND 4, there was a significant effect of sex ($F(1, 42) = 93.12, P \leq 0.0001$) and exposure group ($F(3, 42) = 27.67, P \leq 0.0001$) as well as a significant interaction ($F(3, 42) = 15.35, P \leq 0.0001$) on ER α expression. Within females, ($F(3, 19) = 19.96, P \leq 0.0001$), EB and BPA produced opposite effects, with expression lower in the EB group ($P \leq 0.05$) but elevated in the HBPA group ($P \leq 0.01$). In males ($F(3, 23) = 11.08, P \leq 0.0001$) expression was only elevated in the HBPA group ($P \leq 0.01$). By PND 10 (Figure 1D,F) the significant effect of sex ($F(1, 46) = 48.85, P \leq 0.0001$), and exposure group ($F(3, 46) = 30.06, P \leq 0.0001$) as well as a significant interaction ($F(3, 46) = 25.53, P \leq 0.0001$) remained. In females ($F(3, 23) = 39.09, P \leq 0.0001$), ER α expression was significantly lower in the EB and HBPA groups but unaffected in the LBPA group. The sexually dimorphic expression of ER α was eliminated by HBPA, due primarily to reduced female levels, and reversed by EB. In males, ER α expression trended lower in all exposure groups but only reached statistical significance in the LBPA group ($P \leq 0.01$).

3.3 ER α mRNA expression in the mediobasal hypothalamus

Expression was generally higher in the VMNvl than the ARC at both ages examined and more intense in the caudal subregions (Figure 2 A,F). In all areas examined, expression was sexually dimorphic and higher in females and only EB significantly impacted ER α expression (Figure 2). In EB exposed females, ER α expression was reduced to male-typical levels ($P \leq 0.01$ in all cases) but BPA produced no appreciable effects in either sex with the exception of elevated levels in the cARC of the LBPA females ($P < 0.05$; Figure 2E).

3.4 ER β mRNA expression in the RP3V

ER β mRNA signal (Figure 3) was not as robust as ER α in the anterior hypothalamus, stronger on PND 4 than 10, and mainly distributed in the periventricular portion of the AVPV and MPOA. Among the control animals, expression was sexually dimorphic only on PND 10 in the MPOA.

3.4.1 AVPV—On PND 4 (Figure 3B) two-way ANOVA revealed a significant effect of exposure group ($F(3, 42) = 6.786$, $P \leq 0.0008$) but not sex. One-way ANOVA indicated this effect was primarily attributable to the difference between the EB and LBPA groups ($P \leq 0.05$) rather than a significant difference from control levels. By PND 10, however, two-way ANOVA indicated a significant group effect ($F(1, 20) = 62.49$, $P \leq 0.021$) with levels significantly lower than same sex controls in the EB exposed females, and no visible signal in either sex in the BPA exposed groups (Figure 3E).

3.4.2 MPOA—Two-way ANOVA of PND 4 animals (Figure 4) revealed only a trend for an effect of sex ($F(1, 49) = 0.3519$, $P = 0.056$) or exposure group ($F(3, 49) = 2.736$, $P = 0.054$) with levels lowest in the HBPA females. By PND 10, however, there was a significant effect of sex ($F(1, 42) = 567.1$, $P \leq 0.0001$) and exposure group ($F(3, 42) = 795.2$, $P \leq 0.0001$) as well as an interaction ($F(3, 42) = 366.6$, $P \leq 0.0001$). Both females ($F(3, 21) = 23.45$, $P \leq 0.0001$) and males ($F(3, 21) = 3.314$, $P \leq 0.04$) were affected by exposure. The sex difference in ER β expression was eliminated by EB, due to decreased expression in females ($P \leq 0.01$). Levels were also lower in both sexes following BPA exposure, regardless of dose, compared with same sex vehicle controls, an effect which eliminated sexually dimorphic expression in the HBPA group (Figure 3F).

3.5 ER β mRNA expression in the mediobasal hypothalamus

As expected (Cao and Patisaul, 2011, Hrabovszky et al., 2001), ER β expression was absent in the ARC and much more robust in the cVMNvl compared to the rVMNvl (Figure 4). Expression was sexually dimorphic, with higher levels in females throughout the VMNvl. Only EB significantly altered expression in the PND 4 animals, masculinizing female expression, thereby eliminating the sex difference. BPA had no appreciable effects at either dose in either sex on PND 4. By PND 10 levels remained masculinized in the EB exposed females and again no significant effect of BPA was observed with the exception of slightly higher levels in the cVMNvl of the LBPA exposed males ($P \leq 0.05$).

3.6 Kiss1 mRNA expression in the RP3V and mediobasal hypothalamus

RP3V expression was only quantified in the PND 10 animals (Figure 5) because Kiss1 is not expressed prior to this age (Cao and Patisaul, 2011, Clarkson, Boon, 2009, Clarkson and Herbison, 2006). Among the vehicle controls, only half of each sex showed silver grain clusters indicative of positive neuronal labeling (Figure 5A). No clusters were present in any of the EB exposed animals, regardless of sex (Figure 5B). In the LBPA group, 1 of 8 females and 2 of 7 males had silver grain clusters. None of the HBPA exposed females had clusters, but 3 of 7 males did. Quantification of silver grain clusters revealed a robust sex

difference in the vehicle controls, with levels higher in females, but a reversal of this difference in the BPA exposed groups, with no appreciable labeling in the HBPA females (Figure 5C).

In the ARC, Kiss1 was appreciable from the autoradiograms (not shown) and the emulsion dipped slides (Figure 6 A,C) and sexually dimorphic on both PNDs 4 (Figure 6B) and 10 (Figure 6D) with expression higher in females. Expression levels were quantified in the ARC as a whole because density and intensity is homogenous throughout the ARC (Cao and Patisaul, 2011). EB exposure reduced Kiss1 expression to male-typical levels but BPA had no appreciable impact in females of either age. A significant effect of exposure group was found in the PND 4 males ($F(3, 26) = 4.828, P \leq 0.008$) with expression slightly but significantly higher in the HBPA males ($P \leq 0.05$) but a significant sex difference remained.

4. DISCUSSION

The present study provides comprehensive evidence that neonatal exposure to BPA alters early postnatal, sexually dimorphic expression of ER α , ER β and Kiss1 mRNA in the rat hypothalamus, particularly in the RP3V region. Disruption of ER expression during this time period may have lifelong consequences because it is the age at which the hypothalamus is undergoing steroid hormone directed sexual differentiation. Perturbation of ER expression by BPA presumably alters sensitivity to endogenous estrogen during this critical window of development. Thus, these findings reveal a possible mechanism by which the sex specific ontogeny of hypothalamic pathways may be altered, and highlight that the critical period for disruption within the murine anterior hypothalamus may be as short as 72 hours. This period approximately equates to the third trimester in humans. Further work will be needed, however to confirm this hypothesized link between disrupted early life hypothalamic gene expression and altered adult neuroendocrine physiology.

Neonatal BPA exposure disrupted gene expression throughout the hypothalamus but in a dose, temporal, and region specific manner that did not consistently mirror that of EB exposure in either sex. As anticipated, EB exposure completely masculinized ER expression across the female hypothalamus except for RP3V ER β expression on PND 4, which was largely unaffected by EB. By contrast, in the AVPV, ER α levels were markedly elevated in females following BPA exposure on PND 4, then fell to male-typical levels by PND 10. The drop in ER β expression by PND 10 was even more robust, resulting in no detectable signal at either dose. In the VMNvl, however, ER levels were relatively unaltered by BPA but completely masculinized by EB. These observations are commensurate with prior studies by us and others showing no impact of neonatal BPA exposure on the number of ER α -immunoreactive cells in the adult female VMNvl (Adewale et al., 2011), or in ER α and ER β expression in juvenile rats (Ramos et al., 2003). Similarly, EB exposure resulted in male-typical ER α and ER β levels in the PND 4 and 10 female ARC but BPA had no significant effects on ER expression in this region. These divergent effects of BPA suggest that it alters hypothalamic organization by mechanisms other than by simply acting as an estrogen mimic, and demonstrate that some brain regions are more sensitive to disruption than others.

Our results are consistent with a prior, RT-PCR study, which also found a pronounced sex difference in ER α expression on PND 8 (Monje, Varayoud, 2007) and that sc injection of 50 μ g/kg BPA every 48 hours from PND 1 to 7 abrogates PND 8 female ER α expression to male levels while sc injection of 20 mg/kg produces a modest increase. Collectively, the data suggest that acute administration of high dose BPA initially upregulates ER α throughout the POA of both sexes but this expression pattern changes over time once exposure ceases, declining in the AVPV and MPOA of the HBPA females, but only in the AVPV of the LBPA females. Interestingly, by adulthood, ER α levels are reportedly higher

in the AVPV of females neonatally exposed to 50 µg/kg or 20 mg/kg BPA compared to same sex controls (Monje et al., 2010) but not the MPOA of females neonatally exposed to 50 µg/kg or 50 mg/kg (Adewale, Todd, 2011). Moreover, peripubertal BPA exposure increases female ER immunoreactivity in the MPOA and VMN on PND 37, but this effect is lost by PND 90 (Ceccarelli, Della Seta, 2007). Temporal changes in estrogen sensitive gene expression have also been reported for the selective estrogen receptor Tamoxifen (Patisaul et al., 2003) suggesting that this type of long term regulatory disruption may not be atypical for xenoestrogenic compounds. The mechanisms underlying this effect remain to be determined but altered expression of critical and cell specific ER coactivators or corepressors is one possibility.

The complicated biochemical character of BPA was also demonstrated by the observation that it can augment ER α expression but depress or eliminate ER β expression within the same hypothalamic region. The mechanisms by which BPA, or even endogenous estrogen, can differentially regulate the two ER isoforms remains poorly characterized. One possibility is that epigenetic modification by DNA methylation (Kurian et al., 2010) and/or histone deacetylation (Matsuda et al., 2011) may alter the status of each ER promoter independently thus accounting for the different expression levels of the two ERs. It is now clear that methylation status is dynamic across development, and may play an important role in the mediation of hormone dependent neonatal brain organization (Nugent et al., 2011). Importantly, BPA has previously been shown to be capable of modifying DNA methylation patterns (Dolinoy et al., 2007) suggesting that it at least has the potential to affect ER expression through epigenetic mechanisms.

The specific functional roles ER α and ER β play in the sex specific organization of the hypothalamus have not been fully characterized but it has been hypothesized that ER α is critical for the organization of reproductive neuroendocrine pathways while ER β may facilitate the emergence of sex appropriate behavior (Rissman, 2008). Thus, the diminished ER β levels observed in the anterior hypothalamus of the BPA exposed males may be a potential mechanism by which perinatal BPA exposure disrupts sexual and sociosexual behaviors (Farabollini et al., 2002, Farabollini et al., 1999, Patisaul and Bateman, 2008). Moreover, previous research has established that RP3V neurons expressing tyrosine hydroxylase (TH), a marker for dopaminergic neurons, are co-localized with ER α and, to a lesser degree, ER β and that this co-localization is sexually dimorphic pre-weaning (Orikasa et al., 2002, Patisaul, Fortino, 2006). Both the sex specific level of TH and the co-localization of TH and ER α have been shown to be altered by neonatal BPA exposure in the murine brain (Patisaul, Fortino, 2006, Rubin, Lenkowski, 2006). In mice, this change was associated with the loss of sexually dimorphic locomotor behavior in the open field test. These observations further support the hypothesis that disruption of ER expression in the developing hypothalamus may impact the emergence of sexually dimorphic physiology and behavior. More definitive links, however, between disruption of early life gene expression and adult physiology are needed to confirm the biological significance of the findings reported here.

Kiss1 expression is also sexually dimorphic (Kauffman, 2009, Kauffman, Gottsch, 2007) and tightly coordinated by estrogen (Smith, Cunningham, 2005). Nearly all Kiss1 neurons, in both the anterior and mediobasal hypothalamus, co-express ER α , and more than 20% of RP3V and 10% of ARC Kiss1 neurons co-express ER β (Smith, Popa, 2006). Although neonatal EB exposure decreased Kiss1 expression in the female ARC to male-typical levels, BPA had no effect in either sex, with the exception of a slight increase in the HBPA males. Expression in the RP3V, however, was disrupted in both sexes suggesting that this population may be more vulnerable to endocrine disruption. This postulate is supported by our prior work showing that the ontogeny of Kiss1 signaling pathways can be disrupted in

females by neonatal administration of estradiol benzoate (EB) or the phytoestrogen genistein (Bateman and Patisaul, 2008, Losa, Todd, 2010). The results from the present study further reveal that both doses of BPA were sufficient to eliminate RP3V Kiss1 expression in the PND 10 females, and elevate it in the PND 10 males. Female expression appears to ultimately recover, however, as we have not found adult female RP3V levels of Kiss1 immunoreactivity to be significantly affected by either dose of BPA (Patisaul, Todd, 2009). Moreover, the capacity of GnRH neurons in ovariectomized females to respond to hormone priming does not appear to be affected by neonatal BPA exposure (Adewale et al., 2009), indicating that the organization of the neuroendocrine pathways required to mediate this response appear to be functionally intact. Numerous studies have shown, however, that developmental exposure to BPA results in irregular or absent estrous cycles (Adewale, Jefferson, 2009, Cabaton, Wadia, 2011, vom Saal, Akingbemi, 2007) and compromised fertility (Cabaton, Wadia, 2011) suggesting that HPG regulation may be altered in gonadally intact animals that are not primed with exogenous hormones. This may result from the altered distribution of ER within Kiss1 neurons. Recent studies have revealed that, within the RP3V, the two isoforms play functionally different roles in adult females as antagonism of ER α blocks LH responses to kisspeptin, thereby eliminating the preovulatory LH surge, while antagonism of ER β fails to block the LH surge but instead augments acute LH responses to kisspeptin (Roa et al., 2008). BPA significantly reduced, and completely eliminated, RP3V ER α and ER β expression respectively by PND 10, suggesting that ER expression within Kiss1 neurons is decreased or absent. Future studies will be needed to confirm this possibility and establish how long the effect persists.

The present study provides evidence that the sex specific ontogeny of hypothalamic pathways important for reproductive physiology, behavior and energy balance may be disrupted by BPA exposure. Uncovering the potential mechanisms by which BPA affects neuroendocrine development is important when evaluating whether or not effects observed in rodents can be extrapolated to humans. The possible health consequences of BPA exposure remain controversial (Beronius, Ruden, 2010, Goodman et al., 2009, Vandenberg, Maffini, 2009). Importantly, the health effects of low dose oral exposure remain the subject of considerable interest because human exposure is presumably low but constant and from a variety of sources including food, beverages, the handling of paper receipts, and dust (Biedermann, Tschudin, 2010, Lakind and Naiman, 2010, Vandenberg, Hauser, 2007). Although numerous studies have reported health effects in rodents consistent with disrupted neuroendocrine function including altered pubertal timing, irregular or absent estrous cycles and reduced fertility (Adewale, Jefferson, 2009, Cabaton, Wadia, 2011, Howdeshell, Hotchkiss, 1999, Vandenberg, Maffini, 2009), others have found no effects at all (Howdeshell et al., 2008, Ryan et al., 2010, Tyl et al., 2008, Tyl et al., 2002). Why some studies find significant effects while others do not remains the subject of intense debate but issues like dose, timing of exposure, route of administration, strain, housing conditions, and diet have all been purported to be contributing factors (Beronius, Ruden, 2010, Goodman, Witorsch, 2009, Myers et al., 2009, Thigpen, Setchell, 2007, Vandenberg, Maffini, 2009). Drawing conclusions about safety from this fragmentary and discordant literature has proven to be difficult and contentious. Weight of evidence assessments have been conducted by numerous groups but the results have been inconsistent in their conclusions about the level of concern consumers should have about BPA (Hengstler et al., 2011, NTP, 2009, vom Saal, Akingbemi, 2007). The present, largely mechanistic, study sought to inform this debate by providing information regarding the potential for BPA exposure, during the critical period in which the hypothalamus is undergoing steroid hormone directed sexual differentiation, to alter the expression of genes known to be critical or organizing sex differences.

4.1 Conclusions

The “Fetal Basis of Adult Disease” hypothesis postulates that alterations in gene expression during critical perturbs developmental programming which then manifests as permanently altered gland, organ, or system function (Heindel, 2005). Understanding how BPA affects the sex specific organization of complex, estrogen responsive, neuroendocrine pathways during neonatal life will ultimately help elucidate the mechanisms by which BPA induces a wide range of effects including altered pubertal timing, impaired fecundity, and metabolic syndrome. Our results suggest the POA is more sensitive to disruption by BPA than the mediobasal hypothalamus during neonatal life. The data also clearly show that BPA does not act as an estrogen mimic, and has the potential to selectively alter the expression of the two ER isoforms. The molecular mechanisms underlying these characteristics remain to be elucidated but may include epigenetic modulation, such as methylation or deacetylation (Champagne et al., 2006, Kurian et al., Kurian, Olesen, 2010, Matsuda, Mori, 2011). Sex reversed Kiss1 expression in the RP3V suggest that the ontogeny of sex-specific GnRH feedback pathways may be disrupted, a condition which is consistent with numerous prior studies showing BPA-induced alterations in estrous cyclicity and pubertal onset. Further work will be needed to more definitively determine if early life gene expression changes, such as those reported here, result in significant behavioral or physiological consequences later in life.

Highlights

The hypothalamus undergoes sexual dimorphism in neonatal life

Hormones are critical for this process therefore it may be vulnerable to endocrine disruption

Neonatal exposure to BPA altered the sex specific expression of estrogen receptors

Neonatal exposure to BPA altered the sex specific expression of Kiss1

These gene expression changes may underlie reproductive deficiencies that emerge later in life

Acknowledgments

This work is supported by NIEHS grant RO1 ES016001 to HBP. We thank Karina Todd for assistance with animal care and facilitating ISHH.

Abbreviations

3V	third ventricle
AC	anterior commissure
ANOVA	analysis of variance
ARC	arcuate nucleus
rARC	rostral arcuate nucleus
cARC	caudal arcuate nucleus
AVPV	anteroventral periventricular nucleus
BPA	bisphenol A
ER	estrogen receptor

ERα	ER alpha
ERβ	ER beta
ER beta	gonadotropin-releasing hormone
HPG	hypothalamic-pituitary-gonadal
ISHH	in situ hybridization histochemistry
Kiss1	kisspeptin
LE	Long Evans
LH	luteinizing hormone
MPOA	medial preoptic area
PND	postnatal day
QRT-PCR	quantitative reverse transcription-polymerase chain reaction
RP3V	rostral periventricular area of the third ventricle
VMN	ventromedial hypothalamic nucleus
VMNvl	ventrolateral division of the ventromedial hypothalamic nucleus
cVMNvl	caudal ventrolateral division of the ventromedial hypothalamic nucleus
rVMNvl	rostral ventrolateral division of the ventromedial hypothalamic nucleus

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Figure 1.

Representative autoradiographs of ERα signal in the PND 4 AVPV (upper panels in A) and MPOA (lower panels in A), and in the PND 10 AVPV (upper panels in D) and MPOA (lower panels in D) after vehicle (OIL), EB, LBPA and HBPA treatments (from left to right in both A and D). Optical density analysis of ERα expression in the PND 4 AVPV (B) and MPOA (C), and in the PND 10 AVPV (E) and MPOA (F). ERα expression was significantly upregulated in the PND 4 HPBA females (B,C) then reduced to male-typical levels by PND 10 in both the AVPV (E) and the MPOA (F). LBPA had no effect in either sex on PND 4 but resulted in lower AVPV ERα levels in females by PND 10 (E). Significant differences in expression compared to vehicle are represented by * $p \leq 0.05$, ** $p \leq 0.01$ for the females,

and $^{\#}p \leq 0.05$, $^{\#\#}p \leq 0.01$ for the males. Significant sex differences in expression are represented by $^{\dagger}p \leq 0.05$, $^{\dagger\dagger}p \leq 0.01$, $^{\dagger\dagger\dagger}p \leq 0.001$. The graphs depict mean $\pm$ SEM and the sample size is provided at the bottom. (3V = third ventricle; AC = anterior commissure; scale bar = 500 μm)

Figure 2.

Autoradiographs depicting ERα signal in the caudal VMNvl and ARC on PND 4 (A) and in the rostral VMNvl and ARC on PND 10 (F). Expression was sexually dimorphic within all regions examined and relatively unaltered by BPA exposure. The graphs depict mean $\pm$ SEM on PND 4 (B–E) and PND 10 (G–J) for rVMNvl, cVMNvl, rARC and cARC respectively with the sample size indicated at the bottom. Significant differences in expression compared to vehicle are represented by $**p \leq 0.01$ for the females, and $\#p \leq 0.05$, for the males. Significant sex differences in expression are represented by $^{\dagger}p \leq 0.05$, $^{\dagger\dagger}p \leq 0.01$, $^{\dagger\dagger\dagger}p \leq 0.001$. (3V = third ventricle; scale bar = 500 μ m)

Figure 3.

ERβ mRNA expression in the AVPV and MPOA of PND 4 (A) and PND 10 (D) rats following vehicle (OIL), EB, LBPA and HBPA treatment (A and D, from left to right). Expression was not sexually dimorphic on PND 4 and unaltered by exposure (B,C) but by PND 10, ERβ expression was depleted in the AVPV of both sexes (E) and significantly decreased in the female MPOA, thereby eliminating the sex difference (F). Significant differences in expression compared to vehicle are represented by * $p \leq 0.05$, ** $p \leq 0.01$ for the females, and # $p \leq 0.05$, for the males. Significant sex differences in expression are represented by †† $p \leq 0.01$, ††† $p \leq 0.001$. The graphs depict mean $\pm$ SEM and the sample size

is provided at the bottom. (3V = third ventricle; AC = anterior commissure; scale bar = 500 μm)

Figure 4.

Representative photomicrographs depicting ERβ mRNA labeling in the caudal VMNvl on PND 4 (A) and PND 10 (D). As expected, expression was absent in the ARC. In females, EB exposure reduced ERβ expression to male-typical levels throughout the VMNvl (B,C,E,F) while BPA had no appreciable effect with the exception of a slight elevation in the cVMNvl of the PND 10 males (F). Significant differences in expression compared to vehicle are represented by $**p \leq 0.01$ for the females, and $\#p \leq 0.05$, $##p \leq 0.01$ for the males. Significant sex differences in expression are represented by $^{\dagger\dagger}p \leq 0.01$, $^{\dagger\dagger\dagger}p \leq 0.001$. The graphs depict mean $\pm$ SEM and the sample size is provided at the bottom. (3V = third ventricle; scale bar = 500 μ m)

Figure 5.

Hematoxylin counterstained sections depicting silver grain labeling (black arrows) for Kiss1 in the PND 10 mid-level RP3V (A). Silver grain deposition was discrete and sexually dimorphic in the vehicle treated controls (A). The percentage of individuals with detectable clusters was nearly identical in the control groups but significantly lower or absent in the females exposed to EB or BPA and in the males exposed to EB or the low dose of BPA ($n = 6-8$ per sex per group) (B). Within the individuals displaying clusters, the mean number of clusters was sexually dimorphic in the control group but appreciably lower in females exposed to EB or BPA (C). No statistical analysis was performed because of the limited sample size. (scale bar = 50 μ m)

Figure 6.

Representative photomicrographs (A and C) depicting ARC Kiss1 silver grain clusters (black arrows) on PNDs 4 (A) and 10 (C). ARC expression levels were quantified from the film autoradiographs (not shown). In females, Kiss1 mRNA levels were significantly reduced in the EB exposure females on PNDs 4 (B) and 10 (D) and the sex difference was reversed or eliminated. BPA had no effect in females but Kiss1 levels were moderately upregulated in the HBPA males on PND 4 (B). Significant differences in expression compared to vehicle are represented by $**p \leq 0.01$ for the females, and $\#p \leq 0.05$ for the males. Significant sex differences in expression are represented by $^{\dagger}p \leq 0.05$, $^{\dagger\dagger}p \leq$

0.01, $^{\dagger\dagger\dagger}p \leq 0.001$. The graphs depict mean $\pm$ SEM and the sample size is provided at the bottom. (3V = third ventricle; scale bar = 50 μ m)

Table 1

Effect of BPA on Sexually Dimorphic Expression of ER α , ER β and Kiss1 in the Postnatal Rat Hypothalamus

Gene	Age	Preoptic Area				Mediobasal Hypothalamus			
		Exposure	AVPV	MPOA	rARC	cARC	rVMNvl	cVMNvl	
ER α	PND 4	OIL	F>M	F>M	F>M	F>M	F>M	F>M	
		EB	F<M $\uparrow$	$\downarrow$ F=M	$\downarrow$ F=M	$\downarrow$ F=M $\uparrow$	$\downarrow$ F=M	$\downarrow$ F=M	
		LBPA	F>M	F>M	F>M	$\uparrow$ F>M	F>M	F>M	
	PND 10	HBPA	$\uparrow$ F>M $\uparrow$	$\uparrow$ F>M $\uparrow$	F>M	F>M	F>M	F>M	
		OIL	F=M	F>M	F>M	F>M	F>M	F>M	
		EB	$\downarrow$ F=M	$\downarrow$ F<M	$\downarrow$ F=M	$\downarrow$ F=M	$\downarrow$ F=M	$\downarrow$ F=M	
	PND 4	LBPA	$\downarrow$ F=M	F>M $\downarrow$	F=M	F=M	F>M	F>M	
		HBPA	$\downarrow$ F=M	$\downarrow$ F=M	F=M	F>M	F>M	F>M	
		OIL	F=M	F=M	ND	ND	F>M	F>M	
	PND 10	EB	F=M	F=M	ND	ND	$\downarrow$ F=M	$\downarrow$ F=M $\downarrow$	
ER β	PND 4	LBPA	F=M	F=M	ND	ND	F>M	F>M	
		HBPA	F=M	F=M	ND	ND	F>M	F>M	
		OIL	F=M	F>M	ND	ND	F>M	F>M	
	PND 10	EB	$\downarrow$ F=M	$\downarrow$ F=M	ND	ND	$\downarrow$ F=M	F<M $\uparrow$	
		LBPA	ND	$\downarrow$ F>M $\downarrow$	ND	ND	F>M	F>M $\uparrow$	
		HBPA	ND	$\downarrow$ F=M $\downarrow$	ND	ND	F>M	F>M	
Kiss1	RP3V-PND10	EB	LBPA	HBPA	OIL	EB	LBPA	HBPA	
		F>M	ND	N/A	N/A	F>M	$\downarrow$ F<M	$\downarrow$ F=M	
		ND	N/A	N/A	N/A	F>M	F>M $\uparrow$	F>M	
	ARC-PND 4	EB	LBPA	HBPA	OIL	EB	LBPA	HBPA	
		F>M	ND	N/A	N/A	F>M	F>M $\uparrow$	F>M	
		ND	N/A	N/A	N/A	F>M	F>M $\uparrow$	F>M	
	ARC-PND 10	EB	LBPA	HBPA	OIL	EB	LBPA	HBPA	
		F>M	ND	N/A	N/A	F>M	F>M $\uparrow$	F>M	
		ND	N/A	N/A	N/A	F>M	F>M $\uparrow$	F>M	
	ARC-PND 10	EB	LBPA	HBPA	OIL	EB	LBPA	HBPA	
		F>M	ND	N/A	N/A	F>M	F>M $\uparrow$	F>M	
		ND	N/A	N/A	N/A	F>M	F>M $\uparrow$	F>M	

ND=No Detectable Signal, N/A=Not Available because of insufficient sample size.
 “↓” and “↑” represent signal significantly decreased and increased compared with female vehicle control respectively.
 “↓” and “↑” represent signal significantly decreased and increased compared with male vehicle control respectively.