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

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Received November 28, 2012; accepted February 14, 2013

Bisphenol A (BPA) exposure is ubiquitous, and in laboratory animals, early-life BPA exposure has been shown to alter sex-specific neural organization, neuroendocrine physiology, and behavior. The specific mechanisms underlying these brain-related outcomes, however, remain largely unknown, constraining the capacity to ascertain the potential human relevance of neural effects observed in animal models. In the perinatal rat brain, estrogen is masculinizing, suggesting that BPA-induced perturbation of estrogen receptor (ESR) expression may underpin later in-life neuroendocrine effects. We hypothesized that prenatal BPA exposure alters sex-specific ESR1 (ER α) and ESR2 (ER β) expression in postnatal limbic nuclei. Sprague Dawley rats were mated and gavaged on gestational days (GDs) 6–21 with vehicle, 2.5 or 25 μ g/kg bw/day BPA, or 5 or 10 μ g/kg bw/day ethinyl estradiol. An additional group was restrained but not gavaged (naïve control). Offspring were sacrificed the day after birth to quantify ESR gene expression throughout the hypothalamus and amygdala by *in situ* hybridization. Relative to the vehicle group, significant effects of BPA were observed on ESR1 and ESR2 expression throughout the mediobasal hypothalamus and amygdala in both sexes. Significant differences in ESR expression were also observed in the mediobasal hypothalamus and amygdala of the naïve control group compared with the vehicle group, highlighting the potential for gavage to influence gene expression in the developing brain. These results indicate that ESR expression in the neonatal brain of both sexes can be altered by low-dose prenatal BPA exposure.

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

Human exposure to bisphenol A (BPA), a component of polycarbonate plastics, epoxy resins, dental sealants, thermal receipts, and other products, is indisputably low but widespread (Biedermann *et al.*, 2010; Cooper *et al.*, 2011; FAO/WHO, 2011; Geens *et al.*, 2011, 2012; Vandenberg *et al.*, 2007). More than 90% of the U.S. population have measurable levels of BPA in bodily fluids, with children typically exhibiting higher internal levels than adults (Calafat *et al.*, 2008; Vandenberg *et al.*, 2010). In laboratory animal studies, early-life exposure to BPA has been shown to impact the organization of numerous estrogen-sensitive neural endpoints including the sexual differentiation of hypothalamic regions important for sex-specific reproductive physiology and behavior (reviewed in Wolstenholme *et al.* (2011)). Those observations have elevated concerns regarding the potential for low-dose exposure to result in adverse neural health outcomes in humans (Beronius *et al.*, 2010; Hengstler *et al.*, 2011; Vandenberg *et al.*, 2009).

Understanding the specific molecular and cellular mechanisms through which BPA can alter the developing brain will help establish how such neural effects in rodents may be predictive of similar neural effects in humans (vom Saal *et al.*, 2007; Wolstenholme *et al.*, 2011). The goal of the present study was to quantify the effects of prenatal low-dose BPA exposure on sex-specific estrogen receptor (ESR) expression in the neonatal rat hypothalamus and amygdala (AMYG). Neonatal ESR expression was examined because the density of ESRs is regionally and sexually dimorphic at this age, and this differential sensitivity to neonatal estrogen ultimately confers permanent differences in neural structure and function (McCarthy, 2008; Simerly, 2002). Although there are critical species differences specific to how estrogen organizes the developing brain (McCarthy, 2008; Resko and Roselli, 1997), sex-specific ESR expression is present in perinatal rodents and primates (including humans) (González *et al.*, 2007; Kato *et al.*, 1998; Resko and Roselli, 1997; Wallen, 2005), the distribution of which is highly conserved in vertebrates

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(Brandenberger *et al.*, 1997; Cao and Patisaul, 2012; MacLusky *et al.*, 1979; Walker *et al.*, 2009). Thus, perturbation of neonatal ESR expression by BPA exposure could be a mechanism by which the steroid hormone-dependent organization of these subregions is altered. Moreover, very little is known regarding the impact of prenatal-only BPA exposure on brain sexual differentiation, so the results presented here yield new insights regarding effects resulting from this specific exposure window. Because BPA has long been regarded as estrogenic (Dodds *et al.*, 1938), and prenatal estrogen exposure is masculinizing in female rodents (McCarthy, 2008), ethinyl estradiol (EE) was used as a reference estrogen. Understanding and elucidating the pathways and critical periods in which the vulnerabilities to, and consequences of, low-dose BPA exposure manifest in animal models will contribute mechanistic knowledge requisite to evaluate the potential risk of BPA exposure in humans. Assessing consequences of exposure confined to a specific critical window may also help identify sensitive periods during which humans may be particularly vulnerable to the effects of BPA.

There are numerous sex differences throughout the mammalian brain, particularly within the hypothalamus and surrounding structures (Bonthuis *et al.*, 2010; De Vries, 2004; Simerly, 2002), which underpin physiological and behavioral sexual dimorphisms. These differences are organized primarily by steroid hormones during critical windows of development throughout gestation, neonatal life, and adolescence (Schulz *et al.*, 2009; Simerly, 2002). Accordingly, the distribution of steroid hormone receptors, especially ESRs, during these critical windows of development is sex specific (e.g., in the neonatal rat (Cao and Patisaul, 2011)) and can be manipulated by exogenous administration of steroid hormones such as androgens and estrogens (McCarthy, 2008). Those early-life manipulations can have lifelong effects, including subfertility and behavioral changes (Cooke *et al.*, 1998; McCarthy, 2008; Morris *et al.*, 2004; Simerly, 2002). Therefore, disruption of sex-specific ESR expression during the sexual differentiation process is one molecular mechanism by which developmental BPA exposure might confer lifelong effects on hypothalamic organization and function.

The hypothalamus is the apical coordinator of neuroendocrine activity, sending signals from the central nervous system to the pituitary gland and receiving feedback information from target endocrine glands (such as the gonads, pancreas, and thyroid) to organize, integrate, and maintain homeostatic endocrine function. The hypothalamus is an expansive region comprising numerous sexually dimorphic nuclei. We previously established that expression of the two nuclear ESR subtypes (ESR1, also known as ER α , and ESR2, also known as ER β) is sexually dimorphic within these hypothalamic subregions of the neonatal rat (Cao and Patisaul, 2011). Moreover, we demonstrated that neonatal BPA exposure altered sex-specific ESR expression later in the neonatal period, particularly in the anteroventral periventricular nucleus of the hypothalamus

(AVPV) and the medial preoptic area (MPOA) (Cao *et al.*, 2012). These anterior hypothalamic regions are essential for ovulation in females and coordinate gonadotropin-releasing hormone activity in both sexes. Posterior regions, including the arcuate nucleus (ARC) and the ventrolateral division of the ventromedial nucleus (VMNvl), were not meaningfully altered. Based on those prior studies, here we explored the hypothesis that BPA exposure, confined to the prenatal critical window, would impact ESR1 and ESR2 expression in these same hypothalamic subregions.

An additional region of interest for the present study was the AMYG, a structure lying lateral to the posterior realm of the hypothalamus. The AMYG comprised numerous subregions, each of which has differential and sex-specific expression levels of ESR1 and ESR2 in neonatal and adolescent rats (Cao and Patisaul, 2012; Kühnemann *et al.*, 1994). Moreover, the cytoarchitecture of several amygdaloid subregions, such as total volume and neuronal soma size, is sexually dimorphic in adults and young rodents (Cooke *et al.*, 1999, 2007; Hines *et al.*, 1992; Johnson *et al.*, 2008; Morris *et al.*, 2008b) and under the control of circulating sex hormones (Cooke, 2006; Cooke *et al.*, 1999, 2007). In rats, most of those sex differences are established during the perinatal period (Morris *et al.*, 2008a). The subregions within the amygdaloid complex are highly interconnected (Canteras *et al.*, 1992; Cooke and Simerly, 2005; Maras and Petrulis, 2010; Simerly, 2002) and integrate information sent and received from other brain regions to regulate a wide range of behaviors (Canteras *et al.*, 1992; Simerly, 2002; Swanson and Petrovich, 1998), including sexual behavior (Harris and Sachs, 1975; Newman, 1999), aggression (Wang *et al.*, 1997), anxiety behavior (Toufexis, 2007), and parental behavior (De Vries and Villalba, 1997; Fleming *et al.*, 1980). Subregions of interest for the present study included the posterodorsal portion of the medial amygdala (MePD), the cortical amygdaloid nucleus, posterolateral (PLCo), the posteromedial (PMCo), and the amygdalo-hippocampal area (AHi), all of which play important roles in conferring behavioral sex differences.

Prior studies exploring the impact of early-life BPA exposure on brain development and gene expression have produced inconsistent data (Palanza *et al.*, 2008; Richter *et al.*, 2007; Wolstenholme *et al.*, 2011), likely resulting from differences in exposure duration, dose, route of administration, and critical species differences in neural structure between rats and mice (Bonthuis *et al.*, 2010). Because of those inconsistencies, study design-related recommendations for BPA research have been issued including statistical control for litter effects and the use of oral administration (Goodman *et al.*, 2006; Hengstler *et al.*, 2011; Hunt *et al.*, 2009; Richter *et al.*, 2007). In situations where BPA is thought to act as a weak estrogen, use of a concurrent reference estrogen and minimization of exogenous estrogen exposure are also suggested. All of those were incorporated into the current study, which was a component of a larger project, the methodological details of

which have been published (Ferguson *et al.*, 2011; He *et al.*, 2012). The BPA doses used (2.5 and 25 µg/kg bw/day) are well below the current no observable adverse effect level (NOAEL) of 50 mg/kg bw/day and the current reference dose (tolerable daily intake) of 50 µg/kg bw/day (Chapin *et al.*, 2008; Geens *et al.*, 2012; NTP, 1982). Despite well-recognized metabolic differences between rats and humans, the lowest BPA dose used here is near the top range of estimated daily BPA intake for human infants (FAO/WHO, 2011). Two doses of EE were included as the reference estrogen because gestational estrogen exposure is well recognized to induce region-specific hypothalamic masculinization in female rats (McCarthy, 2008; Simerly, 2002).

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

Collectively, the results from this study yield insight into the mechanisms by which BPA might affect the sex-specific organization of hypothalamic and amygdaloid subnuclei. These results also aid in establishing the degree to which early-life stress from gavage or other stressful procedures can induce significant effects on brain development, independent of the toxicant.

MATERIALS AND METHODS

Animal care, prenatal BPA and EE exposure, brain collection, and section preparation. Postnatal day 1 (PND1) pups were obtained from litters generated for a study described in detail earlier (Ferguson *et al.*, 2011). Briefly, the National Center for Toxicological Research (NCTR) Breeding Colony supplied 364 female and 180 male Sprague Dawley rats (derived from Charles River Crl: COBS CD (SD) BR Rat, Outbred) at weaning (PND21). Upon reaching

breeding age (see below), these rats became the dams and sires (i.e., F₀ generation) of subsequent litters. Upon arrival to the vivarium at PND21, each rat was tail tattooed for identification, weighed, and group-housed (three same-sex/cage). *Ad lib* food (see below for diet information) and water were provided at all times. Housing rooms were maintained on a 12-/12-h light/dark cycle (6:00 a.m.–6:00 p.m.) at 22 ± 1°C (mean ± SE) and 45–55% humidity. All animals were maintained in accordance with the Guide for the Care and Use of Laboratory Animals from the 1996 National Research Council (NRC, 1996), and all studies were approved in advance by the NCTR Institutional Animal Care and Use Committee.

Upon arrival at PND21, rats were housed in polysulfone cages with polysulfone microfilter tops, both of which were purchased new at the beginning of the study (Ancare, Bellmore, NY). Polysulfone caging was chosen as no detectable *in vivo* or *in vitro* estrogenic effects from BPA extracted from new polysulfone cages have been observed (Howdeshell *et al.*, 2003). Glass water bottles with food-grade silicone stoppers (Fisher Scientific, St Louis, MO) and metal sipper tubes were used to further reduce potential environmental estrogen exposure. Metal mop buckets were used in the housing rooms, and all chow was stored in metal containers. This environment began for the F₀ generation at PND21 and was maintained throughout the remainder of the study. Beginning upon arrival from the NCTR Breeding Colony at PND21 and throughout the study, all animals were maintained on a low phytoestrogen chow (TestDiet 5K96 (irradiated pellets), Verified Casein Diet 10 IF, TestDiet, Richmond, IN).

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

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

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

Beginning on the morning of GD6, dams were gavaged with 5.0 ml 0.3% CMC/kg bw/day (vehicle control group), 2.5 µg BPA/kg bw/day (BPA 2.5 group), 25.0 µg BPA/kg bw/day (BPA 25 group), 5.0 µg EE/kg bw/day (EE 5 group), or 10.0 µg EE/kg bw/day (EE 10 group) using a Hamilton Microlab 500

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

Dams were not treated on the day of parturition (typically, GD22) and were left undisturbed on that day. On the day after parturition (PND1), male and female pups ($n = 5\text{--}8/\text{sex}/\text{group}$) were sacrificed by decapitation, the heads rapidly frozen on a flat block of dry ice, and stored at -80°C until cryosectioning at North Carolina State University. All brains were cryosectioned (Leica CM1900, Nussloch, Germany) into four serial sets of 20 μm coronal sections, mounted onto Superfrost plus slides (Fisher Scientific, Pittsburgh, PA), and stored at -80°C until *in situ* hybridization histochemistry (ISHH) processing.

ISHH. For each gene, all sections containing the anterior hypothalamus or the mediobasal hypothalamus (MBH) with the surrounding AMYG subregions were processed simultaneously to avoid batch effects. Thus, two large batches of ISHH were performed for each gene. ISHH was performed using well-established procedures, the details of which (including transcriptional templates for ESR1 and ESR2, radiolabeling, the specificity of the antisense and sense probes, and the ISHH procedures) have been described in detail elsewhere (Cao and Patisaul, 2011, 2012).

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

Image analysis and film quantification. The MCID Core Image software program (InterFocus Imaging Ltd, Cambridge, England) was used to quantify ESR signal on the films using the digital densitometry application and by following conventional procedures as reported previously (Cao and Patisaul, 2011, 2012; Cao *et al.*, 2012). Regions of interest (ROIs) included the AVPV and MPOA within the anterior hypothalamus, the rostral and caudal portions of the VMNvl and ARC (rVMNvl, rARC, cVMNvl, cARC) within the MBH, and the MePD, PLCo, PMCo, and AHi subregions of the AMYG. The borders of each subregion were identified using well-established anatomical landmarks for neonatal rats (Cao and Patisaul, 2011, 2012) and the assistance of a brain atlas (Paxinos *et al.*, 1994).

ROI densities and background levels were measured from anatomically matched sections (two for each hypothalamic ROI and five for the AMYG). The resulting values for each brain section after background subtraction were then averaged to obtain a representative measurement (for that ROI) for each animal. Where there were two or more pups/sex/litter, the results were averaged to obtain a single representative value for that sex in that litter as suggested (Festing, 2006). Optical densities were converted to nCi/g tissue

equivalents using a “best fit” curve (third degree polynomial) generated from the autoradiographic ^{14}C microscales. In all cases, the signal was within the limits of the curve. For quality control, all measurements for the POA and MBH were completed by at least two investigators who were blind to the exposure groups. There was a high degree of concordance among the two data sets, and the results were averaged to obtain the final values for each ROI. AMYG measurements were then made by one of these investigators as described above (because the high degree of concordance between the two evaluators demonstrated with confidence that the remaining analyses could be completed by one person).

Statistics. All statistical analyses were designed in consultation with statisticians at the NCTR and National Institute for Environmental Health and used the litter as the experimental unit for all analyses. In addition to determining whether exposure altered gene expression within each sex, establishing whether exposure altered sex differences in expression was also an experimental goal. Thus, two-way ANOVA with exposure, sex, and the interaction as the factors was used for all analyses. Preliminary inspection of the data indicated obvious differences between the vehicle control and naïve control groups. Thus, for each ROI and gene of interest (ESR1 or ESR2), a two-way ANOVA (with group, sex, and the interaction as factors) compared only the vehicle control and naïve control groups, exclusive of the other exposure groups. Subsequently, the vehicle control, BPA, and EE treatment groups (the naïve control group was excluded) were compared by two-way ANOVA, and Holm-Sidak multiple comparison tests used when a significant main effect of exposure group or sex was identified. Because ESR expression levels in some regions are known to be sexually dimorphic, if there was a significant main effect of sex (even if there was no significant interaction with exposure), Holm-Sidak comparisons were made within sex (i.e., the male vehicle control group was compared with each same-sex BPA and EE group, and the female vehicle control group was compared with each same-sex BPA and EE group). Additionally, to determine whether exposure impacted sexually dimorphic expression, for each gene where a significant main effect of sex was identified in the initial two-way ANOVA, Holm-Sidak comparisons were conducted within the vehicle control group to determine whether this group exhibited sexual dimorphism. If this comparison was not significant, no further between-sex comparisons were done. However, if this comparison was significant, then each exposure group was evaluated individually for sexual dimorphism. All analyses were two-tailed, and results were considered significantly different when $p \leq 0.05$.

RESULTS

Differences in ESR mRNA Levels Between the Vehicle and Naïve Control Groups

For each ROI, the vehicle and naïve controls were compared independent of the other exposure groups to establish whether there was an impact of gavage on gene expression (Figs. 1–7). There were few significant differences in the anterior hypothalamus. There were no significant effects of group on ESR1 or ESR2 levels in the AVPV or MPOA (Figs. 1B and E, 2B and E). However, ESR2 levels in the MePD exhibited a significant group effect ($F(1,23) = 231.88$, $p \leq 0.001$) with expression levels lower in the vehicle control group. Significant main effects of group were found for both ESRs in the VMNvl (ESR1, rVMNvl ($F(1,23) = 53.11$, $p \leq 0.001$); ESR2, rVMNvl ($F(1,22) = 255.22$, $p \leq 0.001$); ESR1, cVMNvl, ($F(1,23) = 30.66$, $p \leq 0.001$); ESR2, cVMNvl, ($F(1,23) = 93.84$, $p \leq 0.001$)) (Figs. 3D and I, 4B and E) and MePD (ESR1 ($F(1, 23) = 37.71$, $p \leq 0.001$); ESR2 ($F(1, 23) = 231.85$, $p \leq 0.001$)) (Figs. 5B and 7B), with

FIG. 1. Representative autoradiographs depicting ESR1 mRNA in the AVPV (A) and MPOA (D) from naïve, vehicle, EE 10, and BPA 2.5 exposure groups (from left to right in both A and D, females on the left side of each panel, males on the right). Optical density of ESR1 expression was analyzed in the AVPV (B and C) and MPOA (E and F). A main effect of sex in ESR1 expression was observed in the MPOA by two-way ANOVA, but no effect of exposure was found for either region. A significant sex difference was observed in the naïve control (represented by *** $p < 0.001$) but not in other groups. Graphs depict mean $\pm$ SEM, and sample size is shown under each group; scale bar = 500 μ m for all panels in A and D; 3V = third ventricle; AC = anterior commissure.

FIG. 2. Representative autoradiographs depicting ESR2 signal in the AVPV (A) and MPOA (D). Optical density analysis of ESR2 mRNA in the AVPV (B and C) and MPOA (E and F) indicated that the levels of ESR2 mRNA were sexually dimorphic in the AVPV, with higher levels in males in all groups. No significant effect of EE or BPA was observed in the AVPV or MPOA. Graphs depict mean $\pm$ SEM, and sample size is shown under each group. Sex differences in expression are represented by * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$; scale bar = 500 μ m for all panels in A and D; 3V = third ventricle; AC = anterior commissure.

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

expression levels lower in the vehicle control group in each case. Significant main effects of group were found for ESR1 expression in the ARC (rARC ($F(1,23) = 25.04$, $p \leq 0.001$); cARC ($F(1,23) = 46.16$, $p \leq 0.001$)) (Figs. 3B and G) and all subregions of the AMYG (MePD ($F(1,23) = 37.71$, $p < 0.001$; PLCo ($F(1,23) = 50.84$, $p \leq 0.001$); PMCo ($F(1,23) = 62.54$, $p \leq 0.001$); AHi ($F(1, 23) = 67.70$, $p \leq 0.001$)) (Figs. 5D, 6B, and 6D), with expression levels lower in the vehicle control group in each comparison.

As expected, ESR expression was sexually dimorphic in several ROIs. Significant main effects of sex on ESR1 expression were identified in the MPOA ($F(1,22) = 15.80$, $p \leq 0.001$), rVMNvl ($F(1,23) = 12.25$, $p \leq 0.002$), cVMNvl ($F(1,23) = 9.48$, $p \leq 0.005$), rARC ($F(1,23) = 7.85$, $p \leq 0.010$), and cARC ($F(1,23) = 15.18$, $p \leq 0.001$), with higher levels in females. A significant effect of sex on ESR2 expression was found in the AVPV ($F(1,22) = 13.30$, $p \leq 0.001$; Fig. 2B) and MePD ($F(1,23) = 6.21$, $p \leq 0.02$), with higher expression levels in males. An additional sex difference was detected in the cVMNvl ($F(1,23) = 12.64$, $p \leq 0.002$; Fig. 4E), with higher ESR2 expression levels in females. Compared with the naïve control group, these sex differences were generally

conserved in the vehicle control group, but the magnitude of the difference was proportionally lower in accordance with the overall decrease in expression levels. Most notably, sex differences in ESR1 expression in the MPOA ($p = 0.07$) and ESR2 expression in the cVMNvl ($p = 0.097$) were marginal in the vehicle control groups compared with the naïve control groups where the sex differences were robust ($p \leq 0.001$ and $p \leq 0.003$, respectively). Additionally, expected sex differences in ESR1 expression in the cARC and VMNvl were present in the naïve controls ($p \leq 0.001$ and $p \leq 0.01$, respectively) but absent in the vehicle controls ($p = 0.17$ and $p = 0.15$, respectively).

Effect of Prenatal BPA or EE Exposure on POA ESR1 and ESR2 Expression

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

FIG. 4. Representative autoradiographs depicting ESR2 mRNA labeling in the rVMNvl (A) and cVMNvl (D). ESR2 signal was absent in the ARC (A and D). Optical density analysis of ESR2 expression (B, C, E, and F) showed that ESR2 signal in the naïve controls was significantly higher than in the vehicle controls. Compared with the same-sex vehicle controls, ESR2 expression in the rVMNvl and cVMNvl was significantly increased in the EE 10 and BPA 25 exposure groups in both sexes and in the EE 5 group in females (C and F). Graphs depict mean $\pm$ SEM and sample size is shown under each group; differences in expression between the naïve and vehicle controls are represented by *** $p \leq 0.001$ and between exposure groups and same-sex vehicle controls by ## $p \leq 0.01$ and ### $p \leq 0.001$ in males, and §§ $p \leq 0.01$ and §§§ $p \leq 0.001$ in females; † $p \leq 0.05$, †† $p \leq 0.01$, and ††† $p \leq 0.001$ represent sex differences in ESR2 expression; scale bar= 500 μ m; 3V= third ventricle.

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

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

ESR2. In the AVPV, significant main effects of sex ($F(1,51) = 40.87, p \leq 0.001$) and exposure group ($F(4,51) = 3.33,$

$p \leq 0.017$) were found in the analysis of ESR2 levels. ESR2 expression was higher in males (Fig. 2C and Table 1). Because a significant effect of sex was detected, within the vehicle control group, expression was analyzed for sexual dimorphism and found to be significant ($p < 0.05$). This dimorphic expression was preserved in all BPA and EE groups ($p < 0.05$ for all). Within-sex analyses for exposure differences did not indicate

FIG. 6. Representative autoradiographs showing ESR1 signal in the PMCo and AHi (A). Optical density analysis of ESR1 expression in the PMCo (B and C) and AHi (D and E) revealed that ESR1 mRNA signal in the naïve controls was significantly higher than in the vehicle controls. ESR1 expression in both regions was significantly increased in all groups, except for EE 5, compared with the vehicle controls. Sexually dimorphic ESR1 expression was not observed in any exposure group in either the PMCo or the AHi. Graphs depict mean $\pm$ SEM, and sample size is shown under each group; differences in expression between the naïve and vehicle controls are represented by *** $p < 0.001$ and between exposure groups and vehicle controls by ### $p < 0.001$; scale bar = 1000 μ m; 3V = third ventricle.

any BPA or EE group differed significantly from its same-sex vehicle control group. In the MPOA, no significant effects were identified (Fig. 2F).

Effect of Prenatal BPA or EE Exposure on MBH ESR1 and ESR2 Expression

ESR1. Results of the two-way ANOVA revealed significant main effects of exposure and sex on ESR1 expression in the rostral and caudal regions of the ARC and VMNvl but

no significant interactions between sex and exposure (Fig. 3) (Exposure effects: rARC ($F(4,53) = 10.87, p \leq 0.001$); cARC ($F(4,53) = 16.40, p \leq 0.001$); rVMNvl ($F(4,53) = 23.16, p \leq 0.001$); cVMNvl ($F(4,53) = 33.83, p \leq 0.001$)). Significant main effects of sex were found in the rARC ($F(1,53) = 6.70, p \leq 0.013$), cARC ($F(1,53) = 12.78, p \leq 0.001$), rVMNvl ($F(1,53) = 5.80, p \leq 0.020$), and cVMNvl ($F(1,53) = 12.45, p \leq 0.001$), with expression levels greater in females. In the rARC, the vehicle control group, ESR1 expression was sexually

FIG. 7. Representative autoradiographs depicting ESR2 signal in the MePD (A). Optical density analysis of ESR2 expression in the MePD (B and C) showed that ESR2 mRNA levels in the naïve controls were markedly higher than in the vehicle controls. ESR2 expression was significantly increased in all exposure groups compared with same-sex vehicle controls in males and was significantly increased in EE and BPA 25 groups in females, and a p value of 0.07 in the BPA 2.5 group. Graphs depict mean $\pm$ SEM, and sample size is shown under each group; differences in expression between the naïve and vehicle controls are represented by $***p \leq 0.001$ and between the exposure groups and vehicle controls by $###p \leq 0.001$ in males and $\$p \leq 0.05$ and $$$$p \leq 0.001$ in females; scale bar = 1000 μ m for all panels in A; 3V = third ventricle.

dimorphic ($p < 0.05$). This sex difference was lost in all but the EE 10 group ($p < 0.01$) (Fig. 3C).

Within-sex analysis of ESR1 expression in the MBH ROIs revealed that prenatal EE and BPA exposure generally elevated expression in both sexes (Table 1). In the rARC, the male BPA 2.5, BPA 25, and the EE 10 groups exhibited significantly higher ESR1 expression levels than the male vehicle control group ($p < 0.01$ for all). Within females, however, only the EE 10 group was significantly higher than the vehicle control group ($p < 0.01$) (Fig. 3C). In the cARC, within-sex analyses of ESR1 expression indicated that males in the BPA 2.5, BPA 25, and EE 10 groups exhibited significantly higher expression levels than the vehicle control group ($p < 0.02$ for all), whereas females in the BPA 2.5 and EE 10 groups had significantly higher expression levels than the vehicle control group ($p < 0.01$ for both) (Fig. 3H). Within males, the BPA 2.5, BPA 25, and EE 10 groups exhibited significantly higher ESR1 expression levels in the rVMNvl than the vehicle control group ($p < 0.02$ for all), whereas within females, the BPA 2.5 and EE 10 groups had significantly higher levels than the vehicle control group ($p < 0.01$ for both) (Fig. 3E). Within males, expression levels of ESR1 in the cVMNvl were significantly higher in the BPA 2.5, BPA 25, and EE 10 groups relative to the same-sex vehicle control ($p < 0.01$ for all), whereas within females, only the BPA 2.5 and EE

10 groups were significantly higher than the same-sex vehicle control group ($p < 0.01$ for both) (Fig. 3J).

ESR2. As expected, ESR2 expression was only detected in the VMNvl (Fig. 4). ESR2 expression was absent in the neonatal ARC, an observation consistent with our previous description (Cao and Patisaul, 2011). The analyses revealed main effects of exposure in the rVMNvl ($F(4,52) = 13.22$, $p \leq 0.001$) and cVMNvl ($F(4,53) = 15.79$, $p \leq 0.001$). A main effect of sex was found in the cVMNvl ($F(1,53) = 33.81$, $p \leq 0.001$), with greater expression in females, but the overall sex difference in the rVMNvl did not reach statistical significance ($F(1,52) = 3.64$, $p \leq 0.062$; Table 1 and Figs. 4C and F). Within the cVMNvl vehicle controls, sex-specific expression did not reach statistical significance ($p < 0.06$) (Figs. 4C and F). For the rVMNvl, comparisons of group differences within sex indicated significantly higher expression levels in males of the BPA 25 and EE 10 groups ($p < 0.01$ for both), and in females, there was an identical pattern ($p < 0.01$ for both) (Fig. 4C). For the cVMNvl, comparisons of group differences within sex indicated significantly higher expression levels in males of the BPA 25 and EE 10 groups ($p < 0.01$ for both), whereas in females, there were significantly higher expression levels in the BPA 25, EE 5, and EE 10 groups relative to same-sex vehicle controls ($p < 0.01$ for all) (Fig. 4F).

TABLE 1
Effects of Prenatal BPA Exposure on Sexually Dimorphic ESR Expression in the Neonatal Rat Brain

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

Notes. Summary of sex-specific ESR1 and ESR2 expression in ROI examined. Comparative expression levels between the sexes (Female [F] and Male [M]) for each gene within a subregion can be read horizontally across the table, whereas expression levels for each exposure group can be read vertically. For each comparison, “=” indicates no statistically significant difference, “>” indicates statistically greater, and “<” indicates statistically lower.

^a*p* value trends toward significance, *p* = 0.055.

Effect of Prenatal BPA or EE Exposure on AMYG ESR1 and ESR2 Expression

As expected (Cao and Patisaul, 2012), ESR1 and ESR2 were coexpressed only in the MePD (Figs. 5A and 7A). For all other amygdaloid ROIs, only ESR1 was detected.

ESR1. Significant main effects of exposure on ESR1 expression were observed in all amygdaloid subregions (MePD ($F(4,54) = 19.13, p \leq 0.001$); PLCo ($F(4,54) = 18.59, p \leq 0.001$); PMCo ($F(4,54) = 23.43, p \leq 0.001$); AHi ($F(4,55) = 29.72, p \leq 0.001$)) (Figs. 5C and E, Figs. 6C and E). A significant main effect of sex on ESR1 expression was observed in the MePD only ($F(1,54) = 4.82, p \leq 0.033$) (Fig. 5C); however, expression was not significantly dimorphic in the vehicle control group. Within males, the BPA 2.5, BPA 25, and EE 10 groups exhibited significantly higher expression levels in the MePD than same-sex vehicle controls ($p < 0.02$ for all), and an identical pattern was apparent for females ($p < 0.01$ for all). Comparisons within males of ESR1 expression levels in the PLCo, the PMCo, and the AHi indicated significant increases in the BPA 2.5, BPA 25, and EE 10 groups ($p \leq 0.031$ for all) compared with the vehicle control group (Figs. 5C and E, Figs. 6C and E). In females, there was a significant increase in ESR1 expression in the BPA 2.5, BPA 25, and EE 10 groups in the AHi and PMCo ($p \leq 0.001$), whereas in the PLCo, only the EE 10 group had increased expression ($p \leq 0.001$).

ESR2. ESR2 signal was robust only in the MePD (Fig. 7A). Significant main effects of sex ($F(1,53) = 27.35, p \leq 0.001$) and group ($F(4,53) = 42.07, p \leq 0.001$) were found but no significant interaction ($F(4,53) = 2.29, p = 0.072$; Fig. 7C). There was no significant sexual dimorphism in the vehicle control group.

Within males, all BPA and EE groups exhibited significantly higher expression levels than the same-sex vehicle control group ($p < 0.01$ for all), whereas within females, only the BPA 25, EE 5, and EE 10 groups were significantly higher than the same-sex vehicle control group ($p < 0.04$ for all).

DISCUSSION

The present results show region-specific alterations in ESR expression within the developing rat brain resulting from prenatal, low-dose, oral BPA exposure. Although prior studies, including three related studies using siblings of the animals described here (Ferguson *et al.*, 2011, 2012; He *et al.*, 2012), have described BPA exposure effects on sexually dimorphic brain morphology and behavior, exposure occurred during both the prenatal and postnatal periods (Ferguson *et al.*, 2011, 2012; He *et al.*, 2012); (Bai *et al.*, 2011; Kubo *et al.*, 2003; Kwon *et al.*, 2000; Rubin *et al.*, 2006; Xi *et al.*, 2010) or the postnatal period only (Monje *et al.*, 2010; Patisaul *et al.*, 2006). Additionally, all but the three related studies (Ferguson *et al.*, 2011, 2012; He *et al.*, 2012) used higher doses and different exposure routes. Thus, the data presented here represent the most comprehensive and detailed evaluation of prenatal BPA exposure effects on ESR expression levels in limbic subnuclei of the neonatal rat. Importantly, this is the first study to provide region-specific information on the sensitivity of ESR expression in the neonatal amygdaloid complex to low-dose prenatal BPA exposure. In general, BPA effects were localized to the MBH and specific subregions of the AMYG. These regions were also sensitive to gavage, suggesting that ESR expression within these areas is particularly responsive to environmental stressors.

To our knowledge, this is the first study to provide data on ESR expression changes potentially related to orogastric gavage. The reported differences between the naïve controls and those animals gavaged with the vehicle were unexpected and could be attributable to one of two primary factors related to gavage: the stress of the gavage procedure itself or unanticipated biological activity of the vehicle. Orogastric gavage is useful because it ensures precise and accurate oral dosing, but potentially stressful to the animals as aspects of it (including restraint technique, vehicle type, and volume) can elevate stress hormones (Balcombe *et al.*, 2004). The volume of gavage used here (5 ml/kg) was below that which has been described to increase stress hormone levels (reviewed in Balcombe *et al.* (2004)). Further, the aqueous nature of the vehicle (0.3% carboxymethylcellulose) is reported to induce less of a stress response after gavage compared with lipid vehicles (Brown *et al.*, 2000). Thus, the ESR expression differences between the naïve and vehicle controls seem unlikely to have resulted from vehicle composition or volume. Additionally, because all subjects, including the naïve controls, were physically restrained, restraint stress is also unlikely to have been a contributing factor. Thus, the ESR expression changes likely resulted from the stress associated with the physical insertion of the feeding needle. Further work specifically focused on this question is needed.

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

We have previously shown that ESRs are expressed in the subregions of the hypothalamus and AMYG in an age- and sex-specific manner in the neonatal rat (Cao and Patisaul, 2011, 2012). The sex differences identified here were consistent with those prior observations although statistical significance was not attained in every region, most notably ESR1 expression in

the AVPV. In the ARC and MPOA, the expected sex differences in ESR1 expression were observed in the naïve, but not the vehicle controls, suggesting that the effect of vehicle gavage on ESR gene expression may have suppressed expected sex differences. In general, sex differences were not significantly altered by prenatal exposure to either EE or BPA, a result which was unexpected given the longstanding recognition that estrogen can be masculinizing in the developing rodent brain (McCarthy *et al.*, 2009; Simerly, 2002). In the anterior hypothalamus, this is likely attributable to timing of exposure, as the early postnatal period (rather than the prenatal period) appears to be the “critical window” for estrogen-dependent masculinization in this region (Cao *et al.*, 2012; Simerly, 2002). Data obtained from female siblings of the pups described here demonstrated that both EE doses significantly increased the volume of the sexually dimorphic nucleus of the preoptic area (SDN-POA) at weaning (He *et al.*, 2012). In that companion study, however, oral dosing (direct to the pup) continued through weaning. This critical difference in exposure duration supports the position that postnatal exposure is required to masculinize gene expression and morphological sex differences in the anterior hypothalamus. Interestingly, although female SDN-POA volumes were significantly increased in the EE-exposed groups, they did not reach male-typical levels. Collectively, the data emphasize the importance of specifically identifying and defining exposure windows for interpretation of BPA effects in the developing brain. In humans, the period encompassing the rodent perinatal period is believed to occur in mid to late gestation (Abbott *et al.*, 2008; Aksglaede *et al.*, 2006; Selevan *et al.*, 2000; Simerly, 2002); thus, the rat perinatal “critical window” is likely to be entirely prenatal in humans.

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

The gene expression changes observed here could reflect either a change in cellular levels of ESRs within each region or a change in the number of cells expressing ESR1 and ESR2. Although transcription levels can be transient (Cao and Patisaul, 2011, 2012), changes in cell numbers would suggest that the effect is likely permanent (McCarthy, 2008). Whether these changes in limbic ESR expression persist beyond the neonatal window is the subject of ongoing studies using related, older cohorts of animals. In the present study, exposure halted

approximately 48 h before the brains were collected, which suggests that altered expression persists for at least a few days. This is consistent with what has been reported for EE and the selective ESR modulator, tamoxifen, the effects of which on ESR2 and related gene expression persist as long as 2 weeks after treatment cessation (Patisaul *et al.*, 2003). Thus, it is plausible that the overall sex-specific, temporal pattern of perinatal ESR expression as perturbed by BPA continues after exposure is terminated.

A potential concern stemming from the results reported here is the possibility that changes in early-life ESR expression following prenatal BPA exposure ultimately contribute to later in-life effects on neuroendocrine function and behavior (Patisaul *et al.*, 2012; Wolstenholme *et al.*, 2011, 2012). One way by which this could occur is via altered sensitivity of these regions to endogenous estrogens as a result of BPA-disrupted ESR expression levels. Because those limbic nuclei undergo steroid hormone-directed sexual differentiation during this period (Baum, 2009; Cooke and Woolley, 2005; McCarthy *et al.*, 2009; Simerly, 2002), altered endogenous hormone sensitivity may confer lifelong effects on neuroendocrine physiology and behavior. A long history of detailed work has shown that even an acute exposure to estrogen during critical windows of perinatal life can have permanent morphological and functional effects on neuroendocrine systems coordinating reproductive physiology and behavior (McCarthy, 2008; Simerly, 2002). How disruption of each ESR subtype individually contributes to potential neuroendocrine and behavioral alteration remains the subject of intense investigation. Although the specific functional role of ESR2 remains unclear, there is growing consensus that it is important for modulating affective and mood-related behaviors, including anxiety, aggression, and social interactions (Handa *et al.*, 2012; Patisaul and Bateman, 2008). Our results reveal that, throughout the limbic system, the expression of ESR2 appeared to be more sensitive to disruption by BPA exposure than ESR1 and was generally diminished by BPA exposure, particularly in the AMYG. We have recently shown in rats that this decreased expression persists through adolescence and is associated with elevated juvenile anxiety (Patisaul *et al.*, 2012), an outcome which is consistent with the emerging consensus regarding the functional role of ESR2 in the brain (Fan *et al.*, 2010; Handa *et al.*, 2012).

Importantly, BPA exposure during development has also been shown to impact mood-related behaviors in other species, including mice and deer mice ((Jašarević *et al.*, 2011) and reviewed in Wolstenholme *et al.* (2011)) and at least one study has associated exposure with hyperactivity and elevated anxiety in young girls (Braun *et al.*, 2009, 2011). Although humans and rodents undoubtedly perceive and express stress and anxiety differently, important core elements of the genetic and neurobiological basis of anxiety phenotypes are evolutionarily conserved across species, particularly in the AMYG and hypothalamus (Donner *et al.*, 2008; Hohoff, 2009; Hovatta and Barlow, 2008; Koolhaas *et al.*, 2007; Landgraf and Wigger,

2002; Millan, 2003). By contrast, other behaviors appear more resilient to BPA exposure. For example, a companion study using siblings of animals in the present study (and directly orally dosed through weaning) found no effects of BPA on righting reflex or slant board behaviors (Ferguson *et al.*, 2011), a not unexpected finding as neither is known to be sensitive to early-life exposure to estrogens, although performances for both assessments are sexually dimorphic. A second companion study identified no consistent or dose-related BPA effects on novelty preference, motor coordination, or spatial learning/memory (Ferguson *et al.*, 2012). Thus, the potential long-term effects of the results reported here remain unclear. The present data, however, contribute critical information regarding the molecular changes to the limbic system, which may underpin previously identified behavioral effects attributed to BPA exposure. Future studies will be needed to more definitively establish the functional and physiological consequences of neonatal gene expression changes within limbic structures, including the AMYG and hypothalamus.

CONCLUSIONS

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

FUNDING

National Institutes of Health (R01-16001-04S1 to H.B.P.).

ACKNOWLEDGMENTS

We thank Katherine A. McCaffrey of NCSU for facilitating ISHH and C. Delbert Law of the NCTR for expert technical assistance. We also appreciate the statistical assistance and guidance from Grace Kissling of the National Institute for Environmental Health Sciences.

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