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Prenatal PCBs Disrupt Early Neuroendocrine Development of the Rat Hypothalamus

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Abstract

Neonatal exposure to endocrine disrupting chemicals (EDCs) such as polychlorinated biphenyls (PCBs) can interfere with hormone-sensitive developmental processes, including brain sexual differentiation. We hypothesized that disruption of these processes by gestational PCB exposure would be detectable as early as the day after birth (postnatal day (P) 1) through alterations in hypothalamic gene and protein expression. Pregnant Sprague-Dawley rats were injected twice, once each on gestational days 16 and 18, with one of the following: DMSO vehicle; the industrial PCB mixture Aroclor 1221 (A1221); a reconstituted mixture of the three most prevalent congeners found in humans: PCB138, PCB153 and PCB180; or estradiol benzoate (EB). On P1, litter composition, anogenital distance (AGD) and body weight were assessed. Pups were euthanized for immunohistochemistry of estrogen receptor α (ERα) or TUNEL labeling of apoptotic cells, or quantitative PCR of 48 selected genes in the preoptic area (POA). We found that treatment with EB or A1221 had a sex-specific effect on developmental apoptosis in the neonatal anteroventral periventricular nucleus (AVPV), a sexually dimorphic hypothalamic region involved in the regulation of reproductive neuroendocrine function. In this region, exposed females had increased numbers of apoptotic nuclei, whereas there was no effect of treatment in males. For ERα, EB treatment increased immunoreactive cell numbers and density in the AVPV of both males and females, while A1221 and the PCB mixture had no effect. PCR analysis of gene expression in the POA identified nine genes that were significantly altered by prenatal EDC exposure, in a manner that varied by sex and treatment. These genes included brain-derived neurotrophic factor, GABAB receptors-1 and -2, IGF-1, kisspeptin receptor, NMDA receptor subunits NR2b and NR2c, prodynorphin, and TGFα. Collectively, these results suggest that the disrupted sexual differentiation of the POA by prenatal EDC exposures is already evident as early as the day after birth, effects that may change the trajectory of postnatal development and compromise adult reproductive function.

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Keywords

Polychlorinated biphenyls (PCBs); endocrine disruption; hypothalamus; sexual differentiation; neuroendocrine development; estrogen receptor; developmental apoptosis; anteroventral periventricular nucleus; medial preoptic nucleus

Introduction

Exposure to environmental endocrine disrupting chemicals (EDCs) during critical developmental periods, particularly late gestation and infancy, are consistently linked to impairments in homeostatic, endocrine, and neurobiological processes in adulthood (Dickerson and Gore, 2007). Amid concerns that chronic low-dose exposures to EDCs may be contributing to a decline in fertility in humans (Diamanti-Kandarakis *et al.*, 2009), recent interest has turned to elucidating how reproductive neuroendocrine systems may be perturbed by EDC exposures during early critical life stages. As the hypothalamic control of reproduction develops in a sexually dimorphic manner due to sex differences in gonadal steroid hormone actions in the brain, it is plausible to hypothesize that some of the links between perinatal EDCs and the diminution in reproductive competency may be due at least in part to reprogramming of the neonatal hypothalamus by these compounds.

Sexual differentiation of the hypothalamus of rodents occurs primarily during the third trimester of gestation through the early postnatal period. During these life stages, the number and phenotype of neurons that arise in the sexually dimorphic nuclei of the hypothalamus are sculpted by a number of neurodevelopmental processes, including programmed cell death, called apoptosis (Davis *et al.*, 1996a; Yoshida *et al.*, 2000). Whereas the exposure of the female rodent brain to circulating gonadal hormones is relatively low, the fetal male testis produces much higher levels of testosterone, which are aromatized to estradiol locally in the male brain (Bakker and Brock, 2010). Sex differences in these neural exposures have profound effects on the numbers of cells that ultimately survive in a region-specific manner.

Importantly, there are links between steroid hormone exposures and apoptosis. Depending upon the brain region, ligand-bound estrogen receptors (primarily ERα) may bind to nuclear response elements that promote transcription of factors that either stimulate or inhibit developmental apoptosis (McCarthy, 2008). This point is exemplified by developmental differences in apoptosis in the neonatal anteroventral periventricular nucleus (AVPV), a region that differs in size and cellular phenotype between males and females and is postulated to mediate estradiol positive feedback onto the GnRH/LH surge in females (Clarkson and Herbison, 2006). In the developing AVPV, estradiol stimulates apoptosis during the early postnatal period (Murakami and Arai, 1989; Sumida *et al.*, 1993; Waters and Simerly, 2009), contributing to the development of a smaller AVPV in adult males (Davis *et al.*, 1996b), and presumably, subsequent differences in reproductive physiology and behavior. Another brain region under investigation in this study is the medial preoptic area (MPN), a region important for adult reproductive behaviors (Wu *et al.*, 2009; Wu and Gore, 2010), sexually dimorphic in size (Madeira *et al.*, 1999), and abundant in ERs (Chakraborty *et al.*, 2003). Evidence that EDCs can act upon ERα and other steroid hormone receptors in the nervous system suggests that they may perturb developmental apoptosis in the AVPV and MPN, a hypothesis tested in this current study.

Our laboratory has been using a class of compounds known as polychlorinated biphenyls (PCBs), a family of persistent chemicals once used widely for industrial applications, as a model for neuroendocrine disruption (Salama *et al.*, 2003; Steinberg *et al.*, 2007; Steinberg *et al.*, 2008; Dickerson *et al.*, 2009). Although banned for decades, PCBs are still detectable

in most humans (Gladen *et al.*, 2003). EDCs rarely exert their effects by a single mechanism, and PCBs have been shown to be weakly estrogenic (Mortensen and Arukwe, 2008; Nomiyama *et al.*, 2010), as well as anti-estrogenic and anti-androgenic, among other mechanisms (Bonefeld-Jorgensen *et al.*, 2001). We utilized two PCB mixtures that differ in their half-life, degree of chlorination, and properties. Aroclor 1221 (A1221; an estrogenic PCB) is a technical mixture once used commercially, and is comprised of lightly chlorinated congeners with a half-life on the order of days (Matthews and Anderson, 1975). We also used a reconstituted mixture of the three most prevalent congeners detected in human and wildlife samples: PCB138, PCB153, and PCB180, which are more heavily chlorinated and have a half-life on the order of years (Milbrath *et al.*, 2009; Seegal *et al.*, 2010). *In vitro* studies have shown that these compounds interact with steroid hormone receptors including ERα at low doses (Bonefeld-Jorgensen *et al.*, 2001). We tested the hypotheses that prenatal EDCs would affect developmental apoptosis and expression of ERα in a sexually dimorphic manner, an effect that could be detectable as early as postnatal day (P) 1. We also used a 48-gene real-time PCR array platform to identify novel gene expression targets of developmental PCB exposure on the P1 hypothalamus.

Methods

Animals and perinatal treatment

All protocols on rats were carried out following guidelines from the National Institute of Health Guide for Care and Use of Laboratory Animals, and performed following protocols approved by the Institutional Animal Care and Use Committee at the University of Texas at Austin. Dams for this study (Harlan Sprague–Dawley Inc.; Houston, Texas; Stock/Strain: Hsd:Sprague–Dawley®™ SD®™) were those described in a sister study on effects of prenatal PCBs on the adult hypothalamus (Dickerson *et al.*, 2011). Rats were housed individually under standard husbandry [12:12 partially reversed light cycled (lights on 2300 h, lights off 1100 h)] and fed low-phytoestrogen Harlan-Teklad 2019 Global Diet *ad libitum* for at least 3 weeks prior to mating. Rats were handled daily to minimize stress.

Dams were mated with sexually experienced male rats (males randomly rotated with females), and the day of sperm positive vaginal smears was termed embryonic day (E) 1. The pregnant rats (n=10-12 per treatment group) were randomly assigned to one of four treatment groups and injected i.p. on E16 and E18 with one of the following: 0.1 ml of vehicle (DMSO 99.5%, Sigma, #D4540, Lot# 037K07663); 50 μg/kg estradiol benzoate (Sigma, #E8515, Lot# 125K1029, serving as an estrogenic positive control); 1 mg/kg Aroclor 1221 (AccuStandard, #C-221N, Lot# 083-166, dose based on our published work showing effects on reproductive function in female rats (Steinberg *et al.*, 2007; Steinberg *et al.*, 2008)); or 1 mg/kg reconstituted PCB mixture (referred to as PCB Mix). Rationale for the PCB Mix is that these are the three most prevalent PCB congeners found in mammalian tissue samples (Gladen *et al.*, 2003; Bentzen *et al.*, 2008): PCB138 (2,2′,3,4,4′,5′-Hexachlorobiphenyl; AccuStandard, #C138N, Lot# 082704MS-AC), PCB153 (2,2′,4,4′,5,5′-Hexachlorobiphenyl; AccuStandard, #C153N, Lot# 111804AG-AC), and PCB180 (2,2′, 3,4,4′,5,5′- Heptachlorobiphenyl; AccuStandard, #C180N, Lot# 013004MT-AC) at equimolar concentration. It should be noted that they are non-coplanar and do not bind the aryl hydrocarbon receptor (Van den Berg *et al.*, 1998). The dose, age and route of exposure were based on the literature on detectable levels of PCBs in humans (Lanting *et al.*, 1998; Lackmann, 2002), the timing of brain sexual differentiation in rats (Murakami and Arai, 1989; Rhees *et al.*, 1990), and for consistency with other neuroendocrine studies including our own (Chung and Clemens, 1999; Chung *et al.*, 2001; Gore *et al.*, 2002; Salama *et al.*, 2003; Woodhouse and Cooke, 2004; Steinberg *et al.*, 2008). Although we have not measured PCB content in the offspring, we have previously predicted that pups were exposed to approximately 2 μg/kg total PCBs for both the A1221 treatment group and the

PCB Mix treatment group (Takagi *et al*., 1986; Steinberg *et al.*, 2007; Steinberg *et al.*, 2008). On the day after birth, P1, the numbers of live and dead offspring were counted, and sex ratio was determined. Anogenital distance (AGD) was measured using a digital microcaliper (Marois, 1968; Steinberg *et al.*, 2008), and the ratio of AGD to the cube root of body weight (AGD index) was calculated to evaluate AGD index (Marois, 1968; Gallavan *et al.*, 1999).

Tissue collection

The offspring were divided into two groups and either sacrificed at P1 between 0900 – 1000 h to evaluate experimental endpoints reported herein, or allowed to mature for a study reported elsewhere (Dickerson *et al.*, 2011). All work in this current study focuses on the P1 offspring to evaluate early life events following prenatal endocrine disruptor exposures. One male and female F1 rat per litter was euthanized for protein and apoptosis studies (N=8 per sex, each from different litters), and 1 male and female per litter were utilized for gene expression studies (N=6 per sex, each from different litters). For immunohistochemistry studies, pups were deeply anesthetized with 0.05 ml ketamine (100 mg/ml) and 0.05 ml of xylazine (20 mg/ml), and trans-cardially perfused with 0.9% saline (5 ml) at a rate of 5 ml/min, followed by 4% paraformaldehyde (50 ml). The brains were removed and postfixed overnight in 4% paraformaldehyde, and then transferred into PBS with 0.2% sodium azide. A vibrating microtome (Leica VT 1000S, Leica Microsystems, Nussloch, Germany) was used to cut 50 μm-thick sections that were stored in PBS with 0.2% sodium azide at 4 °C until use.

For gene expression analyses, animals were rapidly euthanized by decapitation. Brains were quickly removed and the preoptic area-anterior hypothalamus (POA), which contains the AVPV and medial preoptic nucleus (MPN), was dissected on ice, snap frozen (Dickerson *et al.*, 2008) and stored at -80 C until RNA extraction. Terminal trunk blood samples were collected and centrifuged at 5000 × g for five minutes to separate serum, and serum was stored at -80°C until steroid hormone analysis. Because EDC treatment can affect AGD of treated animals, the sex of each animal was confirmed via visualization of the uteri or testes at the time of euthanasia.

Immunohistochemistry for ER alpha

Hypothalamic tissues were recoded for tissue processing and analyses so that the experimenter was blind to treatment group and sex. For each neonatal F1 animal, two AVPV tissues and four MPN tissues per rat were immunolabeled for ERα. Although the number of sections was too great to process in a single run, animals from each sex and treatment group were equally represented in every run. Sections were rinsed in PBS (Phosphate-buffered saline, pH = 7.4) at room temperature on a shaker, followed by treatment with 3:1 methanol: 3% H_2O_2 for 20 minutes at room temperature to eliminate endogenous peroxide activity. Sections were then washed, and incubated in the rabbit polyclonal anti-ERα antibody C1355 (1:10,000; Upstate Biotechnology, Waltham, MA) with 10% normal goat serum (NGS) and 0.1% Triton-X for 72 hours at 4°C on a shaker. This antibody was generated against the last 15 amino acids of ERα, which has no homology to the corresponding region of ERβ. The sections were then washed and incubated in 5% normal goat serum and secondary antibody (biotinylated goat anti-rabbit immunoglobulin (Ig)G, 1:600; BA-1000; Vector Laboratories) for one hour, then subjected to peroxidase reaction with nickel-enhanced diaminobenzidine (DAB). Sections were mounted on gelatin-subbed slides, dried, dehydrated in a graded alcohol series, counterstained with methyl green, and coverslipped with DPX (44581; Fluka, Steinheim, Germany). Controls were also run with the primary antibody omitted, and no specific binding was observed.

Quantification of ERα in the MPN was performed using unbiased stereological analysis according to methods described in detail previously (Chakraborty *et al.*, 2003; Chakraborty *et al.*, 2005). A wet-mount of fresh tissue showed that average tissue thickness was 50.6 μm. The MPN region was identified in Nissl-stained sections by comparing anatomical landmarks to an atlas of the developing rat brain (Ashwell, 2008). Contours were drawn around the MPN at low magnification (10× objective) using an Olympus BX-61 microscope. A buffer zone at the top and bottom of sections was set at 3 μm for all experimental stereology. For each rat, the regional volume was extrapolated based on the contours and tissue thickness (Volume = regional area × thickness). The Stereo Investigator® software (MicroBrightField, Williston, VT) randomly placed 75 μm × 120 μm grids ("dissector frames") within the MPN contour. Within these dissector frames, the DAB-stained ERα-labeled nuclei were counted within a 45 μm × 45 μm counting frame for the MPN ("optical dissectors"). Based on these parameters, the number and density (# immunoreactive cells/ volume of each nucleus) of ERα-immunoreactive (ERα-ir) nuclei falling within the region was quantified. The coefficients of error (Cruz-Orive/Geiser) and variation of the estimates were calculated as described previously (Schmitz and Hof, 2000). Photomicrographs were taken to produce the figures, and images subjected to only minor adjustments of contrast using Adobe Photoshop CS4 (Adobe, San Jose, CA), with any adjustments were applied equally to tissues from rats of different treatment groups. Because ERα labeling was sparse and highly variable in the AVPV and did not enable adequate statistical power, stereological analysis for this region was not possible.

Detection of apoptosis

To stain the nucleosomal DNA fragments in apoptotic cells, we used a modification of the TUNEL method described by Bessert and Skoff (Bessert and Skoff, 1999). All reagents were supplied by the Fluorescein-FragEL DNA Fragmentation Detection Kit (EMD Chemicals Inc., Gibbstown, NJ) and applied according to the kit protocol at room temperature (RT) unless otherwise noted. In brief, tissues were pretreated with sodium citrate buffer (10mM Sodium Citrate, 0.05% Tween 20, pH 6.0) at 60°C for 15 min, then washed in 0.1 M PBS, pH 7.4, incubated in 0.1% Triton X-100 (Sigma-Aldrich, St. Louis, MO) in PBS for 15 min, and washed in 0.1 M PBS. The sections were then treated with 20 μg/ml proteinase K in 10 mM Tris pH 8 for 15 min and washed in PBS. The sections were incubated in equilibration buffer for 30 min, followed by incubation in labeling reaction mixture in a humidified chamber at 37°C for 1.5 hr. Sections were washed in PBS and mounted onto gelatin subbed slides, and coverslipped with Fluorescein-FragEL™Mounting Media. Positive controls for the TUNEL assay were generated by pretreatment of sections with DNAse I (Applied Biosystems Inc., Foster City, CA). Negative controls for the TUNEL procedure were treated in the same manner as the test samples except that the TdT enzyme was omitted from the reaction mixture and was replaced with dH_2O. No labeling was found in the negative controls. Mounted sections were stored in the dark at 4°C until analysis.

A 1:2 series from the AVPV (2 sections total) and 1:2 series from the MPN (4 sections total) were selected from each animal. For apoptosis studies, numbers of labeled cells were counted unilaterally throughout the rostrocaudal extent of the region. Using StereoInvestigator software (MicroBrightField Inc.), contours were drawn for each region at low magnification (10× objective) using anatomical landmarks and a developing rat brain atlas (Ashwell, 2008). Ten counting grids measuring 25 μm × 25 μm per were randomly placed within the left hemisphere for the AVPV and ten counting grids measuring 45 μm × 45 μm were placed on the left side of the third ventricle for the MPN. Total numbers of apoptotic cells were counted for each region at high magnification (100× objective).

Serum Hormone Assays

Because of the small amount of serum obtainable from a P1 rat, serum samples from 2-3 same-sex siblings were pooled for littermates (N=8 pooled samples per sex per assay), and samples run in single testosterone, progesterone, or estradiol RIAs as described previously (Dickerson *et al.*, 2011). Briefly, total serum testosterone was determined using the Active® Testosterone coated well EIA kit (Catalog # DSL-10-4000, Lot # 08035-B, Diagnostic Systems Laboratories, Inc., Webster, TX, USA) on duplicate volumes of 50 µl serum. The assay limit of detection was 0.04 ng/ml, and the intra-assay CV based on duplicate samples for the assay was 2.97%. Progesterone concentrations were determined using the ACTIVE® Progesterone Coated-Tube Radioimmunoassay Kit (Catalog # DSL-3900, Lot # 07076, Diagnostic Systems Laboratories, Inc., Webster, TX, USA), on duplicate volumes of 25 µl. The sensitivity of the assay was 0.12 ng/ml, and the intra-assay CV was 3.26%. Estradiol concentrations were determined using an ultrasensitive double-antibody RIA kit (Catalog # DSL-4800, Lot # 07076, Diagnostic Systems Laboratories, Inc., Webster, TX, USA), on duplicate volumes of 200µL. The assay limit of detection was 2.2 pg/mL, and the intra-assay coefficient of variability based on duplicate samples was 7.97%. For all assays, a few samples for which the CV between duplicates was 10% or greater were excluded from analysis.

RNA extraction

Messenger RNA from frozen POA dissections was extracted using our in-house double detergent lysis buffer system as described previously (Dickerson *et al.*, 2008; Walker *et al.*, 2009). In brief, samples were homogenized and the cytoplasmic RNA was treated with proteinase K, followed by extraction with phenol chloroform and precipitation in isopropanol. Genomic DNA contamination was removed using the TURBO DNA-free kit (Applied Biosystems Inc., Cat. No. AM1907, Foster City, CA) according to the manufacturer's protocol. The concentration of resulting cytoplasmic RNA was determined using a Nanodrop (ND-1000, Nanodrop Technologies, Inc., Wilmington, DE).

Taqman low-density arrays (TLDA)

Samples were run as described by our laboratory (Walker *et al.*, 2009) on a custom rat neuroendocrine TLDA (Applied Biosystems Inc., Foster City, CA), a panel of 48 candidate neuroendocrine genes. Cytoplasmic RNA (2 µg) was converted to cDNA using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems) according to the manufacturer's instructions. The resulting cDNA was diluted 1:5 before PCR reactions were performed using Applied Biosystems' Taqman reagents on an ABI 7900 real-time PCR machine using the following parameters: 50 C for 2 min, 94.5 C for 10 min, 45 cycles of 97 C for 30 sec, and 59.7 C for 1 min. Relative expression for each gene was determined using the comparative Ct method (Pfaffl, 2001). Each sample was normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) expression (Walker *et al.*, 2009), and the data were calibrated to the average change in Ct for the treatment group with the lowest expression.

Statistical Analysis

SPSS statistical software (17.0 for Macintosh, SPSS Inc., Chicago, IL) was used to evaluate the effects of treatment on litter composition, body weight, AGD, serum hormones, AVPV and MPN volume, stereological results of ERα immunoreactive cells, and apoptosis endpoints. We had independent *a priori* hypotheses for each sex; thus, statistics were performed separately for males and females. Datasets were examined for homogeneity of variance and normality. For datasets that met these criteria, comparisons were made by one-way ANOVA (factor: treatment) followed by Fisher's LSD post-hoc analysis when indicated

by a significant main effect. When variance between treatment groups was unequal, datasets were compared using the nonparametric Kruskal-Wallis test. In all these cases *p*-values < 0.05 were considered statistically significant. For the gene expression data, statistics were conducted using the normalized Ct (δ-Ct) for each sample (before transformation to fold change). Because the TLDA measures expression of 48 genes, a Bonferroni correction was used to set the cut off for significance at $p < 0.001$.

Results

Litter composition, birth weight and anogenital distance

On postnatal day P1, litter composition was determined, and birth weight and anogenital distance (AGD) were recorded for all pups in each litter. There was no effect of treatment upon total pups (DMSO: 13.0 ± 0.43; EB: 12.3 ± 0.85; A1221: 12.4 ± 1.0; PCB: 10.8 ± 1.3) or live pups (DMSO: 10.9 ± 0.81; EB: 10.9 ± 0.97; A1221: 11.8 ± 1.0; PCB: 10.2 ± 1.5) per litter, or upon sex ratio (Percentage female: DMSO: 49 ± 3%; EB: 40 ± 3%; A1221: 48 ± 3%; PCB: 48 ± 7%). Likewise, there was no main effect of treatment upon body weight in either sex, although we did observe a trend for increased body weight in females treated with EB or A1221 (Fig. 1A, $p = 0.06$). We observed a sex-dependent effect of treatment on AGD, with EB-, A1221-, and PCB-treated females having reduced AGD ($p<0.001$), and EB- and A1221-treated males having increased AGD ($p<0.05$) compared to their vehicle counterparts (Fig. 1B).

Serum hormones

We observed a significant effect of treatment upon serum estradiol in EB-treated females (Fig. 2A, $p<0.005$), a group with higher estradiol levels than all other groups. A significant main effect of treatment upon serum progesterone was noted in females (Fig. 2B) but not in males, with A1221- and PCB Mix-treated females having reduced progesterone compared to EB- ($p<0.05$) but not DMSO-treated females. However, no group differed from control in progesterone concentrations. There was a main effect of treatment upon serum testosterone in males (Fig. 2C, $p<0.001$), but not females, with a reduction observed in EB and PCB-treated males compared to the control and A1221 males.

Apoptosis in AVPV and MPN

Cells undergoing apoptotic cell death display distinct morphological changes, characterized by cellular shrinkage, nuclear pyknosis, chromatin condensation and membrane blebbing. To ascertain whether prenatal EDC exposure disrupts developmental apoptosis in the neonatal hypothalamus, we used a TUNEL assay and DAPI labeling to visualize apoptotic nuclei in the AVPV and MPN. A representative photomicrograph of TUNEL labeling with DAPI counterstaining is shown for the MPN (Fig. 3A); similar apoptotic cells were seen in the AVPV (not shown). Quantification of apoptotic cell numbers showed that in the female AVPV, EB- and A1221-treatment increased the number of apoptotic nuclei (Fig. 3B, $p<0.001$), while no effect of treatment was observed in the male AVPV. In contrast, no effect of treatment upon apoptosis was observed in either females or males in the MPN (Fig. 3C).

ER alpha expression in the AVPV and MPN

In the AVPV, ERα labeling was sparse and highly variable; thus, stereological analysis for this region was not possible due to inadequate statistical power. However, qualitative observations suggested that ERα immunoreactivity (ir) in the AVPV is greater in control females compared to control males, and it did not appear that either males or females were affected by treatment with EB, A1221, or PCB Mix in this region on P1.

Representative photomicrographs of ERα-ir in the MPN are shown in P1 male and female rats for the four treatments (Fig. 4). Stereological cell counting showed a significant main effect of treatment on ERα-ir cell numbers, with EB-treated males and females having significantly more of these cells than DMSO-, A1221-, and PCB Mix-treated counterparts ($p<0.05$; Fig. 5A). No effect of treatment upon MPN regional volume was observed in males or females (Fig. 5B). Density of ERα was increased by EB-treatment in MPN of P1 males and females ($p<0.05$; Fig. 5C).

Effects of developmental PCB exposure on POA neuroendocrine gene expression

Forty-seven of the candidate genes on the neuroendocrine TLDA were detectable following real-time PCR reactions (Table 1). As described previously (Walker *et al.*, 2009), POA genes were normalized to GAPDH for analyses, as this gene did not vary by treatment. Of the remaining detectable genes, relative gene expression of nine genes was significantly affected by developmental EDC exposure in P1 males or females following the Bonferroni correction (Fig. 6): brain-derived neurotrophic factor (BDNF), GABAB receptors 1 and 2, IGF-1, kisspeptin receptor (GPR54), NMDA receptor subtypes NR2b and NR2c, prodynorphin, and TGFα.

Discussion

We recently reported that prenatal PCB exposure impairs gene and protein expression in the adult female hypothalamus (Dickerson *et al.*, 2011), and alters paced mating behavior (Steinberg *et al.*, 2007). As sexual differentiation begins during late gestation, we hypothesized that PCB exposure at E16 and E18 causes effects on developing sexually dimorphic regions of the neonatal brain that would be manifested very shortly after exposure. In support of our hypothesis, we found that low doses of PCBs, relevant for human exposures (Lackmann, 2002; Lackmann *et al.*, 2004) had sex-dependent effects on developmental apoptosis in the P1 hypothalamus, as well as neuroendocrine gene and protein expression. The implications of these findings are discussed below.

Litter composition, AGD and body weight

In the current study, we selected doses of A1221 and PCB Mix to approximate human and environmental exposures (Lackmann, 2002; Lackmann *et al.*, 2004), anticipating that these sub-toxic levels would not cause any gross morphological effects. Rather, our aim was to assess the early postnatal neuroendocrine outcomes of prenatal PCB treatment. This would enable us to establish just how early the brain is altered and the relationship between these early life outcomes to those we have reported for animals later in life (Steinberg *et al.*, 2007; Steinberg *et al.*, 2008; Dickerson *et al.*, 2011). As predicted, EB, A1221, and PCB Mix had no significant effects on total or live births in the exposed F1 generation, nor did they affect sex ratio or body weight.

In most mammalian species the AGD is sexually dimorphic, with males having a longer AGD than females. Neonatal anogenital distance is largely determined by the action of androgens (Marois, 1968), and thus can be an external indicator of masculinization (Gray *et al.*, 1994). In the current study, we found a sex-dependent effect upon neonatal AGD, although not in the direction that we predicted: EB-, A1221-, and PCB Mix-treatment decreased (hyperfeminized) female AGD, while EB- and A1221- exposure increased (hypermasculinized) male AGD. We are not certain how to interpret this result. One possibility for the observed reduction in female AGD may be related to direct or indirect PCB effects on the androgen receptor (AR), although it is important to note that their relative binding affinity for the receptor is several orders of magnitude lower than endogenous androgens (Portigal *et al.*, 2002; Fang *et al.*, 2003). However, this cannot

explain the larger AGD in EB-treated males. In the context of reduced serum testosterone observed in EB- and PCB Mix-treated males, the increase in male AGD may seem further counterintuitive, but it is consistent with reports by our lab and others of increased AGD following developmental PCB exposure throughout postnatal development (Kuriyama and Chahoud, 2004; Dickerson *et al.*, 2011).

Developmental apoptosis and ERα protein expression in the neonatal POA

In the current study, we investigated the effects of gestational PCB exposure on sexual differentiation and developmental apoptosis in the neonatal hypothalamus of males and females. In the female AVPV, treatment with EB or A1221, but not the PCB mix, increased the number of apoptotic nuclei, while no effect of treatment was observed in the male AVPV. In addition, no effect of treatment was detected in the MPN of either sex. The similar results for EB and A1221 suggest that these effects are likely exerted through an estrogenic mechanism to masculinize the female AVPV via increased apoptosis. In addition, the differences between A1221 and the PCB mix may be attributable to their difference in relative estrogenicity at the ER. While A1221 has been consistently shown to have estrogenic properties, the di-ortho substituted non-coplar congeners that comprise the reconstituted PCB mixture have demonstrated anti-estrogenic properties in several *in vitro* studies (Oh *et al.*, 2007). The lack of treatment effect in males may be attributable to the higher baseline exposure of their developing hypothalamus to steroid hormones. The consequences of altering developmental apoptosis in the female AVPV may affect the AVPV's ability to generate the estrogen-induced GnRH/LH surge and ovulation in adulthood (Wiegand *et al.*, 1978; Wiegand and Terasawa, 1982; Gu and Simerly, 1997; Le *et al.*, 2001). In our companion study (Dickerson *et al.*, 2011), we found that EB- and A1221-treatment reduced AVPV volume in female littermates of the animals used herein who had been allowed to mature to early adulthood and were studied at P60. Together, these results suggest that increased AVPV cell loss at P1 may be a contributing factor to the reduction of the regional AVPV volume observed in adult (P60) females.

There are developmental sex differences in the number of cells in the AVPV that are immunopositive for ERα (Davis *et al.*, 1996b; Orikasa and Sakuma, 2003). Thus, we also ascertained the effect of prenatal EDC exposure on this endpoint in the neonatal AVPV and MPN. In both regions, qualitative observations suggested no clear sex difference in ERα-ir at P1, consistent with reports from other labs (Yokosuka *et al.*, 1997). Although we could not quantify ERα-ir in the AVPV, in the MPN, stereological analysis showed that treatment with EB, but not PCBs, increased the number of cells immunopositive for ERα in both male and female P1 pups. These results were unexpected, as estradiol has been shown to down-regulate ERα at the level of gene expression (Lauber *et al.*, 1991; Simerly and Young, 1991) as well as immunoreactivity in the adult (Koch, 1990) and neonatal (P10) (Orikasa *et al.*, 1994; Orikasa *et al.*, 1995; Orikasa *et al.*, 1996) rodent brain. Other studies investigating the effects of PCBs upon hypothalamic estrogen receptor expression are quite limited. In one, Lichtensteiger *et al.* (2003) found that gestational exposure to A1254 increased ERα gene expression in the ventromedial nucleus of the hypothalamus (VMN), a region important for feminine sexual behavior, in female rat embryos (Lichtensteiger *et al.*, 2003). Because sex differences in ERα in the AVPV and MPN regions do not appear until postnatal day 10, it is likely that future studies evaluating later developmental time points will provide temporal resolution to the endocrine disrupting effects of PCBs upon hypothalamic ERα. Although our experimental design did not enable us to collect tissues from rats at ages other than P1 (this study) and P60 (Dickerson *et al.*, 2011), ongoing work is including a more systematic analysis of developmental postnatal profiling of the POAs of male and female rats prenatally exposed to PCBs.

Neuroendocrine gene expression in the neonatal POA

Using a custom-designed 48-gene real-time PCR array, we identified nine genes whose expression changed significantly with neonatal PCB exposure: brain-derived neurotrophic factor (BDNF), $GABA_B$ receptors 1 and 2, IGF-1, kisspeptin receptor, NMDA receptor subunits NR2b and NR2c, prodynorphin, and TGFα. Each of these identified genes is an important contributing factor to hypothalamic development, differentiation and function (Gore, 2001; Daftary and Gore, 2004; Walker *et al.*, 2009). For instance, BDNF stimulates migration of neurons during development, is highly expressed in discrete regions of the hypothalamus during postnatal development, and its release from certain cell types is regulated by gonadal steroid hormones [reviewed in (Tobet *et al.*, 2009)]. In our study, expression of BDNF was reduced 50% by EB treatment in males, while females were not affected by treatment. Similarly, several neurotransmitters, including GABA, are thought to act as neurotrophic factors in hypothalamus (McClellan *et al.*, 2008). The $GABA_B$ receptor subunits B1 and B2 guide cell migration and positioning in the ventromedial nucleus (VMN). In females prenatally exposed to either EB or A1221, we observed an increase in the expression $GABA_{B1}$, while a decrease in the expression of $GABA_{B2}$ was observed in males treated with the PCB mix. This observation could have implications for proper establishment of sexually dimorphic circuitry and connections within this region.

Other significantly affected genes are involved in brain sexual differentiation by modulating cell survival and developmental apoptosis. For example, the growth hormone IGF-1 plays a crucial role in somatic growth, as well as proliferation and inhibition of apoptosis [reviewed in (D'Ercole and Ye, 2008)]. Similarly, NR2b and NR2c are each subunits of the ionotropic NMDA receptor, whose activation not only has actions on GnRH neurons and the reproductive axis (Gore, 2001; Maffucci *et al.*, 2008), but also play an important role in sexually dimorphic apoptosis (Hsu *et al.*, 2000; Hsu *et al.*, 2001).

Kisspeptin signaling is important for many aspects of reproductive maturation and function, including a recently discovered role in the guidance of GnRH neurites to the median eminence at the base of the hypothalamus (Fiorini and Jasoni, 2010). In males treated with either EB or PCB Mix, we observed a decrease in POA kisspeptin receptor expression. Interestingly, in littermates of these animals used for a related study, we observed a delay in male pubertal onset (Dickerson *et al.*, 2011) that may reflect improper targeting of kisspeptin fibers. The neuropeptide dynorphin is co-expressed in kisspeptin neurons, and also acts to modulate GnRH secretion (Navarro *et al.*, 2009). In females treated with EB or PCB Mix, we observed a decrease in prodynorphin gene expression, and a non-significant trend ($p=0.006$) in A1221-treated females. This result is consistent with our related study, in which we observed decreased kisspeptin protein expression and impaired GnRH neuron activation in adult female littermates in the EB, A1221, and PCB treatment groups (Dickerson *et al.*, 2011). It is possible that disruption of this neuronal circuitry begins during neonatal development. Finally, TGFα, expression of which was increased in male-A1221 rats, is a cytokine involved in a number of cellular functions such as cell growth, proliferation, differentiation, and apoptosis (Galbiati *et al.*, 2003). Moreover, this growth factor is released by hypothalamic glial cells, and plays a role in regulation of GnRH release. As a whole, these gene expression data identify a group of candidates for further study as early developmental targets of EDCs.

The neural, neuroendocrine, and/or behavioral changes observed in the adult offspring of EDC-exposed females may be the result of indirect effects secondary to changes in maternal care (Cummings *et al.*, 2005; Cummings *et al.*, 2010). Although we did not assess maternal behavior in the current study, our results provide evidence that suggest a direct effect of PCBs on the developing neuroendocrine system of exposed neonatal offspring, independent of the effects of PCBs on maternal care. Ongoing studies in our lab are evaluating the effect

of these PCBs on aspects of maternal behavior, and will help clarify the contribution of changes in maternal care to neuroendocrine deficits in the developing offspring.

Comparison of Treatment Effects

PCBs are typically found as complex mixtures in environmental, human, and wildlife samples. We therefore utilized two mixtures of PCBs that differ in degree of chlorination, half-life, and chemical properties to study the effects of PCBs on the developing neuroendocrine system. Although this experimental approach has inherent limitations, including difficulty in discerning individual mechanisms of action, we selected these compounds in an effort to represent ecologically relevant conditions. While A1221 is thought to be mainly estrogenic with regard to its interaction with the ER, congeners that comprise the reconstituted PCB mixture have been shown to be estrogenic, anti-estrogenic as well as anti-androgenic (Bonefeld-Jorgensen *et al.*, 2001). Moreover, while A1221 contains congeners of all three classifications (coplanar, dioxin-like coplanar, and non-coplanar), the reconstituted PCB mixture congeners are all non-coplanar, and do not bind the arylhydrocarbon receptor with significant affinity (Van den Berg *et al.*, 1998).

We noted some similarities and other differences in the actions of the three treatment groups upon the neuroendocrine and somatic endpoints. The profiles of these effects are summarized in Table 2. Prenatal EB treatment was associated with the greatest number of effects in both sexes, followed by A1221 and finally the PCB Mix. There were more similarities between profiles of endpoints affected by A1221 and EB, especially in females, consistent with A1221's greater estrogenic potential compared to the PCB Mix. The relatively few effects of the reconstituted PCB mixture on the endpoints evaluated may reflect a weaker interaction with neuroendocrine components. It is clear that further work is necessary to understand the underlying mechanisms of these EDCs on targets in the hypothalamus.

Summary and Conclusions

Collectively, these data show that endocrine disruption by gestational PCBs causes changes to the hypothalamic neural circuitry controlling reproduction in the exposed offspring, a process that is detectable as early as the day after birth. We found that PCBs cause changes to sexually dimorphic brain regions underlying sex-specific reproductive physiology and behavior through the perturbation of normal developmental apoptosis, and by altering gene expression of neurotransmitters and receptors known to play important roles in differentiation and migration of hypothalamic neurons. Although there may not be an easy or obvious interpretation of the nine genes affected by prenatal PCB exposure, the gene expression data add to the story of the effects of developmental EDC exposure on reproductive neuroendocrine function. In fact, the sex differences in gene expression profiles and differential regulation by the various EDCs suggest that subtle mechanistic differences underlie the physiological phenotypic differences such as hormonal regulation, reproductive development, and adult reproductive function.

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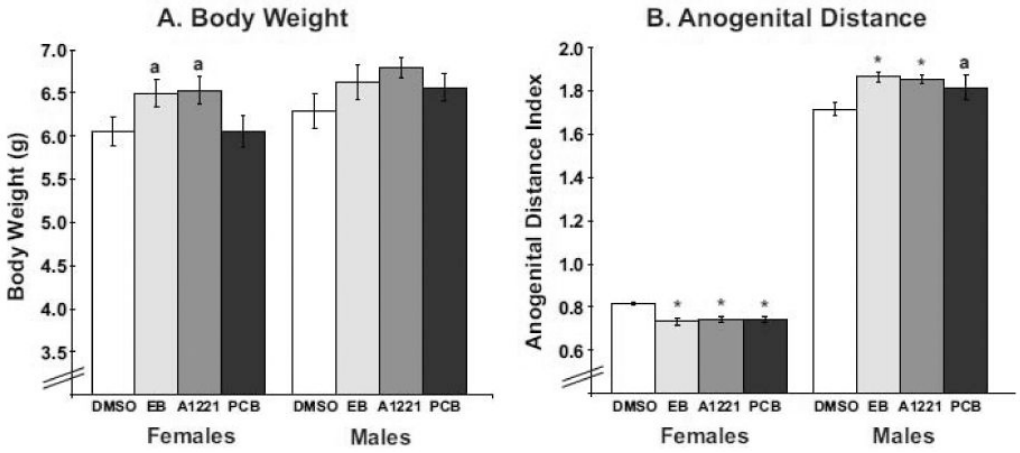

Figure 1.

Data for body weight (A) and anogenital distance (B) on P1 are shown for female and male rats (mean ± S.E.M). Treatment with estradiol benzoate (EB) or Aroclor 1221 (A1221) caused a non-significant trend for increased body weight in females ($^{a}p=0.06$). For anogenital distance (AGD), treatment with EB, A1221, or PCB Mix caused a decrease in female AGD (**$p<0.001$), while EB- or A1221-treatment increased male AGD (*$p<0.05$). Treatment with PCB Mix caused a non-significant trend for increased AGD in males ($^{a}p=0.06$). AGD index is the ratio of AGD (mm) to the cube root of body weight. On the x-axis of this and other figures, the label PCB refers to the PCB Mix group.

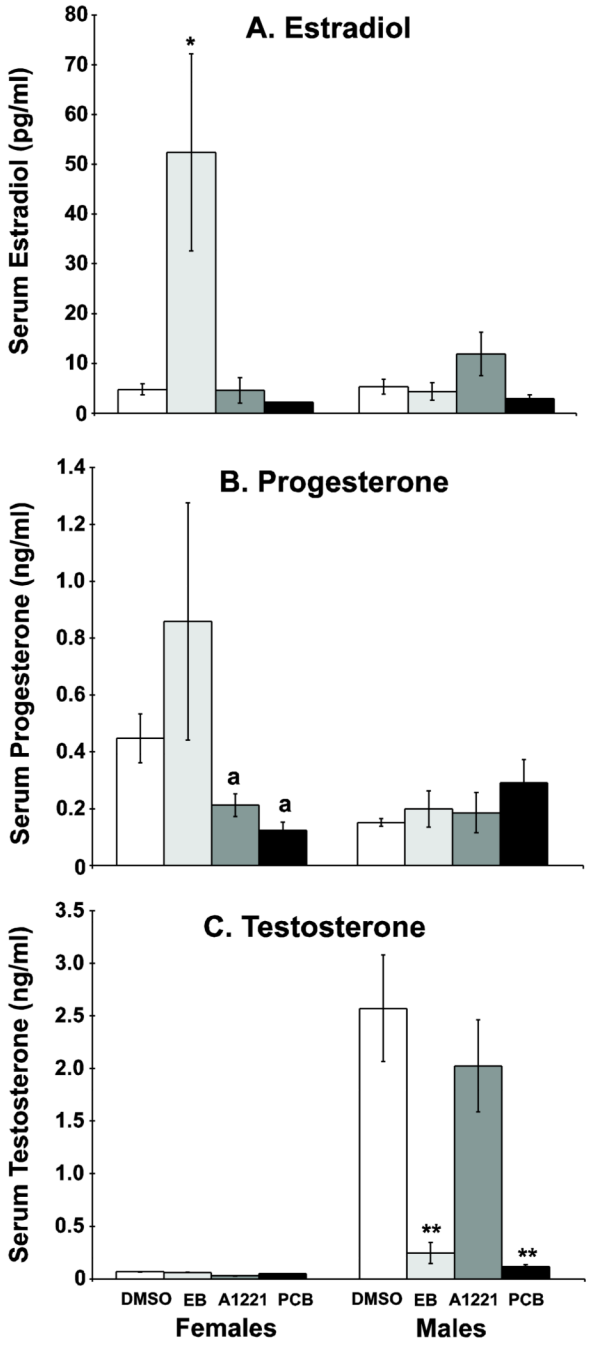

Figure 2.

Serum levels of estradiol (A), progesterone (B), and testosterone (C) in neonatal F1 animals. For estradiol, EB-treatment caused an increase in estradiol levels in females (*$p<0.005$), while males were unaffected by any treatment. No effect of treatment upon progesterone was observed in males or females, although the A1221- and PCB Mix-treated females had lower progesterone levels than the EB females (a, $p<0.05$). For testosterone, EB- and PCB Mix-treated males had lower serum testosterone compared to DMSO- and A1221-treated males (**$p<0.001$ vs. both). N=8 pooled samples per sex per assay. The bars are the mean ± S.E.M.

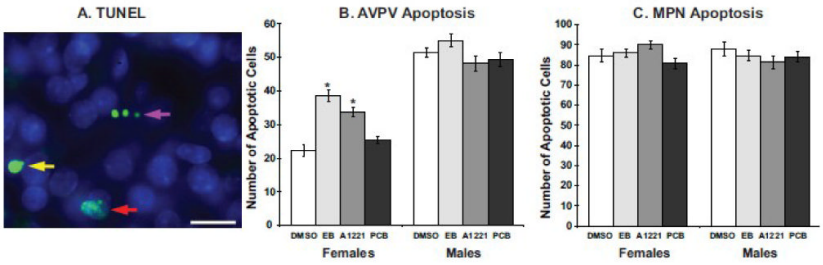

Figure 3.

A representative photomicrograph shows TUNEL labeling results for early (red arrow), middle (yellow arrow), and late (purple arrow) phase apoptosis the MPN (A). TUNEL-positive cells appear bright green (arrows) and DAPI counterstained nuclei appear blue. Scale bar = 25 μm. Quantification of apoptotic nuclei was performed in the AVPV (B) and MPN (C). In females, EB- and A1221-treated females have a higher incidence of apoptotic nuclei compared to DMSO control (*$p<0.01$), while males were unaffected by treatment. No effect of treatment upon MPN apoptosis was observed in either sex.

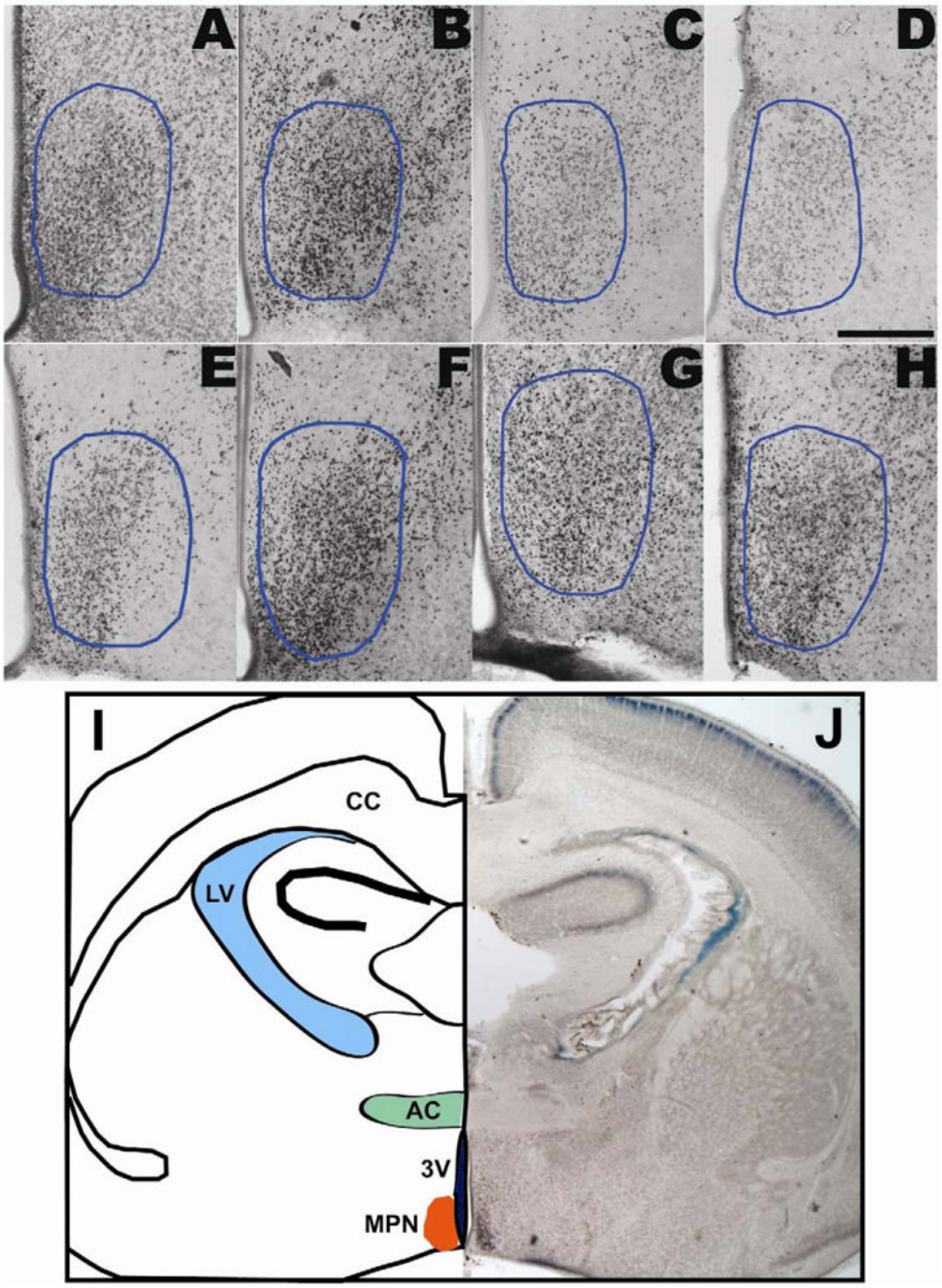

Figure 4.

Photomicrographs of estrogen receptor alpha (ERα) immunoreactivity in the MPN (A-H; outlined in blue) of P1 female and male rats prenatally exposed to DMSO vehicle (A,E), EB (B,F), A1221 (C,G), or PCB Mix (D,H). The third ventricle is at the lower left of each micrograph. Although the images presented here were photographed at low magnification (10×), quantification and analysis of ERα-immunoreactivity was performed at high-power magnification (40×). ERα nuclei are labeled with dark brown nickel-enhanced DAB product. Scale bar = 250 μm. I, J: A lower magnification micrograph at the level of the MPN was reflected and drawn to indicate landmarks. Abbreviations: 3V, third ventricle; AC:

anterior commisure; CC: corpus callosum; LV: lateral ventricle; MPN: medial preoptic nucleus.

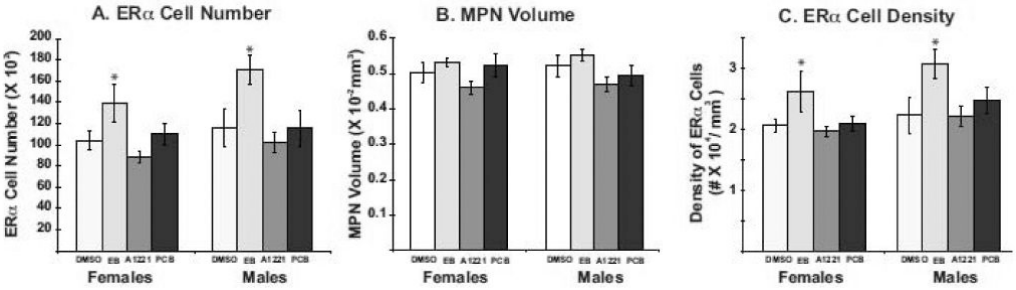

Figure 5.

Stereologic analysis results for ERα-immunoreactive cell numbers in the MPN (A), regional volume of the MPN (B) and ERα-immunoreactive cell density (C) are shown for neonatal P1 rats (n = 6-8 rats per treatment group). Data are the mean ± S.E.M. ERα cell number was significantly increased in EB-treated males and females compared to DMSO control. MPN volume was unaffected by EDC treatment in either sex. Cell density was increased by EB treatment in both sexes. *$p < 0.05$.

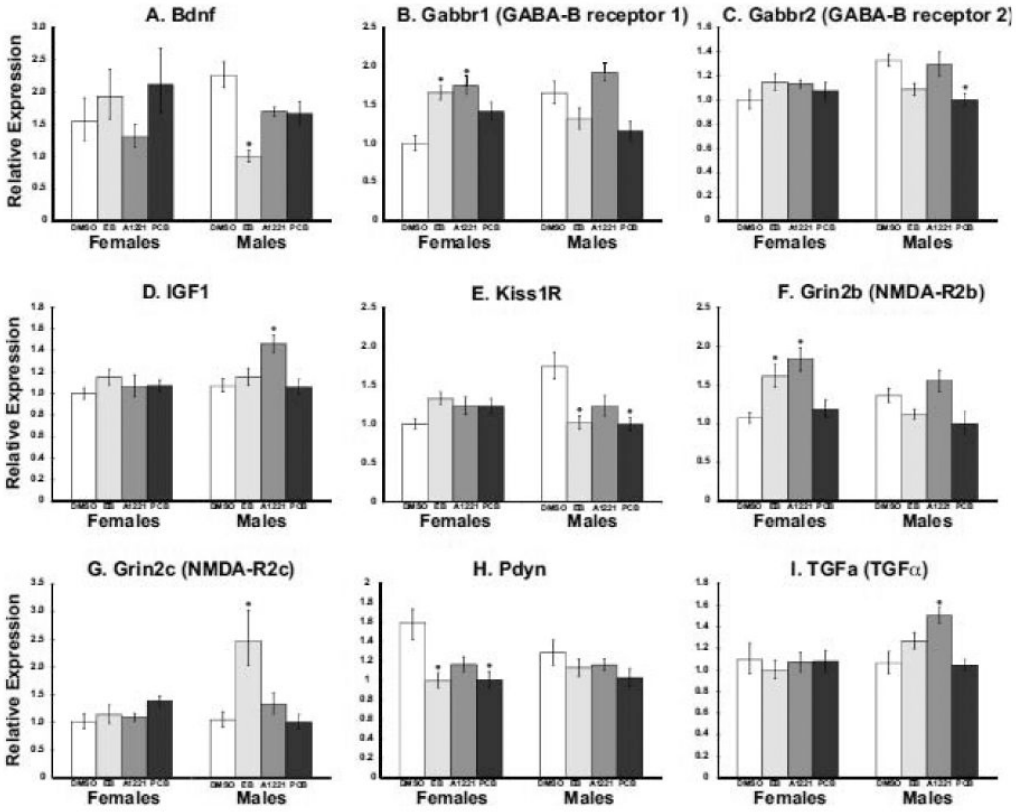

Figure 6.

Gene expression data are shown for the nine neuroendocrine genes that were significantly affected in the POA of neonatal P1 male or female rats. Data shown are mean ± S.E.M. Relative expression for each gene was determined using the comparative Ct method, with each sample normalized to GAPDH, and data calibrated to the average change in Ct for the treatment group with the lowest expression. N = 6 rats per group; *$p<0.001$ vs. DMSO control of the same sex. Gene abbreviations appear in Table 1.

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Table 1

Low-density PCR array gene expression results

		Males			Females		
Gene	**Name**	**EB**	**A1221**	**PCB**	**EB**	**A1221**	**PCB**
Ahr	Aryl hydrocarbon receptor	0.01 ↓	--	0.05 ↓	--	--	0.009 ↑
AR	Androgen receptor	--	--	--	--	--	--
Arnt	Aryl hydrocarbon nuclear translocator	--	--	0.06 ↓	0.004 ↑	0.022 ↑	0.026 ↑
Bdnf	Brain-derived neurotrophic factor	**<0.001** ↓	0.01 ↓	0.01 ↓	--	--	--
Cyp 17a1	Cytochrome P450, 17a1 (17-alpha hydroxylase)	--	--	--	--	--	--
Cyp 19a1	Cytochrome P450, 19a1 (aromatase)	--	--	--	0.037 ↑	--	0.026 ↑
Cyp 1b1	Cytochrome P450, 1b1	-- --	--	0.04 ↑	0.075 ↓	0.062 ↓	0.078 ↑
Esr1	Estrogen receptor alpha	--	--	--	0.067 ↑	--	0.079 ↑
Esr2	Estrogen receptor beta	--	--	--	--	--	--
Gab br1	GABA-B receptor 1	0.07 ↓	--	0.028 ↓	**<0.001** ↑	**<0.001** ↑	<0.004 ↑
Gab br2	GABA-B receptor 2	0.011 ↓	--	**<0.001** ↑	--	--	--
Gal	Galanin	--	--	--	--	--	--
Gnrh 1	Gonadotropin-releasing hormone 1	--	--	--	--	--	--
Gnrh r	Gonadotropin-releasing hormone receptor	0.037 ↓	--	--	--	--	--
Gper	G-protein coupled receptor 30	--	--	--	--	--	--
Gria 1	GluR1	--	--	--	--	--	--
Gria 2	GluR2	0.072 ↓	--	0.042 ↓	--	--	--
Gria 3	GluR3	--	--	--	--	--	--
Grik 2	Kainate 2 receptor	0.01 ↓	--	0.07 ↓	--	--	--
Hsd 17b1	Hydroxysteroid 17-beta dehydrogenase 1	--	--	--	--	--	--
Hsd 17b2	Hydroxysteroid 17-beta dehydrogenase 2	--	--	--	--	--	--
Hsd 17b3	Hydroxysteroid 17-beta dehydrogenase 3	--	--	--	--	--	--
Hsd 17b8	Hydroxysteroid 17-beta dehydrogenase 8	--	--	--	--	--	--
Igf1	Insulin-like growth factor 1	--	**<0.001** ↑	--	--	--	--
Igf1r	Insulin-like growth factor 1 receptor	0.03 ↓	--	0.04 ↓	--	--	--
Kiss 1	Kisspeptin	--	--	--	--	--	--

		Males			Females		
Gene	**Name**	**EB**	**A1221**	**PCB**	**EB**	**A1221**	**PCB**
Kiss 1r	Kisspeptin receptor (GPR54)	**<0.001** ↓	0.01 ↓	**<0.001** ↓	0.009 ↑	0.047 ↑	0.05 ↑
Pgr mc1	Membrane progesterone receptor	--	--	--	--	--	--
Nmd ar1	N-methyl-D-aspartate receptor (NMDAR) subunit 1 (NR1)	--	--	--	--	--	--
Grin 2a	NMDAR subunit 2a	0.066 ↓	--	0.033 ↓	--	--	--
Grin 2b	NMDAR subunit 2b	--	--	--	**< 0.001** ↑	**< 0.001** ↑	--
Grin 2c	NMDAR subunit 2c	**0.001** ↑	--	--	--	--	0.03 ↑
Grin 2d	NMDAR subunit 2d	--	--	--	--	--	--
Pdyn	Prodynorphin	--	--	--	0.008 ↓	--	0.01 ↓
Pgr	Progesterone receptor	--	--	--	--	--	--
Slc1 7a1	Vesicular glutamate transporter 1	--	--	0.006 ↓	--	--	--
Slc1 7a6	Vesicular glutamate transporter 2	--	--	--	--	--	--
Srd5 a1	Steroid 5-alpha reductase 1	--	--	--	--	--	--
Stat5 b	Signal transducer and activator of transcription 5B	--	--	--	--	--	--
Sts	Steroid sulfatase	--	--	--	--	--	--
Tac2	Neurokinin B	--	--	--	--	--	--
Tgfa	Transforming growth factor alpha	--	--	**< 0.001** ↑	--	--	--
Tgfb 1	Transforming growth factor beta-1	--	--	--	--	--	--
Ucp 2	Uncoupling protein 2	--	--	--	--	--	--
Vdr	Vitamin D receptor	--	--	--	--	0.005 ↑	0.016 ↑

Low-density PCR arrays were used to measure expression of 48 genes in whole POAs of P1 female and male rats treated prenatally with EDCs. The full list of detected neuroendocrine genes is shown. Statistical results are shown for significant effects and for trends in data. Because of the Bonferroni correction, the cut-off for a significant effect was set at $p < 0.001$. Not shown are results for internal controls 18S, glyceraldehyde-3-phosphate dehydrogenase (GAPDH). In addition, one gene was not detected in the assay: 3ß-hydroxysteroid dehydrogenase (HSD3b).

Table 2
Summary of treatment effects at P1 for each significant endpoint

Endpoint	Females	Males
Body weight	EB, A1221 > DMSO, PCB Mix	No effect
Anogenital distance	EB, A1221, PCB Mix < DMSO	EB, A1221(trend for PCB Mix) > DMSO
Serum Estradiol	EB > DMSO, A1221, PCB Mix	No effect
Serum Progesterone	A1221, PCB Mix < DMSO	No effect
Serum Testosterone	No effect	EB, PCB Mix < DMSO, A1221
Apoptotic cells (AVPV)	EB, A1221 > DMSO, PCB Mix	No effect
ERα cell number and density (MPN)	EB > DMSO	EB > DMSO
BDNF mRNA	No effect	EB < DMSO
$GABA_B$-R1 mRNA	EB, A1221 > DMSO	No effect
$GABA_B$-R2 mRNA	No effect	PCB Mix < DMSO
IGF-1 mRNA	No effect	A1221 > DMSO
Kiss1R mRNA	No effect	EB, PCB Mix < DMSO
NMDA-R2b mRNA	EB, A1221 > DMSO	No effect
NMDA-R2c mRNA	No effect	EB > DMSO
Prodynorphin mRNA	EB, PCB < DMSO	No effect
TGFα mRNA	No effect	A1221 > DMSO