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Two-hit exposure to polychlorinated biphenyls at gestational and juvenile life stages: 2. Sex-specific neuromolecular effects in the brain

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Abstract

Exposures to polychlorinated biphenyls (PCBs) during early development have long-lasting, sexually dimorphic consequences on adult brain and behavior. However, few studies have investigated their effects during juvenile development, a time when increases in pubertal hormones influence brain maturation. Here, male and female Sprague Dawley rats were exposed to PCBs (Aroclor 1221, 1 mg/kg/day) or vehicle prenatally, during juvenile development, or both, and their effects on serum hormone concentrations, gene expression, and DNA methylation were assessed in adulthood. Gene expression in male but not female brains was affected by 2-hits of PCBs, a result that paralleled behavioral effects of PCBs. Furthermore, the second hit often changed the effects of a first hit in complex ways. Thus, PCB exposures during critical fetal and juvenile developmental periods result in unique neuromolecular phenotypes, with males most vulnerable to the treatments.

Keywords

endocrine-disrupting chemical; preoptic area; dopamine receptor; mu opioid receptor; androgen receptor; estrogen receptor

1. Introduction

Polychlorinated biphenyls (PCBs) are some of the most widespread environmental endocrine-disrupting chemicals (EDCs), as they persist in the food chain and are detectable in tissues of virtually all humans (Agency for Toxic Substances and Disease Registry, 2000).

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While banned in the 1970s, recent epidemiological data show that PCB body burdens continue to be associated with impaired reproductive and neurobiological health in humans (Boucher et al., 2009; Buck Louis et al., 2013; Engel and Wolff, 2013). In addition, rodent studies demonstrate that PCBs exert subtle but chronic effects on a range of social and anxiety related behaviors (Elnar et al., 2012; Jolous-Jamshidi et al., 2010; Reilly et al., 2015; Tian et al., 2011). Many of these behaviors are sexually dimorphic and organized by neonatal exposure to steroid hormones (Adler et al., 1999; Auger and Olesen, 2009; Bitran, 1993; Henley et al., 2011; Mora et al., 1996). As some PCB congeners, including those in the current study (Aroclor 1221, A1221) are weakly estrogenic (Jansen et al., 1993), the majority of behavioral studies focus on gestational or neonatal exposure, a life stage when brain sexual differentiation occurs, and when hormonal perturbations were predicted to have the most profound effects (McCarthy et al., 2009).

Juvenile development is also a time of continued sensitivity to organizational effects of gonadal hormones, as well as activation of neural pathways that were organized earlier in life (Schulz et al., 2009; Sisk and Foster, 2004). We recently demonstrated that exposing rats to PCBs during juvenile development, with or without prior prenatal exposure, affected several types of behavior in a sex- and age-specific manner (Bell et al., 2015). Two hits of PCBs, the first in late gestation and the second in juvenile development, resulted in abnormal levels of play and anxiety-like behavior in adolescent females, and caused disruptions of opposite-sex partner preference in adult males. In some cases, juvenile exposure modified or unmasked the effects of a previous prenatal exposure, especially in the male rats.

The goals of this study were to determine how two hits of PCB exposure, given during prenatal or juvenile development, or both, interact to change expression of genes in the adult brain as potential molecular substrates related to the observed behavioral changes. Neural regions were selected based on their roles in sexually dimorphic sociosexual and anxiety-like behaviors and in mediating the rewarding qualities of these social interactions (Burgdorf et al., 2007; Davis et al., 2010; Gordon et al., 2002; Harding and McGinnis, 2005; Newman et al., 1997; Pfaff and Sakuma, 1979). Genes that were studied within these regions included those involved in dopaminergic and endogenous opioid signaling, the vasopressin and oxytocin systems, and steroid hormone receptors that regulate social and anxiety-like behaviors (Bale et al., 2001; Bielsky et al., 2004; Buck et al., 2014; Bychowski et al., 2013; Egashira et al., 2007; Ferguson et al., 2000; Harding and McGinnis, 2004; Lim and Young, 2006; Matochik et al., 1992; Trezza et al., 2010; Veenema et al., 2013). We hypothesized that changes in gene expression would be correlated with changes in behavior, that a second PCB hit would change the developmental trajectory of gene expression in the brain in a manner not predicted by either hit alone, and that the sexes would differ in their sensitivity to PCB effects.

2. Methods

2.1 Animals and Husbandry

All animal protocols were conducted in accordance with the Guide for the Care and Use of Laboratory Animals and approved by The University of Texas at Austin's Institutional

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Animal Care and Use Committee. Sprague-Dawley rats were purchased from Harlan Laboratories (Houston, Texas) and were housed in a humidity- and temperature-controlled room with a 12:12 reversed light cycle (lights out at noon) at 21–23°C. 2–3 animals were group-housed in polycarbonate cages (43 × 21 × 25 cm) with aspen bedding (PJ Murphy Forest Products, Sani-Chip), a PVC tube for enrichment, and weekly cage changes. Rats were fed low phytoestrogen Harlan-Teklad 2019 Global Diet (Harlan-Teklad, Indianapolis, Indiana) *ad libitum* for the duration of the experiment. Upon arrival, rats were handled daily to acclimate them to their new housing conditions, and mating began at least two weeks later.

Females (3–4 months old, virgin) were mated with sexually experienced untreated male rats (~6 month old); for balance, each stud male sired two litters, one that was subsequently treated with the vehicle and the other with PCBs. The morning after successful mating (sperm-positive vaginal smear), termed embryonic day (E) 1, dams were singly housed. Dams were provided with nesting materials several days prior to expected parturition on E23. On the day after birth [postnatal day (P) 1], litters were culled to equal sex ratios, with final litter size ranging from 6 to 8 pups. Weaned pups were housed with same sex littermates (2–3 per cage), and were weighed and handled for at least 5 minutes weekly. Animals were tested for social and anxiety behaviors in adolescence and adulthood, with results published in a sister paper (Bell et al., 2015). Because of the large number of animals necessary for both studies, the animals were raised in 3 cohorts over 1.5 years, with treatments equally distributed across each cohort.

2.2 Treatments

Aroclor 1221 (A1221, AccuStandard, New Haven, CT, Cat No: C-221N-50MG, Lot: 23683) is a mix of ~45 lightly chlorinated PCB congeners with known estrogenic (Layton et al., 2002; Shekhar et al., 1997), anti-aromatase (Woodhouse and Cooke, 2004), and anti-androgen (Schrader and Cooke, 2003) actions, but without effect on aryl dhydrocarbon receptor (Poland and Glover, 1977). It was dissolved in a 4% dimethylsulfoxide vehicle (Veh, Cat No D4540; Sigma, St Louis, Missouri in sesame oil) for intraperitoneal injection at 1 mg/kg dam body weight. Dams were randomly assigned to either Veh (n = 6) or A1221 (n = 6), and each litter contributed no more than two animals per group. On E16, E18, and E20, during the period of sexual differentiation of the rat brain (Breedlove, 1992; Ramaley, 1979; Rhees et al., 1990; Tobet and Fox, 1989; Wagner et al., 1998), dams were weighed and injected with 0.1 ml of Veh or A1221 solution using a 1 ml syringe with a 25 gauge needle, 3 hours prior to lights out. This mixture and dosage is not toxic to dams, does not cause fetal loss, and was selected so that outcomes of the current study could be compared with findings from several other previous studies using a very similar exposure regime (Dickerson et al., 2011a; Reilly et al., 2015; Steinberg et al., 2008; Walker et al., 2014). Although we did not measure body burden, we estimated that each pup is exposed to approximately 2 μg/kg A1221 based on (Takagi et al., 1976). This is within the range of human exposure according to levels found in maternal serum, cord blood and milk fat (Agency for Toxic Substances and Disease Registry, 2000; Karmaus et al., 2002; Lackmann, 2002; Law et al., 2005; Longnecker et al., 2005; Matthews and Anderson, 1975; Patterson et al., 2009; Schantz, 1996).

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Rats were given an additional set of juvenile injections, either Veh or A1221 (1 mg/kg), again at 0.1 mL volume, ip, on P24, 26, and 28, when puberty is beginning, estrogen-positive feedback is being established (Andrews et al., 1981) and the brain is highly sensitive to organizational and activational effects of gonadal steroids (Döhler and Wuttke, 1975; Saksena and Lau, 1979; Schulz et al., 2009; Smyth and Wilkinson, 1994; Vetter-O'Hagen and Spear, 2011). Littermates within a cage were given the same treatment to prevent cross-contamination. With both gestational and juvenile exposures, there were four experimental groups in a 2×2 balanced design (first hit prenatal, second hit juvenile): Veh-Veh, A1221-Veh, Veh-A1221, and A1221-A1221. Final Ns per group were between 9 and 12 for all measures, from 6 litters per treatment. The experimenters were blind to treatment throughout the duration of the experiment.

2.3 Tissue collection

Rats were euthanized in adulthood (between P93-P108) by rapid decapitation 1–3 hours before lights out, on proestrus in females and 3–9 days after the last behavioral test (reported in the companion study; Bell et al., 2015) in both sexes. Brains were immediately removed and placed in ice for 5 minutes prior to placing in an ice-cold stainless steel brain matrix. After cutting the optic chiasm, a razor blade was inserted through the center of this landmark, and three 2-mm (rostral) and two 1-mm (caudal) coronal sections were taken. Sections were placed on an ice-cold microscope slide, and snap frozen on dry ice. One to 11 months later, frozen sections were placed on a freezing stage, allowed to equilibrate to −18°C, and micropunches (0.98 mm diameter) were taken from each region of interest according to Paxinos and Watson, 2009 (Paxinos and Watson, 2009). Photographs were captured of sections before and after punching to ensure consistency across the cohorts (Figure 1). Samples were placed in a cold Eppendorf tube and stored at −80°C for 2–9 months until nucleic acid isolations. Trunk blood samples were collected and allowed to clot for 30 minutes before centrifugation (1500 × g for 5 minutes). Sera were collected and stored at −80°C until use, 1–2 years later.

2.4 Serum hormone quantification

Total serum testosterone (T) was determined in male animals via radioimmunoassay (ImmuChem Double Antibody ^{125}I RIA kit, Cat No 07-189105, Lot# RTK1420, MP Biomedicals, Costa Mesa, CA), according to manufacturer directions. All samples were run in a single assay, and duplicate volumes of 50 μl serum were used. The assay limit of detection was 0.03 ng/mL, and the intraassay C.V. was 1.41%. This assay is not sensitive enough to run T in females. Total serum estradiol (E_2) was determined in male and female rats via radioimmunoassay (UltraSensitive Estradiol RIA, Cat No DSL4800, Lot# 150622 C, Beckman Coulter, Pasadena, CA), according to manufacturer directions. Samples were run in a single assay and duplicate volumes of 200 μl of serum were used. Assay sensitivity was 2.2 pg/ml and intraassay C.V. was 1.30%. Progesterone (P_4), triiodothyronine (T3), and thyroxine (T4) were also determined from a separate serum aliquot from the same male and female rats via a magnetic bead panel (Milliplex Steroid/Thyroid Hormone Magnetic Bead Panel, Cat No STTHMAG-21K, Lot# 2484258, EMD Millipore Corp, Billerica, MA) according to manufacturer directions. Samples were precipitated with acetonitrile before reconstitution with assay buffer, and were run in duplicate volumes of 25 μl. The limits of

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detection were 0.09, 0.04, and 0.28 ng/ml and the intra-assay CVs were 2.53%, 4.99%, and 4.16% for P_4, T3, and T4, respectively.

2.5 Nucleic acid extraction

Frozen tissue punches were lysed and homogenized using 22 gauge needles and syringes. DNA and RNA were extracted using an Allprep DNA/RNA mini kit (Qiagen Cat No 80204) according to manufacturer instructions and the RNA column was treated with DNase (Qiagen Cat No 79254). RNA was eluted with 100 μl of nuclease free water (Applied Biosystems Cat No AM9937) and DNA was eluted with 200 μl of buffer included in kit. Samples were stored at −20°C in 66% ethanol and 0.5M NaCl for 1–4 weeks before being concentrated as follows. Samples were placed at −80°C for 10 minutes before they were centrifuged at 14000 × g for 20 minutes at 4°C to pellet the nucleic acid. The pellet was washed with 70% ethanol and centrifuged again for 10 minutes before supernatant was discarded and samples were dried via inversion at room temperature for 10 minutes and then in a speedvac at 43°C for 5 minutes. Pellets were resuspended in 12 μl water and quantity was determined via Promega QuantiFluor Systems on the Glomax Multi + Detection System (RNA: Cat No E3310, dsDNA: Cat No E2670), according to manufacturer instructions. 150–1500 ng of RNA, and 15–75 ng of DNA, were isolated, depending on the region. RNA quality was assessed by randomly selecting ~10% of the samples to run on a Bioanalyzer 2100 (Agilent RNA 6000 Pico Kit, Cat No 5067-1513, Agilent Technologies, Santa Clara, California); all tested samples had RNA integrity numbers of 9 and above.

2.6 Gene expression quantification

RNA samples (200 ng) were converted to cDNA in 20 μl reactions using a high-capacity cDNA reverse transcription kit with RNase inhibitor (Life Technologies Cat No 4374966) according to the manufacturer directions. Samples were held at 25°C for 10 minutes, 37°C for 120 minutes, and 85°C for 5 minutes on Applied Biosystems 2720 Thermocycler. Product was stored at −20°C for up to 10 months until use. Predesigned gene expression primer and probe assays were purchased from LifeTech to identify genes of interest (FAM/ MGB-NFQ, Cat No 4351370) and reference genes (VIC/MGB-NFQ, Cat No 4448490), described in Table 1. Assays were prevalidated for duplexing to run a target and calibrating gene together, and reaction efficiency was confirmed in the lab to be within an acceptable range (90–110%). Taqman Gene expression master mix (Cat No 4370074) was used in a 20 μl reaction with 10 ng of cDNA, and each sample was run in triplicate. qPCR was carried out on an Applied Biosystems ViiA 7 (Software version 1.2.4) in the Gore Lab and with the following run parameters: 50°C for 2 minutes, 95°C for 10 minutes, 45 cycles of 95°C for 15 seconds, and 60°C for 1 minute. Quantification cycle (Cq) was determined automatically by the software. *Rpl13a* and *Gapdh* were chosen as reference genes because of previous experience in our lab demonstrating that they are not significantly affected by similar PCB treatments. In the current experiments, reference Cqs differed less than 2% between groups. Relative expression was determined for each sample using the comparative Cq method: reference Cqs were subtracted from target Cqs to determine delta Cq within each sample well. Triplicate delta Cqs were averaged together, removing any technical outliers, and the median Delta Cq of the same sex Veh-Veh group was subtracted to determine fold change in expression for each individual.

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2.7 DNA methylation quantification

Because expression of the mu opioid receptor (*Oprm1)* was affected by PCBs in the prefrontal cortex (PFC) and preoptic area (POA), and expression of androgen receptor (*Ar*) was affected in the POA, these targets were selected for follow-up of DNA methylation of cytosine-guanine dinucleotides (CpG) sites within 250 bases of the transcription start sites (TSS) of each gene. 200 ng of DNA in 45 μl water from the PFC and POA of each rat was shipped to EpigenDx (Worcester, MA) for bisulfite conversion and pyrosequencing of *Oprm1* (3 CpG sites, −241 to −209 bp from the TSS), and *Ar* (8 CpG sites, −70 to +39 bp from the TSS) regulatory regions. CpG sites were analyzed by pyrosequencing for percentage of methylation.

2.8 Analysis and statistics

When tested as a covariate, no effects of litter were detected for any of the significant effects, so individual rats were used as the unit of analysis for statistical purposes. Any outliers were identified via Grubbs test and were removed (maximum of one per group unless notes taken while performing the experiment indicated a technical reason for exclusion, e.g., poor dissection or errors in isolation). Individual hormonal, gene expression, and methylation measures were analyzed using a 2 × 2 analysis of variance (ANOVA) within each sex to determine any main effects of prenatal or juvenile treatment and interactions, with appropriate follow-up t-tests to identify the source of detected interactions. If measures failed to meet normality or homogeneity assumptions (as indicated by Shapiro Wilks and Levene's tests), a non-parametric Kruskal-Wallis test was used, indicated by KW. In this case, an interaction was identified by testing for effects of one treatment while holding the other constant and vice versa. Prior to tissue collection, the sociosexual behavior of these same animals was assessed (Bell et al 2015). PCB exposure affected the production of ultrasonic vocalizations after being placed in a novel testing apparatus prior to the introduction of a stimulus animal and the time spent with a hormone- or a no-hormone-treated opposite sex stimulus animal in a three chamber test of sociosexual partner preference in adult male animals. Pearson correlations were used to determine relationships between hormone and gene expression outcomes and 1) significant behavioral changes and/or 2) related methylation measures. All endpoints were selected for analysis according to a priori hypotheses and, accordingly, significance levels were not adjusted. Analysis was completed using SPSS (Version 18, IBM), with significance defined as $*p < 0.05$ and $**p < 0.01$.

3. Results

3.1 Hormones

Serum hormone concentrations are shown in Figure 2. P_4 and E_2 concentrations were much higher in females than males, as expected (Fig 2). The only main effect of prenatal PCB exposure was detected for circulating P_4 concentrations, and was limited to males (Fig 2A, KW, $p = 0.007$). Specifically, males exposed to PCBs during prenatal development had lower serum P_4 concentrations than males exposed to vehicle at that time.

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3.2 Gene Expression

The greatest number of PCB effects on gene expression was found in the preoptic area (POA), and exclusively in males (Figure 3). An interaction between prenatal and juvenile exposure was found in male rats for expression of androgen receptor (*Ar*, Fig 3A, $F_{(1,35)} = 9.48$, $p = 0.004$), estrogen receptor alpha (*Esr1,* Fig 3B, KW, $p < 0.001$), mu opioid receptor (*Oprm1*, Fig 3C, $F_{(1,33)} = 9.26$, $p = 0.005$), and oxytocin receptor (*Oxtr,* Fig 3D, $F_{(1,34)} = 4.61$, $p = 0.039$). The interactions can be explained by multiple comparisons where the effect of exposure at one age depended on the exposure status at the other age. Prenatal exposure decreased expression of *Ar* ($F_{(1,19)} = 7.35$, $p = 0.014$), *Esr1* ($F_{(1,18)} = 6.19$, $p = 0.023$), *Oprm1* ($F_{(1,18)} = 5.84$, $p = 0.026$), and *Oxtr* ($F_{(1,19)} = 6.00$, $p = 0.024$) only in animals that received the vehicle as juveniles. Juvenile exposure to PCBs also reduced expression of *Ar* ($F_{(1,20)} = 4.54$, $p = 0.046$) and *Oprm1* ($F_{(1,19)} = 4.98$, $p = 0.038$) only in animals unexposed prenatally. In contrast to effects found in animals exposed at only one developmental period, prenatal exposure increased expression of *Esr1* ($F_{(1,15)} = 8.74$, $p = 0.010$) in juvenile-exposed animals, and juvenile exposure increased expression of *Ar* ($F_{(1,15)} = 5.45$, $p = 0.034$), *Esr1* ($F_{(1,14)} = 20.01$, $p = 0.001$), and *Oprm1* ($F_{(1,14)} = 4.86$, $p = 0.045$) in prenatally exposed animals. Thus, two developmental hits affected gene expression in the opposite direction from one developmental hit.

Significant effects of PCBs on gene expression were also found in the prefrontal cortex (PFC, *Oprm1*), nucleus accumbens (NA, *Drd2*), and lateral septum (LS, *Ar* and *Avpr1a*), again only in male rats (Figure 4). In the PFC (Fig 4A), a main effect of juvenile exposure was found to increase expression of *Oprm1* in the PCB-exposed rats ($F_{(1,34)} = 4.44$, $p = 0.042$). In the NA (Fig 4B), a main effect of prenatal exposure was found to increase expression of dopamine receptor D2 (*Drd2*) in the PCB-exposed males ($F_{(1,37)} = 6.35$, $p = 0.016$). In the LS (Fig 4C and 4D), a Pre x Juv interaction was found for *Ar* ($F_{(1,37)} = 6.829$, $p = 0.013$) and vasopressin receptor 1a (*Avpr1a,* $F_{(1,37)} = 8.40$, $p = 0.006$). Follow-up tests revealed that the effect of prenatal exposure was present only if animals were unexposed in juvenile development (*Ar*: $F_{(1,20)} = 7.48$, $p = 0.013$; *Avpr1a*: $F_{(1,20)} = 5.47$, $p = 0.030$). An effect of juvenile exposure on *Avpr1a* expression in the LS was also found, but only if animals were unexposed in prenatal development ($F_{(1,20)} = 7.44$, $p = 0.013$). No effects of treatment were found for any genes measured in the bed nucleus of the stria terminalis (BNST), medial amygdala (MeA), paraventricular nucleus (PVN), or ventromedial hypothalamus (VMH) as summarized in Table 2 and presented in Supplemental Table 1.

3.4 Gene-Behavior Correlations

In a companion study, we reported the behavioral phenotype of these same rats, and showed that two types of behaviors were significantly affected in the adult male, but not female, rats (Bell et al., 2015). These behaviors included 1) the production of USVs when placed into a novel testing apparatus prior to an interaction with a stimulus animal and 2) the preference for spending time near a hormone- or no-hormone-treated opposite sex stimulus animals. To relate whether the molecular changes described above could be responsible for PCB-induced changes in adult male behavior in the same animals, we conducted Pearson's correlations between expression of genes and hormone concentrations with significantly affected behaviors in males (Table 2). Results showed that the number of flat USVs was positively

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correlated with *Drd2* expression in the NA (r = 0.338, p = 0.031), both of which were increased by prenatal PCB exposure. Numbers of pre-stimulus USVs were negatively correlated with *Avp* expression in the BNST (r = –0.425, p = 0.006), and *Oprm1* in the NA (r = –0.350, p = 0.027), but these were not affected by PCBs. Time spent near the no-hormone stimulus animal (no-hormone stimulus time) in the sociosexual preference test was positively correlated with *Oprm1* in the PFC (r = 0.546, p < 0.001), both of which were increased by juvenile PCB exposure. No-hormone stimulus time was also positively correlated with serum P concentrations (r = 0.345, p = 0.027), both of which were decreased by prenatal PCB exposure, independent of juvenile effects. No-hormone stimulus time was also positively correlated with MeA *Esr1* (r = 0.354, p = 0.025) and *Oprm1* (r = 0.336, p = 0.048), but these genes were not affected by PCBs. The significant increase in time spent with no-hormone stimulus animal, paired with a non-significant increase in time with the hormone stimulus animal, resulted in an overall increase in time spent with both stimulus animals in animals exposed to PCBs as juveniles. As with no-hormone stimulus time, this measure was positively correlated with *Oprm1* expression in the PFC (r = 0.346, p = 0.033), which was also increased by juvenile PCB exposure. The behavior was also positively correlated with MeA *Ar* (r = 0.511, p = 0.001) and MeA *Esr1* (r = 0.427, p = 0.006), but these gene targets were not affected by PCBs. Finally, the preference for the hormone stimulus animal over either stimulus animal was negatively correlated with *Oprm1* in the PFC (r = –0.341, p = 0.036).

3.5 DNA Methylation

Because PCBs affected expression of *Ar* and *Oprm1* in the POA and *Oprm1* in the PFC of male rats, and the expression of these genes are modulated by DNA methylation (Vucetic et al 2011, Nielsen et al 2009, Hao et al 2011, Keil et al 2014), epigenetic analyses were performed on these targets. We decided to conduct work in both sexes as the *Ar* is on the X-chromosome and should have much higher methylation in females than males (Walker et al., 2014). As expected, % methylation of *Ar* in the POA was much higher in females than males (Figure 5). Effects of PCBs were found in female (Figure 5B) but not male (Figure 5A) animals. At two of seven sites (Figure 5B, D, E), a main effect of juvenile exposure was found: –70 ($F_{(1,31)} = 8.13$, p = 0.008) and –57 ($F_{(1,33)} = 5.88$, p = 0.021). However, this main effect was qualified, as it appears to be driven by low levels of methylation in the A1221-Veh group only. This general pattern was found across several sites, and was detected as a significant Pre x Juv interaction in the average percent methylation combined for the seven sites (Figure 5B, C; $F_{(1,32)} = 5.08$, p = 0.031). Follow-up tests revealed that juvenile exposure increased methylation levels, but only in prenatally exposed animals, $F_{(1,16)} = 5.78$, p = 0.029. Methylation at –51 was positively, albeit weakly, correlated with expression of *Ar* mRNA in female POAs (r = 0.352, p = 0.033), but not males. No significant effects of PCBs were found on *Oprm1* CpG sites –241, –217, and –209 in the POA or PFC in either sex (Figure 5F and G), and methylation patterns at these sites were not correlated with gene expression.

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4. Discussion

The results of the current study provide novel information about how two periods of PCB exposure interact to affect the neuromolecular phenotype. As with the performance of social and anxiety-like behaviors in a companion study, the effects of prenatal and juvenile PCB exposure on gene expression in the brain were relatively small and sex-dependent. In adulthood, males but not females showed effects of PCB exposure on brain and behavior. As hypothesized, the majority of effects were due to an interaction between the two hits: juvenile exposure masked or revealed effects of prenatal exposure depending upon the endpoint measured. In some cases, similar effects of prenatal and juvenile exposures were observed, suggesting that the juvenile period represents an extension of the developmental processes initiated during prenatal period. Finally, the POA is a key part of the neural network involved in sexual behaviors in male rats (Hull and Dominguez, 2007; Sakuma, 2008) and was particularly responsive to PCBs. These findings are consistent with previous effects of PCBs, known hormone sensitivity, and the fact that these same P90–100 males had sociosexual behavioral changes in adulthood (Gore et al., 2011; Gorski et al., 1980; Bell et al., 2015). The lack of effects of PCBs on POA gene expression (here) or sexual behaviors (Bell et al., 2015) in adult females is consistent with the postulated lesser role of the POA in feminine sexual behavior in rats (Sakuma, 2008; Veening et al., 2014).

4.1 Progesterone, but not other hormones, was affected by A1221

Of the 5 serum hormones measured, only P_4 showed an effect of treatment: concentrations of P_4 were decreased in males exposed to prenatal A1221 irrespective of the second hit. To our knowledge, this is the first such observation in male animals, as a previous study demonstrated prenatal A1221-induced reductions in P_4 at P1 and P60 in female but not male rats (Dickerson et al., 2011a; 2011b). However, a different PCB mix (1mg/kg of PCB #138, 153, and 180, 1:1:1) increased serum P_4 in P1 males (Dickerson et al., 2011a). Progesterone treatment for three days prior to testing impaired social recognition in adult male rats, at least in part by reducing vasopressin expression in the BNST, MeA and LS (Bychowski et al., 2013; Bychowski and Auger, 2012). However, the decrease in P_4 in the current study was not accompanied by a change in *Avp* expression, nor was there evidence of better social recognition memory in male animals when tested as juveniles (Bell et al., 2015). Progesterone is also involved in male sociosexual behavior, as PR knockout mice exhibited reduced mount latency (Schneider et al., 2005). Therefore it is possible that reduced circulating P_4 levels are related to the moderate and partial increases in sociosexual preferences observed in adult animals in the sister study (Bell et al., 2015). Indeed, P_4 and no-hormone stimulus time were positively correlated within animals. The lack of change in serum concentrations of E_2 or T is in agreement with some studies (Dickerson et al., 2011b; Steinberg et al., 2008; Walker et al., 2014). Other studies have shown that more highly chlorinated PCB congeners tended to reduce E_2 and T concentrations (Hany et al., 1999; Kaya et al., 2002; Muto et al., 2003; Yamamoto et al., 2005). Similarly, A1221 had no effect on T3 or T4 in the current study, but more highly chlorinated congeners are well known to decrease T3 and T4 (Giera et al., 2011; Khan et al., 2002; Sauer et al., 1994; Ulbrich and Stahlmann, 2004).

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4.2 Reward-related genes were sensitive to A1221 in male rats

While several genes involved in the regulation of sexually dimorphic social behaviors were assayed, the opioid and dopaminergic genes in reward-related neural circuits were among the most affected in the current study. We demonstrated that *Oprm1* expression in the POA was reduced by either prenatal or juvenile PCB exposure. Mu opioid receptor agonists delivered centrally and directly to the POA inhibited sexual behavior and blocked the normal preference to interact with a receptive over a non-receptive female in male rats (Hughes et al., 1990; Parra-Gámez et al., 2009). Therefore, a reduction in *Oprm1* expression in the POA could promote sociosexual interests and be a mechanism for the increased preference for hormone-treated stimulus animals observed in adult male rats treated with PCBs prenatally in our companion paper (Bell et al., 2015). However, this possibility is not supported by correlation analysis showing that the two measures were not related within animals. An increase in time spent performing general social investigation in males treated with PCBs during juvenile development was also observed in that study (Bell et al., 2015). However, this cannot be explained by a parallel reduction in POA *Oprm1* in juvenile-treated males, as central mu opioid receptor agonists have been shown to increase non-sexual affiliative behavior in male rats (Meyerson, 1981).

In addition to effects in the POA, *Oprm1* expression in the PFC was increased by juvenile exposure to PCBs. This effect was not observed in juvenile mice exposed to a mix of six moderately chlorinated and non-dioxin like PCBs during juvenile development (P0-P21) (Elnar et al., 2012), which could be the result of the different congeners used, species tested, or age at analysis. The different directionality of effects between PFC and POA, and the lack of effect on *Oprm1* expression in the NA, indicates site-specific effects of PCBs that may depend on specific cell types or coexpression of other PCB-responsive receptors or transcription factors. While no studies, to our knowledge, have specifically demonstrated the role of mu opioid receptor in the PFC in the regulation of social behavior, there is partial evidence suggesting involvement. Opioid receptor stimulation in the PFC is associated with other reward seeking behaviors such as drug and food consumption and craving (Colasanti et al., 2012; Gorelick et al., 2005; Mena et al., 2011; Mitchell et al., 2012) and, in the NA, mu opioid receptors mediated social reward (Trezza et al., 2011). The mPFC is also important in social play (Bell et al 2009), and social isolation affected expression of opioid receptors in the PFC (Vanderschuren et al 1995). Time spent near a no-hormone stimulus animal, total social time (Bell et al 2015), and PFC Oprm1 expression were all increased by juvenile PCB exposure and positively correlated. Therefore, PFC Oprm1 changes could be a mechanism behind the pro-social behavioral effects.

Of the two dopamine receptors assayed, *Drd2* but not *Drd1* was affected by PCBs. More specifically *Drd2* expression was upregulated in the NA by prenatal A1221 in male animals. Noncoplanar PCBs, including PCB4, a component of A1221, cause long-term decreases in dopamine levels in the striatum (Choksi et al., 1997; Seegal et al., 1997; 1994; 1990; Shain et al., 1991). While not measured in the current study, it is possible that DA levels were reduced and resulted in a compensatory upregulation of *Drd2* expression. Indeed, 3 mg/kg Fox River PCB mix throughout gestation and weaning increased D2 autoreceptor sensitivity (Fielding et al., 2013). These effects may be specific to the PCB mixture or to the brain

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region, as A1254 during juvenile development did not affect D1 or D2 receptor binding in the dorsal striatum of female mice (Tian et al., 2011). Dopamine action in the NA is consistently linked to play and USV production in juvenile animals (Vanderschuren et al., 1997); however, no effects of PCBs were observed in these behaviors in male animals in our companion paper (Bell et al., 2015). In addition, D2 receptor agonist action in the NA caused an increase in the production of USVs in adult male rats alone in a novel recording chamber (Brudzynski et al., 2012). Therefore, the increased *Drd2* expression in response to prenatal PCBs in the current study could be a mechanism behind the parallel increase in flat USV production in prenatal PCB-exposed adult males in our other study (Bell et al., 2015). Indeed, these measures were positively correlated within animals. Given the importance of corticolimbic opioid and dopaminergic action in substance abuse, these findings may also be a mechanism by which PCBs increased sensitivity to cocaine and amphetamine (Fielding et al., 2013; Poon et al., 2013).

4.3 Steroid hormone receptor expression was affected by A1221 in male but not female rats

In the current study, *Ar* gene expression was decreased in the POA and LS by prenatal exposure, and in the POA by juvenile exposure. In the MeA, *Ar* expression was unaffected. The sensitivity of *Ar* expression to A1221 agrees with previous findings using a similar exposure regime; however there are some age, sex, and region-specific effects. While A1221 increased AR expression in the anteroventral periventricular (AVPV) region of the hypothalamus in P90 female rats (Walker et al., 2014), it reduced *Ar* mRNA in the POA of P60 females (Dickerson et al., 2011b) and the arcuate nucleus (ARC) of aged female (Walker et al., 2013) and P90 male rats (Walker et al., 2014). Protein levels of AR were also reduced in embryonic female rat hypothalamus in response to gestational treatment with another PCB mixture, A1254 (Colciago et al., 2006). It is not clear why effects in females were not detected in the current study.

Esr1 was affected in the POA but not in the other six brain regions in which it was assayed. In the POA, either prenatal or juvenile A1221 exposure reduced expression in males, but not females. The literature shows mixed effects of PCBs on *Esr1* expression: prenatal A1221 reduced ERα cell number in the adult female AVPV while having no effect on *Esr1* expression in the adult female POA (Dickerson et al., 2011b), or adult male or female AVPV or ARC (Walker et al., 2014). Mixed effects were also found in aged female animals, with A1221 increasing expression in the ARC and decreasing expression in the median eminence (Walker et al., 2013). While, human studies show both estrogenic and anti-estrogenic effects of PCBs (Bonefeld-Jørgensen et al., 2001; Tavolari et al., 2006) no relationship was detected between several classifications of PCBs and *Esr1* expression itself (Warner et al., 2012). Therefore, the current finding is the first evidence for effects of A1221 on *Esr1* expression in male animals, and requires replication.

4.4 The effects of A1221 on social nonapeptides, vasopressin and oxytocin, were limited

In the current study, *Avp* mRNA was not affected in the BNST, PVN or MeA. This is the first study to assess PCB effects on gene expression of vasopressin; only one other study tested for effects of PCBs on vasopressin action, where dehydration-induced vasopressin

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release from the supraoptic nucleus is blunted in rats treated with high doses of A1254 for 15 days in adulthood (Coburn et al., 2007). In contrast to *Avp*, expression of *Avpr1a* was reduced by either prenatal or juvenile exposure to A1221 in the LS. In the LS, the vasopressin receptor 1a is important for social play in juvenile rats (Veenema et al., 2013) and social recognition memory (Ferguson et al., 2002); however, female, but not male, juvenile animals showed changes in play behavior and no changes in social memory in our sister paper, and therefore the functional implications of these *Avpr1a* effects in males is unknown.

Expression of the oxytocin receptor, *Oxtr*, was reduced by prenatal exposure to A1221 in the male POA. This is in agreement with findings from a similar exposure paradigm in adult male ARC (Walker et al., 2014), and was region-specific; no changes in *Oxtr* expression were seen in the PFC or MeA. *Oxt* was also not affected by A1221 in the PVN. In a similar studies, prenatal A1221 increased *Oxt* expression in P90 male and female AVPV (Walker et al., 2014) and aged female ARC (Walker et al., 2013), suggesting regional specificity of A1221 effects within the brain. Oxytocin facilitates sexual behavior, with actions in the PVN, VMH and POA (Argiolas and Melis, 2013). However, PCBs tended to increase sociosexual preferences and general social interest in adult male animals, so, like the *Avpr1a* effects, the behavioral relevance of the *Oxtr* change is undetermined.

4.5 DNA methylation was not a major mechanism of PCB-induced change in gene expression

Hormones and early life influences affect long-term brain development by modifying DNA methylation (Champagne et al., 2006; Hao et al., 2011; Vucetic et al., 2010). Early life PCB exposure is also known to reduce global DNA methylation and expression of a DNA methyltransferase (Desaulniers et al., 2009) and histone demethylase (Casati et al., 2012) in liver. Therefore, in order to determine whether the mRNA changes in *Ar* and *Oprm1* were due to epigenetic programming caused by the A1221 exposure, we measured DNA methylation in regulatory regions of these genes.

Our prediction was not substantiated, as juvenile PCB exposure significantly increased methylation in only two of seven CpG sites on the *Ar* in female POA and there were no effects in males. When all sites were averaged together, an interaction was detected, such that juvenile PCB exposure increased methylation only when females were also exposed prenatally. These effects were distinct from those observed in a similar study, in which prenatal PCBs increased methylation at one different site in males and not females (Walker et al., 2014). DNA methylation of CpG sites is typically thought to reduce accessibility to transcriptional factors and ultimately decrease mRNA expressed (Razin, 1998), and this classic inverse relationship appears to true for *Ar in vivo* and in reproductive tissues (Jarrard et al., 1998; Keil et al., 2014; J. Tian et al., 2012). However, this might not be the case in heterogeneous neural cells. Indeed, methylation and gene expression were positively correlated at only one of seven sites in POA *Ar*. The same positive relationship at CpG site −51 was found in a similar study (Walker et al., 2014), suggesting that other epigenetic and non-epigenetic regulatory factors may be more important in regulating gene expression than methylation at these specific sites.

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No effects of PCBs were seen on methylation patterns in *Oprm1* in the PFC or POA. *Oprm1* is epigenetically regulated via DNA methylation in a variety of *in vivo* models (Andria and Simon, 1999; Hwang et al., 2007), and its expression is inversely correlated with methylation status in other studies (Chorbov et al., 2011; Doehring et al., 2013; Nielsen et al., 2009; Vucetic et al., 2010). Moreover, dietary methyl donor supplementation reverses the effects of high fat diet on PFC *Oprm1* expression (Carlin et al., 2013). Therefore, the lack of correlation between methylation levels and gene expression in any of the 3 CpG sites in *Oprm1* was surprising. This may be explained by the CpG sites assayed, which were chosen based on the quality of the assay and are different from those in the other published studies.

5. Conclusion

This study demonstrates that neural gene expression was affected by discrete exposures to PCBs during prenatal or juvenile development. Moreover, the two exposure periods interacted to either mask or reveal effects of exposure at one developmental period. These effects were modest and occurred in male but not female animals, in parallel with the behavioral changes observed in adulthood in the same male animals (Bell et al., 2015). Specifically, changes in NA *Drd2*, PFC *Oprm1*, and serum P_4 might moderate those behavioral effects. We demonstrated that the brain is differentially sensitive to PCBs during prenatal and juvenile periods of development depending on the age and previous exposure history. While our treatment windows occurred during periods of prenatal and postnatal life critical for gonadal hormone action, there may be other periods of development sensitive to long-lasting PCB effects that were not explored herein. Indeed, it is possible that gene expression in the brain remains sensitive to effects of PCBs throughout the lifespan, especially if PCBs are acting via non-hormonal mechanisms, a testable hypothesis. With that said, the effects of PCBs would likely differ depending on the regulatory processes that are occurring at that time, and is an interesting question for future study. Overall, these results demonstrate the need for toxicological testing to take sex, developmental stage, and lifetime exposure history into account when considering adverse outcomes of EDCs.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Highlights

- Prenatal and juvenile PCB exposures affect gene expression in adult rat brains.
- The effect of a single exposure is often reversed by a second hit.
- Males are more sensitive to long-term effects of PCBs than females.
- Gene expression in the medial preoptic area was the most affected by PCBs.

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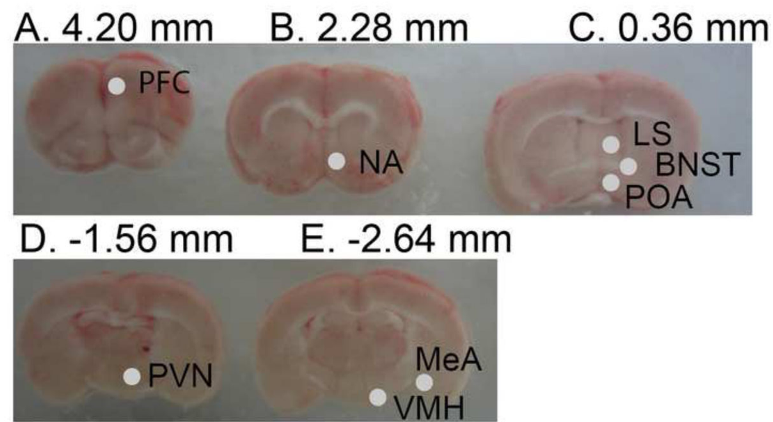

Figure 1.

Images of 5 brain sections from rostral to caudal (A–E) showing locations of 0.98 mm diameter punches. Punches are shown only on one hemisphere for ease in viewing, but bilateral punches were used for RNA extraction. Sections A–C are 2 mm thick, D–E are 1 mm thick. Abbreviations: Prefrontal cortex (PFC), nucleus accumbens (NA), lateral septum (LS), bed nucleus of the stria terminalis (BNST), preoptic area (POA), paraventricular nucleus (PVN), ventromedial hypothalamus (VMH), and medial amygdala (MeA).

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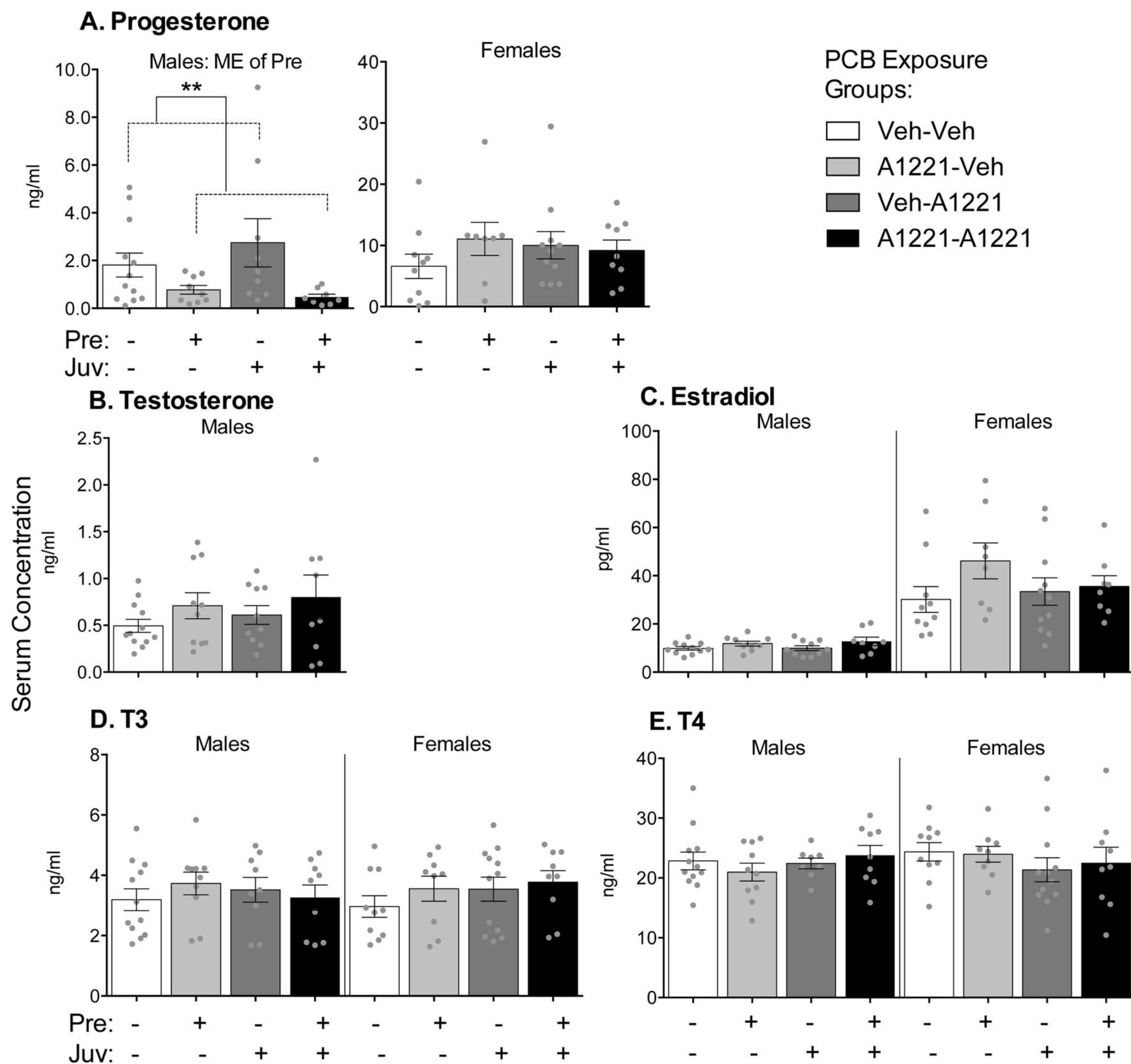

Figure 2.

Serum concentrations of progesterone (A), testosterone (B), estradiol (C), T3 (D), and T4 (E) are shown as mean ± SEM, with dots indicating data points from individual rats. Note different y-axis scales between graphs. Within-sex main effects (ME) of prenatal or juvenile exposure, or interactions (Pre x Juv) between the two, are described in each subtitle, with specific group differences indicated by $^{**}p < 0.01$.

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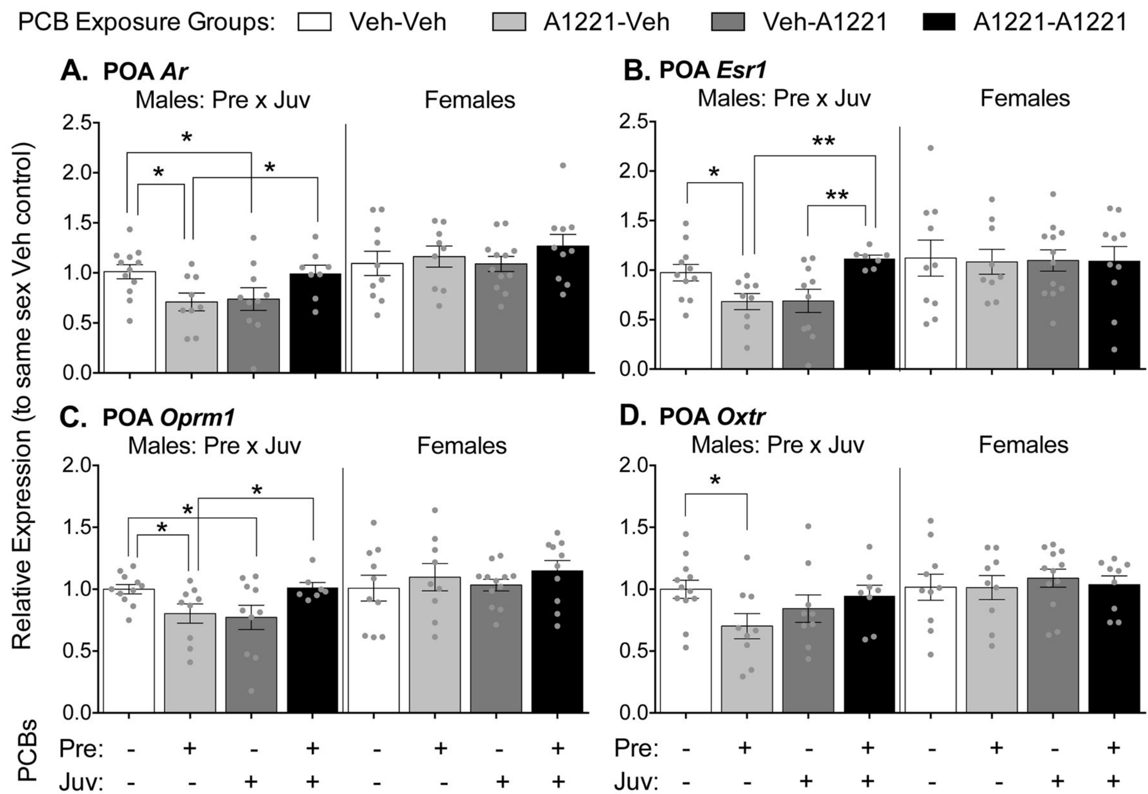

Figure 3.

Gene expression levels in the preoptic area (POA) are shown. Levels of *Ar* (A), *Esr1* (B), *Oprm1* (C) and *Oxtr* (D) are shown as mean ± SEM, with dots indicating individual data points. Note different y-axis scales between graphs. Within-sex main effects (ME) of prenatal or juvenile exposure, or interactions (Pre x Juv) between the two, are described in each subtitle, with specific group differences indicated by * $p < 0.05$, ** $p < 0.01$.

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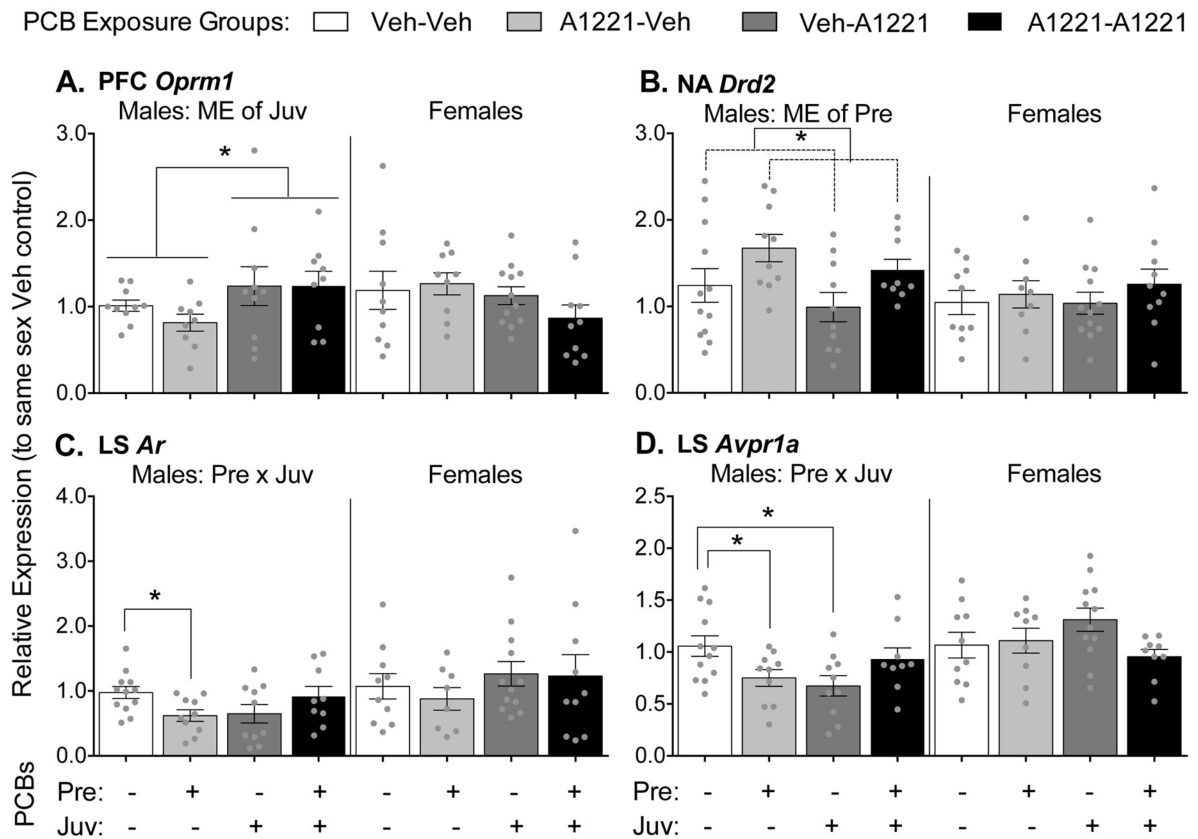


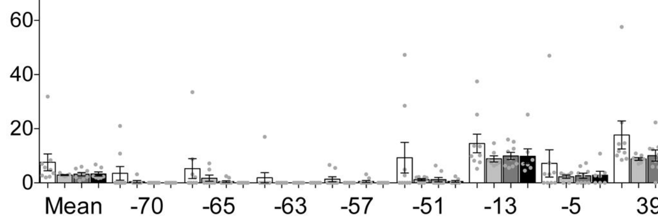
Figure 4.

Gene expression levels in the prefrontal cortex (PFC; A), nucleus accumbens (NA; B), and lateral septum (LS; C and D) are shown. Levels of *Oprm1* (A), *Drd2* (B), *Ar* (C) and *Avpr1a* (D) are shown as mean ± SEM, with dots indicating individual data points. Note different y-axis scales between graphs. Within-sex main effects (ME) of prenatal or juvenile exposure, or interactions (Pre x Juv) between the two, are described in each subtitle, with specific group differences indicated by * $p < 0.05$.

Rat Androgen Receptor, *Ar*, ENSRNOG00000005639,
Transcript ID: Ar-201, EpigenDX Assay ID: ADS070-FS2re

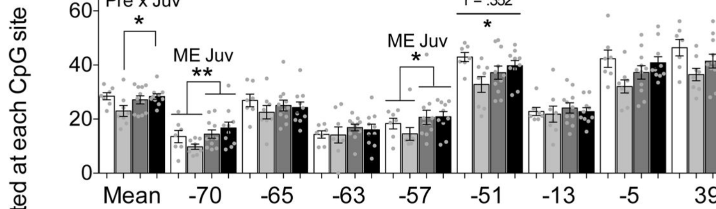
Veh-Veh
A1221-Veh
Veh-A1221
A1221-A1221

A. POA, *Ar* Male

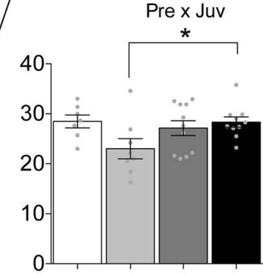


B. POA, *Ar* Female

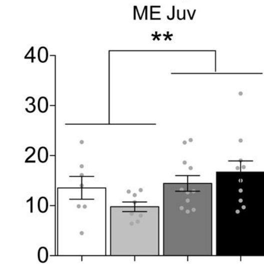
Percent of copies methylated at each CpG site



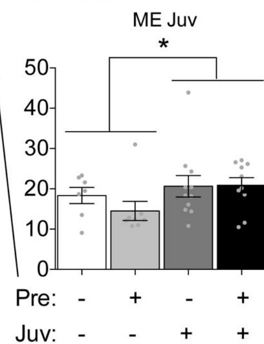
C. Female POA, *Ar* Mean



D. Female POA, *Ar* -70

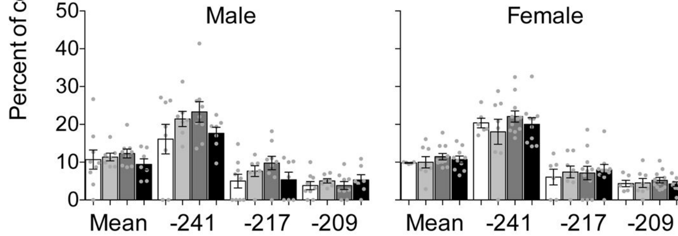


E. Female POA, *Ar* -57



Rat mu 1 Opioid receptor, Oprm1, ENSRNOG00000018191
Transcript ID: Oprm1-203, EpigenDX Assay ID: ADS1905-RS1re

F. POA, *Oprm1*



G. PFC, *Oprm1*

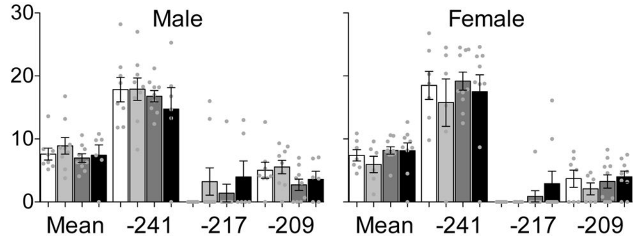


Figure 5.

DNA methylation (%) averaged across all (Mean) or at individual CpG sites in the promoter region of *Ar* (A–E) and *Oprm1* (F–G) is shown in the POA (A–F) and PFC (G). Insert graphs (C–E) show sites within *Ar* in female POA where significant effects of treatment were found. CpG locations are given relative to the transcriptional state site (TSS), which is −183 and −995 upstream from ATG in *Ar* and *Oprm1*, respectively. Bars show means ± SEM, and dots show individual data points. Note different y-axis scales. Within-sex main effects (ME) of prenatal or juvenile exposure, or interactions (Pre x Juv) between the two, are described in each subtitle, with specific group differences indicated by * $p < 0.05$ and **

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$p < 0.01$. R values and significance (* $p < 0.05$ and ** $p < 0.01$) for correlation with gene expression results across all animals are shown by a horizontal line above the CpG site.

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Table 1

PCR Target and assay information

Drd1 and Oprm1 assays target more than one transcript variant.

Gene Symbol	Gene name	Brain Regions assayed	Life Technologies Assay ID	Amplicon Length	Probe Location	Accession Number
Gapdh	Glyceraldehyde 3-phosphate dehydrogenase	MeA, NA	Rn01775763_g1	174	Exon 8	NM_017008.4
Rpl13a	ribosomal protein L13a	BNST, LS, PFC, POA, PVN, VMH	Rn00821946_g1	66	Exons 4–5	NM_173340.2
Ar	androgen receptor	LS, MeA, POA	Rn00560747_m1	75	Exons 3–4	NM_012502.1
Crh	corticotropin releasing hormone	BNST, PVN	Rn01462137_m1	112	Exons 1–2	NM_031019.1
Esr1	estrogen receptor alpha	BNST, LS, MeA, NA, POA, PVN, VMH	Rn01640372_m1	67	Exons 6–7	NM_012689.1
Avp	vasopressin	BNST, MeA, PVN	Rn00690189_g1	78	Exons 2–3	NM_016992.2
Avpr1a	vasopressin receptor 1a	LS	Rn00583910_m1	65	Exons 1–2	NM_053019.2
Oxt	oxytocin	PVN	Rn00564446_g1	78	Exons 2–3	NM_012996.3
Oxtr	oxytocin receptor	MeA, PFC, POA	Rn00563503_m1	60	Exons 1–2	NM_012871.2
Drd1	dopamine receptor D1	NA	Rn03062203_s1	83	Exon 2	XM_006253599.2, XM_006253600.2
Drd2	dopamine receptor D2	NA	Rn00561126_m1	64	Exons 2–3	NM_012547.1
Oprm1	mu opioid receptor	MeA, NA, PFC, POA, VMH	Rn01430371_m1	64	Exons 2–3	NM_001038597.2, NM_001038599.2, NM_001038600.2, NM_001038601.2, NM_013071.2

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Table 2

Within-animal Pearson correlations between male genes, hormones and behaviors

The four behaviors that were significantly affected by PCBs in adulthood (all in males, Bell et al 2015) were correlated with gene expression and hormone concentrations from the same animals. These behaviors were the production of ultrasonic vocalizations (USVs) prior to interacting with a receptive female stimulus animal, and time spent near a hormone- or no-hormone treated female and preference for the hormone-treated female (time with hormone-treated female/time with both females) in sociosexual partner preference test. Effects of PCBs to increase (↑), decrease (↓), or interact (Pre x Juv) are shown. Significant correlations between measures that were affected by PCB exposure are highlighted.

		USVs prior to female	Time near no-hormone female	Time near both females	Preference for hormone female
	PCB Effect	**Pre ↑**	**Pre ↓, Juv ↑**	**Juv ↑**	**Pre ↑**
BNST *Avp*		−0.425 **	−0.202	−0.139	0.158
BNST *Crh*		−0.039	0.218	0.298	−0.010
BNST *Esr1*		−0.272	0.239	0.094	−0.157
LS *Ar*	Pre x Juv	−0.050	0.028	0.068	−0.082
LS *Esr1*		0.250	0.051	0.112	0.019
LS *Avpr1a*	Pre x Juv	−0.086	0.059	0.174	−0.095
MeA *Ar*		−0.011	0.314	0.511 **	−0.021
MeA *Avp*		−0.137	−0.024	−0.043	−0.031
MeA *Esr1*		0.072	0.354 *	0.427 **	−0.131
MeA *Oprm1*		−0.202	0.336 *	0.287	−0.238
MeA *Oxtr*		0.000	0.102	0.275	0.029
NA *Drd1*		0.178	−0.062	0.003	−0.111
NA *Drd2*	Pre ↑	0.338 *	−0.243	−0.293	0.079
NA *Esr1*		−0.285	0.195	0.220	0.004
NA *Oprm1*		−0.350 *	−0.113	−0.016	0.180
PFC *Oprm1*	Juv ↑	−0.116	0.546 **	0.346 *	−0.341 *
PFC *Oxtr*		−0.229	0.017	−0.056	−0.035
POA *Ar*	Pre x Juv	−0.284	−0.057	−0.062	−0.022
POA *Esr1*	Pre x Juv	−0.188	−0.101	0.003	0.055
POA *Oprm1*	Pre x Juv	−0.198	−0.079	0.067	0.013
POA *Oxtr*	Pre x Juv	−0.302	0.118	0.031	−0.127

		USVs prior to female	Time near no-hormone female	Time near both females	Preference for hormone female
	PCB Effect	**Pre ↑**	**Pre ↓, Juv ↑**	**Juv ↑**	**Pre ↑**
PVN *Avp*		−0.312	0.053	0.011	−0.061
PVN *Crh*		−0.131	0.030	0.027	−0.083
PVN *Esr1*		0.177	−0.032	0.031	0.113
PVN *Oxt*		−0.151	−0.084	−0.035	0.104
VMH *Oprm1*		−0.148	0.261	0.072	−0.242
VMH *Esr1*		−0.187	−0.140	−0.067	0.147
P	Pre ↓	−0.043	0.345 *	0.296	−0.215
T3		0.201	−0.212	−0.190	0.100
T4		−0.008	0.107	0.134	−0.077
T		0.259	0.111	−0.100	−0.307

** $p < 0.01$,

* $p < 0.05$

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