HHS Public Access

Author manuscript

*Toxicol Appl Pharmacol*. Author manuscript; available in PMC 2021 January 30.

Published in final edited form as:

*Toxicol Appl Pharmacol*. 2018 August 15; 353: 55–66. doi:10.1016/j.taap.2018.06.002.

# Sex differences in effects of gestational polychlorinated biphenyl exposure on hypothalamic neuroimmune and neuromodulator systems in neonatal rats.

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## Abstract

Polychlorinated biphenyls (PCBs) are ubiquitous in the environment and exposure to them is associated with immune, endocrine and neural dysfunction. Effects of PCBs on inflammation and immunity are best described in spleen and blood, with fewer studies on neural tissues. This is an important gap in knowledge, as molecules typically associated with neuroinflammation also serve neuromodulatory roles and interact with hormones in normal brain development. The current study used Sprague-Dawley rats to assess whether gestational PCB exposure altered hypothalamic gene expression and serum cytokine concentration in neonatal animals given an immune challenge. Dams were fed wafers containing a mixture of PCBs at an environmentally relevant dose and composition (20 ug/kg, 1:1:1 Aroclor 1242:1248:1254) or oil vehicle control throughout their pregnancy. One day old male and female offspring were treated with an inflammatory challenge

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Supplementary Data Description

Supplementary Table 1 includes a list of all the gene targets, including a brief description of the protein they encode, the NCBI reference sequence, Life Technologies Assay IDI, and Probe Sequence.

Conflict of Interest Statement

• None of the authors (M Bell, A Dryden, R Will, or A Gore) have any conflicts of interests regarding any of the following items:
• third-party financial support for the work in the submitted manuscript.
• financial relationships with any entities that could be viewed as relevant to the general area of the submitted manuscript.
• sources of revenue with relevance to the submitted work who made payments to us, or to our institution on our behalf, in the 36 months prior to submission.
• interactions with the sponsor of outside of the submitted work.
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(lipopolysaccharide, LPS, 50 ug/kg, sc) or saline vehicle control approximately 3.5 hours prior to tissue collection. Across both basal and activated inflammatory states, PCB exposure caused greater expression of a subset of inflammatory genes in the hypothalamus and lower expression of genes involved in dopamine, serotonin, and opioid systems compared to oil controls. PCB exposure also altered reactions to inflammatory challenge: it reversed the normal decrease in *Esr2* hypothalamic expression and induced an abnormal increase in IL-1b and IL-6 serum concentration in response to LPS. Many of these effects were sex specific. Given the potential long-term consequences of neuroimmune disruption, our findings demonstrate the need for further research.

### Keywords

Endocrine disrupting chemicals; sex difference; dopamine; estrogen receptor beta; cytokine; lipopolysaccharide

## Introduction

Communication between neural, endocrine, and immune systems is essential for an organism's ability to respond to, and interact with, its environment. While the neuroimmune system was initially described in mediating behavioral and pathological responses to inflammatory challenges, we are now appreciating its active role in normal, hormone-sensitive developmental processes (Bilbo et al., 2012). Numbers of microglia, the resident immune cells in the brain, and their expression of cytokine signaling molecules are developmentally regulated and sexually dimorphic (Gendron et al., 1991; Young et al., 1995; Ortega et al., 2011; Bilbo et al., 2012; Schwarz et al., 2012; Crain et al., 2013; Hanamsagar et al., 2017). They mediate basic processes like synaptic pruning and formation, neuronal survival, differentiation, and programmed cell death (Hanamsagar and Bilbo, 2017). These processes drive learning and cognition in the hippocampus (Zhao et al., 2015; Torres et al., 2016), later sexual behavior in the hypothalamus (Amateau and McCarthy, 2002; Amateau and McCarthy, 2004; Lenz et al., 2013) and social behavior in frontal cortex (Filiano et al., 2016; Nelson and Lenz, 2017b). As such, disruption by perinatal immune challenges can result in an increased incidence of schizophrenia, depression, bipolar disorder, epilepsy, and autism spectrum disorders (Bilbo and Schwarz, 2012; Knuesel et al., 2014; Reisinger et al., 2015; Hanamsagar et al., 2017).

Neuroimmune signaling functions during development can also be disrupted by environmental challenges other than inflammatory ones, including effects of maternal stress, high fat diet, and diesel exhaust on microglial action (Bolton et al., 2013; Bolton et al., 2014; Bolton et al., 2017; Hanamsagar and Bilbo, 2017). Effects of hormone-active environmental contaminants are just beginning to be characterized (Rebuli et al., 2016; Meadows et al., 2017; Takahashi et al., 2018). One group of widespread environmental contaminants with great potential to affect developing neuroimmune systems are polychlorinated biphenyls (PCBs). PCBs are present in the food chain due to prior industrial contamination (Anderson et al., 1998; Kostyniak et al., 1999; Turyk et al., 2006; McGraw and Waller, 2009) and exposure can be high during early development: fetuses are exposed to PCBs via placental transfer (Newsome et al., 1995), infants are exposed to concentrated PCBs via breastmilk,

and biological detoxification systems are still immature in developing individuals (Hansen, 1998; Landrigan, 2001; Blake et al., 2005; de Zwart et al., 2008). Indeed, negative correlations are found between degrees of exposure to PCBs *in utero* and fetal and childhood growth and neurodevelopmental milestones in populations from Michigan, New York, Netherlands, Germany, and the Yu-cheng disaster (Fein et al., 1984; Jacobson et al., 1990; Gladen and Rogan, 1991; Chen et al., 1994; Guo et al., 1995; Darvill et al., 2000; Schantz et al., 2003; Stewart et al., 2003; Stewart et al., 2008; Berghuis et al., 2013; Nowack et al., 2015)

The possible mechanisms of PCB action on neuroimmune processes are potentially diverse. PCBs are well-recognized as dopaminergic cell neurotoxicants (Seegal et al., 1986; Bell, 2014) and endocrine-disrupting chemicals (EDCs) (Soto et al., 1995; Portigal et al., 2002; Gore et al., 2015). Given that neural and peripheral immune cell populations are affected by estradiol (Lenz et al., 2013; Rebuli et al., 2016) and dopamine (Yan et al., 2015), they are possible targets of PCB action. PCBs are also suspected peripheral immunotoxicants (Weisglas-Kuperus, 1998). For example, exposure to PCBs at 18 months of age is associated with blunted vaccine efficacy at 5 and 7 years of age in children (Heilmann et al., 2010). In peripheral tissues of adult rodents, exposure to industrial mixtures of different PCBs, called Aroclors (A) 1242, 1248, and 1254, suppressed adaptive immune responses (Exon et al., 1985; Davis and Safe, 1989; Silkworth et al., 1989; Hamers et al., 2011), while other specific PCB congeners increase innate immune responses (Choi et al., 2012). PCBs may also affect inflammatory molecules in the neural tissues, but this phenomenon has not been well-studied (Voie and Fonnum, 2000; Mariussen et al., 2002; Sipka et al., 2008). Most recently, (Hayley et al., 2011) demonstrated that gestational exposure to a PCB mixture increased select cytokine expression in adult female brains. Importantly, the above studies were on adult animals, *in vitro*, or with just one sex. As sex differences are present in peripheral and neural immune responses to an bacterial endotoxin in rodents and humans (Moxley et al., 2002; Marriott et al., 2006; Loram et al., 2012; Everhardt Queen et al., 2016), neuroimmune developmental patterns (Schwarz et al., 2012; Crain et al., 2013; Lenz et al., 2013; Crain and Watters, 2015; Nelson and Lenz, 2017a), and neuroimmune responses to stressors and drugs (Pyter et al., 2013; Hudson et al., 2014; Doyle et al., 2017), it is essential to compare males and females directly.

Given the importance of inflammatory molecules in the brain for normal development and later health status, we sought to determine neonatal effects of PCBs on the hypothalamus, a region previously demonstrated to be PCB sensitive (Bell, 2014) and essential for the inflammatory fever response. We quantified basal and lipopolysaccharide (LPS)-challenge-induced expression of genes related to inflammatory signaling, nuclear receptors, and neurotransmitter systems that are sensitive to environmental contaminants and/or hormones (Grossman, 1984; Safe et al., 1985; Hestermann et al., 2000; Vegeto et al., 2003; Beischlag et al., 2008; Warner et al., 2012; Busbee et al., 2013). This study tests the hypothesis that early life PCB exposure will alter basal neuroimmune signaling and reactivity to an immune challenge, which could be mechanisms by which PCBs affect a range of hormone-sensitive social behaviors and neurodevelopmental outcomes.

## Materials and Methods

### 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 Institutional Animal Care and Use Committee. Sprague Dawley rats were used, as is common in studies of PCBs and neuroimmune effects in early development. They were purchased from Harlan Laboratories (Houston, Texas) and were housed in a humidity- and temperature- controlled room with a 12-hour light / 12-hour dark light cycle, lights off at noon, at 21–23 °C. Two to three 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. While not tested for PCB content, no fish meal is used in production thereby reducing the chance of contamination. 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 month old, virgin) were mated with sexually experienced male rats (~6 months old) overnight after confirming receptivity. The morning after successful mating, as indicated by a sperm positive vaginal smear and 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], pups were weighed, anogenital distances were measured, and tissue was collected.

During gestation, dams were exposed to either Oil vehicle (n = 12) or PCB mixture, described below (n = 11). After birth, male and female offspring were injected with either lipopolysaccharide (LPS) or saline (Sal) vehicle (see below). This created four experimental groups per sex, in a 2×2 design (Figure 1). Each litter contributed no more than one male and female per treatment group. However, not all litters were large enough to contribute one animal to every group, as some pups were also assigned to other experiments. Number of pups per group were as follows: female Oil-Sal (n = 11); female Oil-LPS (n = 11); female PCB-Sal (n = 8); female PCB-LPS (n = 8); male Oil-Sal (n = 10); male Oil-LPS (n = 10); male PCB-Sal (n = 11); male PCB-LPS (n = 10). Litter treatments were evenly represented across three cohorts run over the course of a year. Cohort did not affect expression of genes significantly altered by PCBs or LPS when tested as a fixed variable or covariate. The experimenters were blind to treatment throughout the duration of the experiment.

### PCB Treatment

A mixture of PCBs (1:1:1 ratio of Aroclor 1242, 1248, and 1254) was used. They were all purchased from AccuStandard, New Haven, CT, and catalog, lot, and CAS numbers are as follows: C-242-N-50MG, Lot# 01141, CAS# 53469–21-9; C-248N-50MG, Lot# F-110, CAS# 12672–29-6; C-254N-50MG, Lot# 5428, CAS# 11097–69-1. Together, these commercially created blends of PCBs are composed of predominately noncoplanar congeners (only ~10% coplanar and ~6% dioxin like) and lightly- and heavily-chlorinated PCBs, per the authors calculations from (Frame et al., 1996) and United States

Environmental Protection Agency congener classification (2003), and were chosen to mimic the range of mixtures found in natural populations in the US (Hites et al., 2004; Kostyniak et al., 2005). PCBs were diluted in vegetable oil vehicle, chosen because the fatty acid profile matches that of the regular chow (10% palmitic, 4% stearic, 23% oleic, 51% linoleic, 7% linolenic). Approximately 100 µl of vehicle or PCB oil (500 µg fat per oil dose) was applied to a quarter of palatable wafer ('Nilla Wafer, Nabisco, ~0.9 g wafer; 180 µg fat and 330 µg sugar per wafer serving), adjusted to provide a dose of 20 µg PCB / kg body weight (BW) per individual dam. Given high concentrations of PCBs in breastmilk and other studies indicating that lactational transfer is actually higher than placental (Takagi et al., 1976; Takagi et al., 1986), this exposure route, timing, and dose mimics exposure of a breastfeeding human baby in North American populations exposed to moderately high amounts of PCBs. This is estimated based on estimates of transfer of PCBs from dam to offspring and additional routes of PCB exposure, and is generally lower than many other studies of this same nature (Grandjean et al., 1995; Lanting et al., 1998; Stellman et al., 1998; Dewailly et al., 1999; Dekoning and Karmaus, 2000). Dams were randomly assigned to either Oil or PCB groups in a counterbalanced design. Dams were fed oil or PCB treated wafers every weekday morning from E1 until parturition. Prior to mating, dams were trained to take the wafer out of the experimenter's hand so that they generally consumed wafers immediately upon receipt. PCBs were handled with necessary personal protective equipment in a ventilation hood, and chemical and animal waste is disposed of via the Environmental Health and Safety office on campus. No effects of PCBs were seen on birth outcomes such as P1 body weights or anogenital index, and there were no effects on number of pups on P1 (live or dead). Dam body weight gain over pregnancy was also not affected.

## LPS Treatment

Lipopolysaccharide (LPS, *E. coli* 0111:B4, Sigma, L4391, Lot 014M4019V) was used to induce a temporary and non-infectious inflammatory response. LPS binds toll-like receptors on immune cells, which then activates nuclear factor of kappa light poly-peptide gene enhancer in B-cells (NFκB), induces cytokine expression, and results in sickness behaviors. Within two hours before lights out, pups were injected (~0.1 ml, i.p., 28-gauge needle) with sterile normal saline (Sal) vehicle or LPS (50 µg/kg as per (Schwarz and Bilbo, 2011)) and immediately returned to their litter or home cage. The litters were observed to confirm that the injected pups remained in the nest.

## Tissue Collection

Rats were euthanized and brains were collected three to four hours after injection, at the peak of the LPS-induced cytokine expression (Ortega et al., 2011) and in the first third of the dark period. Animals were taken from their home cage and rapidly decapitated in an adjacent room under dim red light. Brains were quickly removed from the skull, chilled on ice, and the whole hypothalamus dissected out with razor blades. Trunk blood samples were collected and allowed to clot for 30 minutes before centrifugation (1500 × g for 5 minutes) and serum stored for subsequent cytokine and hormone assays. All samples were transferred into individual RNase-free eppendorf tubes, and stored at −80C.

## Gene Expression

RNA was isolated as previously described (Bell et al., 2016). Briefly, frozen tissue was lysed and homogenized using 22 gauge needles and syringes. RNA was extracted using an RNeasy Mini Kit (Qiagen Product Number 74104, Lot # 154011565) with RNase Free-DNase set (Qiagen Product Number 79254, Lot # 151045293). RNA quantity was assessed via Promega QuantiFluor Systems on the Glomax Multi + Detection System (RNA: Cat No E3310, Lot # 0000156608), according to manufacturer instructions, and 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. RNA samples (200 ng) were converted to cDNA in 20 μl reactions using a high-capacity cDNA reverse transcription kit (Applied Biosystems, Cat No 4368814, Lot # 00372558) with RNase inhibitor (Applied Biosystems, Cat No N8080119, Lot # 1634300) 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.

Quantitative PCR was completed with cDNA samples on custom-designed microfluidic 48-gene Taqman Low Density Array (TLDA) cards (Applied Biosystems, Cat No 4342253), using Taqman Gene Expression Mastermix (Applied Biosystems, Cat No 4369016) according to manufacturer's directions. Targets were selected for their involvement in xenobiotic responses, inflammation, hormone signaling, and neurotransmitter signaling (listed in Table 1 and described in Supplemental Table 1). qPCR was carried out on an Applied Biosystems ViiA 7 (Software version 1.2.4) 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 and used to determine relative expression. A geometric mean of 18s, Rpl13a and Gapdh was determined and used as the reference Cq and subtracted from target Cqs to determine delta Cq within each sample. The median Delta Cq of the same sex Veh-Veh group was subtracted to determine fold change in expression for each individual. Any outlier within each group was identified by a Grubbs test, but no more than one animal was removed per group. Five animals were deemed outliers from all gene expression analysis due to hypothalamic brain sections being slightly too rostral or caudal. Ten other individual data points (out of 3552 data points from 48 genes per 74 remaining animals) were removed because they were highly significant outliers ($p < 0.01$) on a gene-by-gene basis. Of these ten, only three were removed from genes where a significant effect was found, and their removal actually reduced the likelihood of an effect being detected.

## Serum Cytokines

Serum samples were thawed on ice and diluted in assay buffer. The Milliplex Cytokine / Chemokine Hormone assay (RECYTMAG-65K, Cat No) was run according to manufacturer directions. This assay contains Interleukin (IL)- 1a, 4, 1b, 6, 10, interferon gamma (IFNγ), and tumor necrosis factor (TNF, also known as TNFα), which were selected based on literature and availability in the assay. Samples (25 μl) were run in duplicate across two plates. Quality controls fell within appropriate range; intraassay variabilities for the seven assays were between 0.9 and 7.4; interassay variability of QCs and standards were 14.2

(IL-1a), 8.9 (IL-4), 24.2 (IL-1b), 8.9 (IL-6), 7.5 (IL-10), 32.6 (IFNγ), 12.2 (TNF). While the interassay variability is high for IL-1b and IFNγ, groups were represented evenly across the plates and there were not significant differences in serum concentration between the two runs. Two animals were identified as outliers with a Grubbs test across several serum cytokine analysis and were removed from all cytokine analysis; eight other data points were removed on an individual basis because they were also highly significant outliers.

## Serum Corticosterone

Corticosterone was determined via radioimmunoassay according to manufacturer instructions (ImmuChem Double Antibody $^{125}$I RIA Kit, MP Biomedicals LLC, Orangeburg NY) in duplicate 100 μl serum samples. Intra-assay CV was 2.18% and minimum detectability was 7.7 ng/ml. One outlier was identified with a Grubbs test and was removed from analysis.

## Statistics

Analysis was completed using SPSS (Version 23, IBM) and GraphPad Prism, with significant differences demarcated in figures and tables as *, $p < 0.05$ and **, $p < 0.01$. Hypothalamic gene expression, serum cytokine concentrations, and serum corticosterone concentrations were analyzed using a two-way analysis of variance (ANOVA) to determine main effects of PCB treatment, LPS treatment, and interactions between PCB and LPS treatment within a sex. Males and females are treated as independent units of analysis because of known sex differences in neuroimmune outcomes, which may overwhelm any PCB or LPS treatment effects within a statistical model. Accordingly, qPCR data was normalized to same sex-controls. Appropriate follow-up t-tests were used 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) even after square root transformation, non-parametric Mann-Whitney U tests were used within a sex, and are indicated in the results as a 'MW'. The non-parametric equivalent of a main effect of PCB or LPS was identified by collapsing across the other treatment levels, and an interaction was identified by testing for effects of one treatment within the other and *vice versa*.

For four genes (*Il6, Ifna1, Cyp1a1,* and *Cxcl9*) and two serum cytokines (IL-1α and IFNγ), at least one group had fewer than 30% of its samples fail to amplify (defined as $C_q < 35$) or reach detectable levels; as such, group differences were identified with a $\chi^2$ goodness of fit test. As with non-parametric tests, the equivalent of main effects and interactions were tested by collapsing across or testing within the other treatment levels. *Il4* failed to amplify in all hypothalamic samples. Because of the large number of genes analyzed, the Benjamini-Hochberg False Discovery Rate (Benjamini and Hochberg, 1995) correction was applied. For these calculations, p values for effects of PCBs were drawn together from 2×2 ANOVAs, nonparametric tests, or $\chi^2$ analysis within a sex. No outcomes survived the correction. However, because each target was chosen based on an a priori hypothesis, group differences were considered statistically significant at $p < 0.05$ level.

To determine relationships among gene expression patterns and serum hormones and cytokines, hierarchical cluster analysis and heatmaps were generated within a sex using R

(version 3.0.3). Dependent variable columns with 20 or fewer observations per sex (*Cxcl9, Cxcl22, Cyp1a, Idol, Ifna1, Il4, Il6*, serum IL-1a, and serum IFNγ, due to low levels of expression) were removed and data were transformed into z-scores. A correlation matrix was created using Pearson's correlations with pairwise complete functions to deal with missing data. This was transformed into a dissimilarity matrix by subtracting the absolute value of the pairwise correlation from one, and then a distance matrix by removing self-correlations. The distance matrix was used to build hierarchical clusters with the R package hclust, with each object beginning as its own cluster and iteratively adding the two most similar clusters with a complete agglomerative method. The quality of the clusters was tested with the R package pvclust via multiscale bootstrap resampling of the same distance measures, and 10,000 bootstrap replications. The package calculates an 'appropriately unbiased' p-value for each cluster; when $p > 0.95$, the hypothesis that "the cluster does not exist" is rejected with significance level 0.05 (Shimodaira, 2004). A similar process was used to quantify relationships between groups across all measures; z-score transformed averages of target levels were determined per group, and hclust and pvclust were again used to determine p values for clusters of groups within a sex.

## Results

### Gene Expression

A summary of PCB and LPS effects within each sex on gene expression are presented in Table 1, with full gene names. Animals exposed to PCBs showed higher expression of several proinflammatory genes compared to Oil exposed controls, independent of LPS challenge (Figure 2). These genes included *Ikbkb* in females (MW, $p < 0.05$); *Ccl22* in females ($F_{(1,16)} = 28.58$, $p < 0.01$); and *Tnf* in males (square root transformed, $F_{(1,32)} = 6.91$, $p < 0.05$). PCB exposure also affected genes that modulate neurotransmission (Figure 3). For one gene, *Esr2*, PCBs altered the effect of LPS in females, as indicated by a PCB × LPS interaction ($F_{(1,33)} = 6.24$, $p < 0.05$). Expression was greater in LPS- than Sal- exposed animals, but only in females exposed to PCBs ($F_{(1,14)} = 7.66$, $p < 0.05$). This is opposite of the pattern observed in control females, as well as the main treatment effect in males ($F_{(1,33)} = 4.93$, $p < 0.05$), in which LPS exposure reduced expression. Animals exposed to PCBs also showed lower expression of *Slc6a3* in females ($F_{(1,27)} = 4.68$, $p < 0.05$) and males ($F_{(1,29)} = 5.73$, $p < 0.05$); *Slc6a4* in females (MW, $p < 0.05$) and males ($F_{1,32} = 7.50$, $p = 0.01$); *Th* in females ($F_{(1,33)} = 4.15$, $p = 0.05$) and males ($F_{(1,33)} = 5.41$, $p < 0.05$); and *Pomc* in females ($F_{(1,27)} = 4.54$, $p < 0.05$).

The LPS challenge produced the expected increases in proinflammatory molecules, independent of PCB exposure, and more so in males than females (Figure 4). Animals exposed to LPS showed greater expression of *Ptges* in males ($F_{(1,30} = 8.86$, $p < 0.01$); *Ptgs2* in females ($F_{(1,32)} = 7.40$, $p = 0.01$) and males (MW, $p < 0.01$); *Il1b* in males (MW, $p < 0.01$); and *Tnf* in males (see Figure 2A, MW, $p < 0.01$). Additionally, *Cxcl9* expression was detectable in more LPS (84%) than Sal (26%) treated females ($\chi^2 = 14.01$, $p < 0.01$); and LPS (85%) than Sal (36%) treated males ($\chi^2 = 11.91$, $p < 0.01$). LPS also caused small but significant decreases in expression of molecules known to modulate, but not directly

mediate, inflammatory responses, and only in males (**data not shown**): *Arntl* ($F_{(1,33)}$ = 7.82, p < 0.01); *Arrb1* ($F_{(1,33)}$ = 4.45, p < 0.05); and *Tgfb2* ($F_{(1,32)}$ = 4.57, p < 0.05).

The inflammatory challenge also reduced expression of some modulators of neurotransmission and hormone signaling (Figure 5). These genes included *Esr1* in males ($F_{(1,33)}$ = 6.93, p < 0.05); *Esr2* in males ($F_{(1,33)}$ = 4.93, p < 0.05, see Figure 3A); *Htr1a* in females ($F_{(1,32)}$ = 4.60, p < 0.05); *Oprm1* in females ($F_{(1,33)}$ = 4.83, p < 0.05) and males ($F_{(1,33)}$ = 6.91, p < 0.05); *Oprk1* in males ($F_{(1,33)}$ = 6.50, p < 0.05); and *Pomc* in females (see Figure 3E, $F_{(1,27)}$ = 4.41, p < 0.05).

## Serum cytokines

There was a significant main effect of LPS on concentrations of the cytokines IL-10 and TNF in neonatal females and males; both were greater in LPS than Sal exposed rats (MW, all p < 0.01) (Figure 6). IL-6 was also significantly higher in LPS than Sal treated males. In males exposed to PCBs but not Oil, IL-1b was significantly elevated in LPS compared to Sal (MW, p < 0.01). In females exposed to PCBs, there was also an IL-6 response to LPS (MW, p < 0.01) that was not present in Oil group. No effects were detected for IL-4 or IL-1a. IFNγ had some groups in which fewer than 30% of the samples were detectable and so were analyzed with a $\chi^2$ instead of ANOVA. For IFNγ, a greater proportion of male LPS treated samples (65%) were above levels of detection than Sal treated samples (21%, $\chi^2$ = 7.653, df = 1, p < 0.01). No effects of LPS on IFNγ were detected in females. No main effects of PCBs were detected for any serum cytokine.

## Serum hormones

Corticosterone concentrations were higher in LPS-challenged females and males than in Sal-exposed females ($F_{(1,32)}$ = 27.28, p < 0.01) and males ($F_{(1,36)}$ = 30.95, p < 0.01). No effect of PCB exposure on corticosterone was detected when analyzed within a sex with a two-way ANOVA, but one was detected when analysis was collapsed across sex: Corticosterone concentrations were higher in animals exposed to PCBs than Oil ($F_{(1,68)}$ = 6.02, $p$ < 0.05) (Figure 7).

## Hierarchical Cluster Analysis

Hierarchical cluster analysis was conducted separately for the sexes. In females, Oil LPS and PCB LPS groups clustered together (p = 0.96), but Oil and PCB groups treated with saline did not (Figure 8). In males, Sal and LPS treated groups appeared to cluster together, independent of PCB treatment (Figure 9), however these clusters are just approaching statistical validation (p = 0.94 and 0.91). In females (Figure 8), clusters A and B included targets related to hormones and neurotransmitters in which PCB and LPS exposure interacted to affect expression. By contrast, expression of targets in cluster E was predominantly increased by LPS and included inflammatory molecules and corticosterone. In males (Figure 9), clusters A and B again included targets related to neurotransmission modulation that were decreased by PCBs, whereas expression of genes and concentrations of serum cytokines in clusters C and E were decreased and increased by LPS, respectively.

## Discussion

Neuroimmune processes are important mediators of normal brain development, and perturbations in their developmental trajectories have been implicated in later behavioral alterations and disease outcomes (Hanamsagar et al., 2017). Previous work has demonstrated that PCBs can influence peripheral immune systems and neurodevelopment, independently. However, this study is one of the first to indicate that exposure to environmentally relevant concentrations and mixtures of PCBs can affect neuroimmune systems, which may be a mechanism of the effects on hormone-sensitive and sexually differentiated behavior. These effects of PCBs are generally independent of LPS exposure. However, PCB exposure also induced a response to LPS that was not present otherwise: heightened release of serum IL-1b and IL-6, and expression of hypothalamic *Esr2*. Immune challenge with LPS affected expression of genes related to neuroimmune signaling, and both PCBs and LPS affected expression of genes involved in serotonin and opioid neurotransmission, but there was minimal overlap in molecular targets between the two insults. These results may suggest that PCBs and LPS affect neuroimmune and neuromodulator signaling via distinct intracellular cascades that converge at a few key targets.

Importantly, many of the effects of both PCBs and LPS were sex-specific. This may be the result of known sex differences in peripheral and neural immune systems. In the hypothalamus, there are more overall and activated microglia in males compared to females in the preoptic area at this age in an estradiol-sensitive manner (Lenz et al., 2013; Pyter et al., 2013). As such, exposure to perinatal hormones could alter responses to LPS and/or be affected by PCB exposure. Peripheral immune cells are also hormone sensitive and show sex-differences in expression of TLR4 proteins that could explain sex-specific responses to LPS in the current study (Marriott et al., 2006; Temple et al., 2008; Aomatsu et al., 2013; Nelson and Lenz, 2017a). However, discrete populations of cells at different developmental states could show unique responses to LPS; indeed, dendritic and macrophages show dramatic changes during neonatal periods (Kuper et al., 2016). Clearly, more research is needed to follow-up on this interesting possibility.

### PCB effects on inflammation

Independent of LPS challenge, PCBs affected expression of a limited set of genes involved in the transmission of neuroimmune signaling molecules. Because of the importance of *Ikbkb* and *Ccl22* in a host of inflammatory responses, the PCB-induced upregulation in females could have major consequences on health. *Ikbkb* encodes a kinase subunit (IKKβ) that responds to pro-inflammatory stimuli, such as LPS, IL-1, and TNF, to release NFκB from inhibition by IκB (Hacker and Karin, 2006). NFκB is a critical mediator of transcriptional responses to LPS and cytokines in innate immunity and inflammation (Schutze et al., 1995). The PCB-induced upregulation of *Ikbkb* in the current study is in agreement with a suggestion that PCB153 effects on cytokine induction may be mediated by degradation of IkB to activate NFκB (Kwon et al., 2002).

In contrast to *Ikbkb*, chemokine (C-C motif) ligand 22 (CCL22, the product of *Ccl22*) is associated with M2-polarized macrophages that promote tissue remodeling (Biswas and Mantovani, 2010) and the fever response to prostaglandins in the hypothalamus (Osborn et

al., 2011). *Ccl22* undergoes dramatic shifts in sexually differentiated expression across development within the hippocampus and cortex (Schwarz et al., 2012) and this dynamic regulation might make it a more vulnerable target to perturbation. Exposure to diesel exhaust particles reduced concentrations of CCL22 in M2 polarized human blood derived macrophages (Jaguin et al., 2015), while the current study demonstrates that PCBs increased expression of *Ccl22* in hypothalamic tissues; these differences could result from unique cell populations, developmental periods, and/or contaminant mechanism of action, but highlight CCL22 as a molecule of continued interest for future study.

PCB exposure induced a distinct, yet also pro-inflammatory response in males: greater basal hypothalamic expression of *Tnf* than oil exposed controls. *Tnf* encodes tumor necrosis factor (TNF), a proinflammatory signaling molecule that acts as a cytokine and induces apoptosis (Sedger and McDermott, 2014). Beyond this, TNF is also involved in neural developmental processes, including synaptic plasticity and pruning (McCoy and Tansey, 2008). If TNF protein levels are indeed disrupted in the current model, altered synaptic plasticity could be a mechanism for potential long-term deficits from developmental PCB exposure. This PCB-induced *Tnf* upregulation is in agreement with previous studies of acute PCB exposure on circulating concentrations in adult male rodents (Choi et al., 2012; Abliz et al., 2016).

## LPS effects on inflammation and interactions with PCBs

In validation of our experimental model, LPS treatment induced the expected inflammatory responses in both Oil and PCB treated animals, including greater expression of proinflammatory hypothalamic genes in LPS than Sal exposed animals, including *Cxcl9*, *Il1b* (males only) and *Tnf* (males only). This was accompanied by elevated concentrations of serum cytokines IL-6 (males only), IL-10, TNF, and the stress hormone corticosterone. As such, males appear to be more sensitive to LPS at this developmental period, in direct agreement with studies on serum cytokine responses to LPS (Marriott et al., 2006; Everhardt Queen et al., 2016) and analogous to prefrontal cortex responses to an acute stressor (Hudson et al., 2014) in adult mice. Effects of LPS to increase expression of genes involved in prostaglandin $E_2$ could also be a mechanism by which early life LPS treatment alters hormone sensitive behaviors in adulthood (Nelson and Lenz, 2017a).

LPS challenge also revealed peripheral proinflammatory effects of PCBs that were otherwise undetected at basal states and sex-specific. In females, PCB exposure exacerbated the serum IL-6 response to LPS, such that the female-typical response was now male-like. IL-6 is thought of as a classic M1 monocyte activation marker (Mantovani et al., 2004) and is also important in activating T cells and differentiating B cells (Hunter and Jones, 2015). These findings are in agreement with other studies demonstrating effects of PCBs on IL-6 in rat serum (Miller et al., 2010) and human mast cells *in vitro* (Kwon et al., 2002) but not in brain (Hayley et al., 2011).

An interaction between PCB exposure and LPS challenge on a different serum cytokine was also observed in males: the LPS challenge induced greater serum concentrations of IL-1b, but only in PCB exposed animals. A similar response pattern is observed in gene expression (Figure 4c), however only a main effect of LPS was detected statistically. IL-1b is a prototypic pro-inflammatory cytokine involved in the fever response, hypothalamic-

pituitary-adrenal axis activation, and sickness behaviors (Shaftel et al., 2008) and implicated in autoinflammatory syndrome (Guarda et al., 2011) and neurodegeneration (Shaftel et al., 2008). The male-specific PCB-induced response is in agreement with findings on PCB 153 from adult male rat serum (Abliz et al., 2016), human mast cells (Kwon et al., 2002), and P1 microglia primary culture (Loram et al., 2012).

## PCB and LPS effects on neuromodulators

PCBs and LPS interacted to potentiate expression of a single gene: *Esr2*. *Esr2*, which encodes estrogen receptor beta (ERβ), was upregulated in response to LPS, but only in PCB-exposed females. This is in contrast to effects observed in other studies where LPS reduced *Esr2* in endothelial cell culture, (Holm et al., 2010) and in male animals in the current study, but similar to effects in estradiol treated primary microglia from rats of undisclosed sex (Liu et al., 2005). Estrogens and their receptors can have both pro- or anti-inflammatory effects depending on the dose, duration of treatment, and target tissues and cell types (Arevalo et al., 2015; Kovats, 2015; Nelson and Lenz, 2017a); therefore the PCB-induced change could have important effects on downstream responses to immune challenge. ERβ is also affected by other EDCs including chlorpyrifos (Venerosi et al., 2015), BPA (Cao et al., 2014), and PCBs (Warner et al., 2012; Qu et al., 2014), however PCB effects are somewhat inconsistent (Salama et al., 2003; Dickerson et al., 2011; Walker et al., 2014). Expression of *Esr1* or *Ahr*, potential receptors for PCBs, was not affected by PCBs in this study.

PCBs and LPS both affected dopaminergic, serotonergic, and opioidergic pathways, but in non-interacting ways. PCB-exposed male and female animals showed lower expression of genes encoding tyrosine hydroxylase (*Th*), the rate-limiting enzyme in dopamine production, and dopamine transporter (*Slc6a3*), but not receptors. Striatal dopamine is a well-established target of PCBs (Bell, 2014) and these data indicate that the hypothalamic population is also sensitive. PCB exposed male and female animals also showed a reduction in expression of *Slc6a4*, the gene encoding the serotonin transporter, adding to the list of mechansism by which PCBs can affect serotonergic regulation (Boix and Cauli, 2012). In contrast to PCB-responsive amines, the opioid system appears to be less sensitive to PCBs, with the only effects being a slight decrease in *Pomc* expression in females. Instead, this system responded to LPS, as expected from our understanding of endogenous opioids role in inflammation (Hua, 2016).

## Questions for continued study

Whether PCBs act peripherally and/or centrally is an open question. PCBs do accumulate in lipophilic brain tissue, and could therefore have direct actions at hypothalamic receptors. Of interest is the concept that PCBs acutely alter the permeability of the blood brain barrier in adult male mice (Seelbach et al., 2010; Choi et al., 2012), thus increasing effects of peripheral inflammatory signals in the brain. As such, PCBs could indirectly affect hypothalamic gene expression by affecting peripheral signals which then reach brain to induce slightly different responses to LPS. This possibility is supported by the clustering of serum IL-6 and hypothalamic *Esr2* in females (Figure 9, clusters A and B) and serum IL-1b and hypothalamic *Tnf* in males (Figure 10, cluster E). While these correlations certainly do

not indicate a causal relationship between serum cytokine levels and neural gene expression, they do indicate potential targets for continued investigation of sex-specific PCB effects.

Effects of PCBs were observed in only a small portion of targets assessed, which could indicate a limited and specific effect of PCBs on inflammation. Alternatively, it could be a result of our experimental design, as PCB effects might be localized to a subset of specific cell types (Loram et al., 2012) or subregions within the hypothalamus (Pintado et al., 2011), or become detectable later post-LPS injection. The 3.5 hour delay between LPS challenge and tissue collection was chosen as a compromise between the time when the effects of LPS on gene expression peaks in the rodent brain between neonatal, adolescent, and adult animals (Ortega et al., 2011), as these ages were assessed in a separate study. Ongoing studies using immunohistochemistry are seeking to determine if the effects in gene expression translate to alterations in protein levels, and numbers and localization of cells expressing those proteins in the brain.

Overall, this study demonstrated that neonatal exposure to PCBs can induce limited, but potentially important, basal and LPS challenge-induced alterations in the brain and body's inflammatory response. These findings reinforce the concept that the study of exposure to chemicals in isolation, without additional environmental challenges, may be insufficient to assess their full effects (Desaulniers et al., 2013). Early life inflammation, either *in utero* or postnatally, is associated with neurological disorders and other adverse long-term consequences (Nawa and Takei, 2006; Fatemi and Folsom, 2009). Therefore, understanding effects of PCBs during this early developmental period is essential in appreciating potential long-lasting effects into adulthood in accordance with the concept of developmental origins of health and disease (Barker, 1990).

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

## Acknowledgements

The authors wish to thank Spurthi Tarugu, Tejaswi Marri, Michael Reilly, Dr. Weiling Yin, Dr. Alexandra Garcia and Lindsay Thompson for their assistance with tissue collection and molecular work.

Funding Information

This work was supported by the National Institutes of Environmental Health and Science [R01 ES020662 and R01 ES023254 to ACG; T32 ES07247 and F32 ES023291 to MRB] and DePaul University to MRB.

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## Highlights

- Perinatal polychlorinated biphenyls (PCBs) alter neonatal hypothalamic gene expression
- PCBs reduce expression of dopamine-related targets in males and females
- PCBs alter serum cytokine responses to inflammatory LPS differently between sexes
- PCBs increase expression of selected neuroimmune signals independent of LPS effect

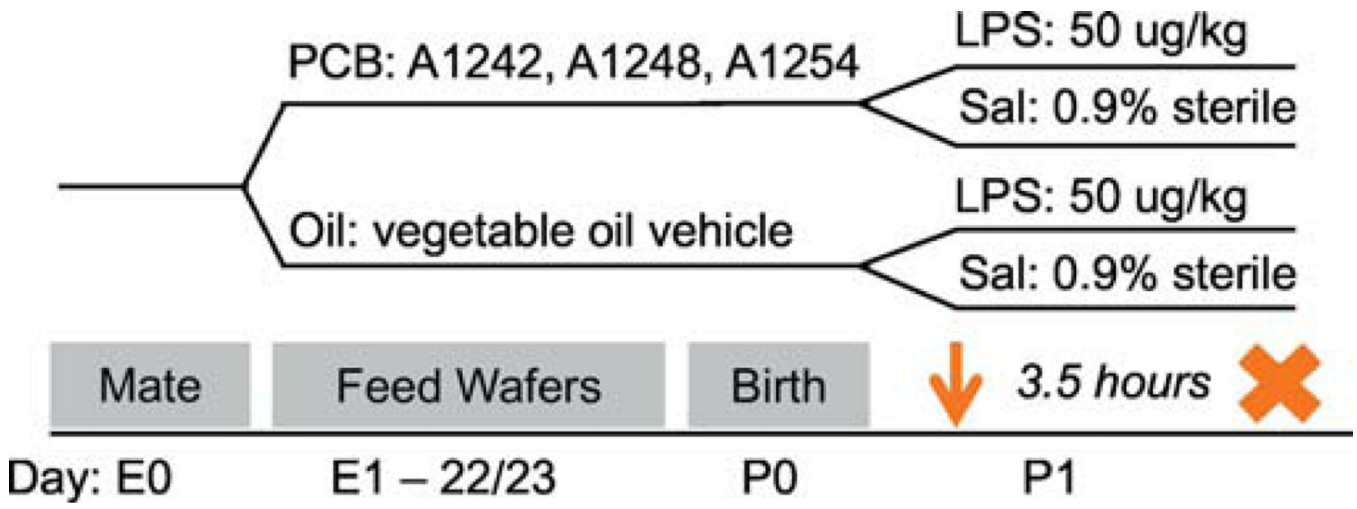


**Figure 1.**

Experimental Design. Pregnant dams were fed pieces of wafers treated with ~100ul of either Oil (n=12) or PCBs (n = 11) throughout their pregnancy, embryonic day (E)1 to E22 or 23. One male and one female from each litter was injected (i.p.) with lipopolysaccharide (LPS) or saline (Sal) on postnatal day (P) 1 and brains were collected 3.5 hours later.

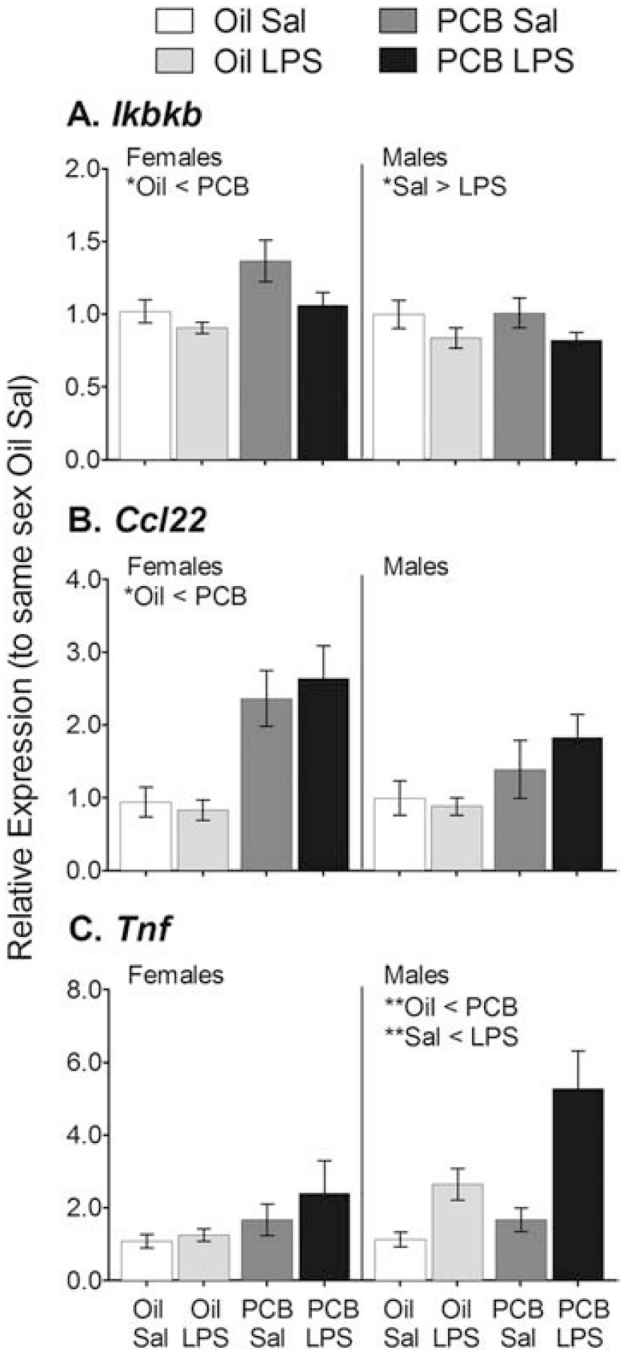


**Figure 2.**

PCBs increased gene expression of inflammatory molecules in the hypothalamus. Expression of *Ikbkb* in females (A), *Ccl22* in females (B), and *Tnf* in males (C); were all greater in PCB than Oil exposed rats. LPS also reduced expression of *Ikbkb* (A) and increased expression of *Tnf* (C) in males relative to Sal controls. Data shown are mean +/− SEM. Within-sex main effects of PCB or LPS treatment are noted in each subtitle, *$p < 0.05$ and **$p < 0.01$.

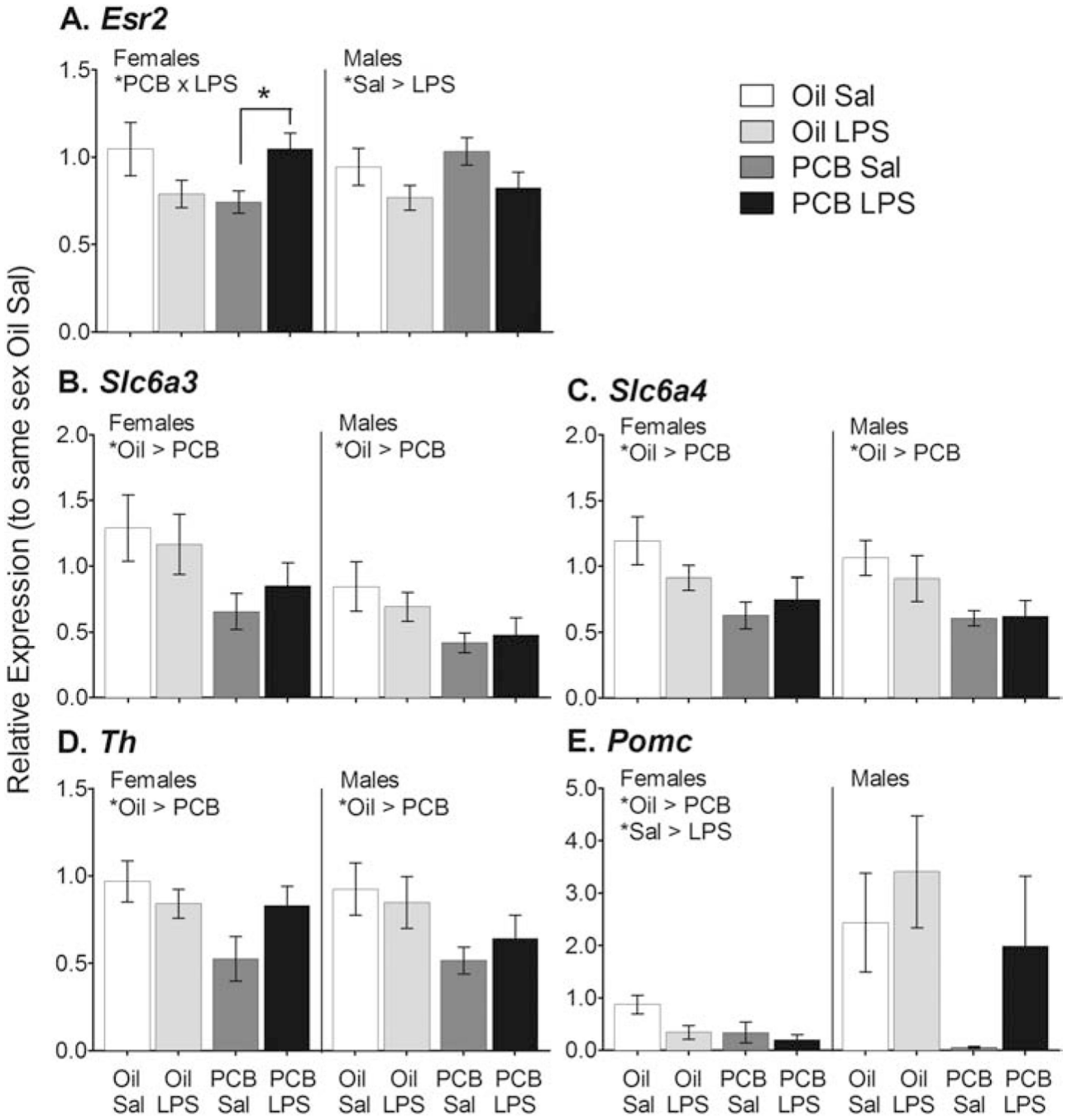


**Figure 3.**

PCBs reduced gene expression of neurotransmission modulators in the hypothalamus. PCB exposed females showed an increase in *Esr2* expression in response to LPS that was not present in controls, while LPS reduced expression in males (A). PCBs also downregulated expression of *Slc6a3* in males and females (B); *Slc6a4* in males and females (C); *Th* in females and males (D); and *Pomc* in females (E). Data shown are mean +/− SEM. Within-sex main effects of PCB or LPS treatment and PCB × LPS interaction are noted in each subtitle, *$p < 0.05$.

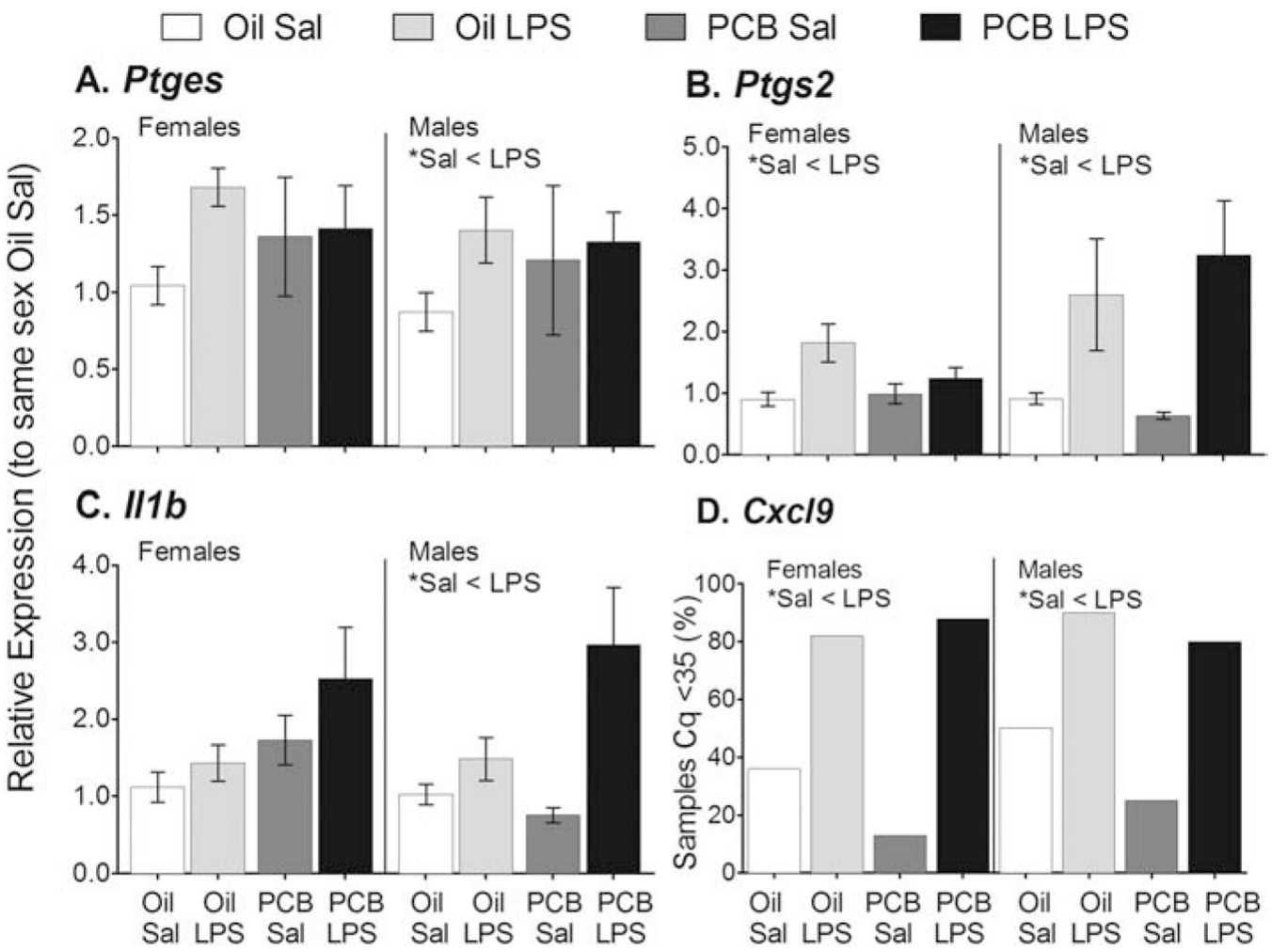


**Figure 4.**

LPS increased expression of four inflammatory molecules in the hypothalamus. Expression of *Ptges* in males (A); *Ptgs2* in females and males (B); and *Il1b* in males (C); were all greater in LPS than Sal treated rats. Data shown are mean +/− SEM. LPS exposure also increased the percent of samples with detectable levels of *Cxcl9* in males and females (D). No effects of PCBs were observed in these targets. Within-sex main effects of LPS treatment are noted in each subtitle, *$p < 0.05$.

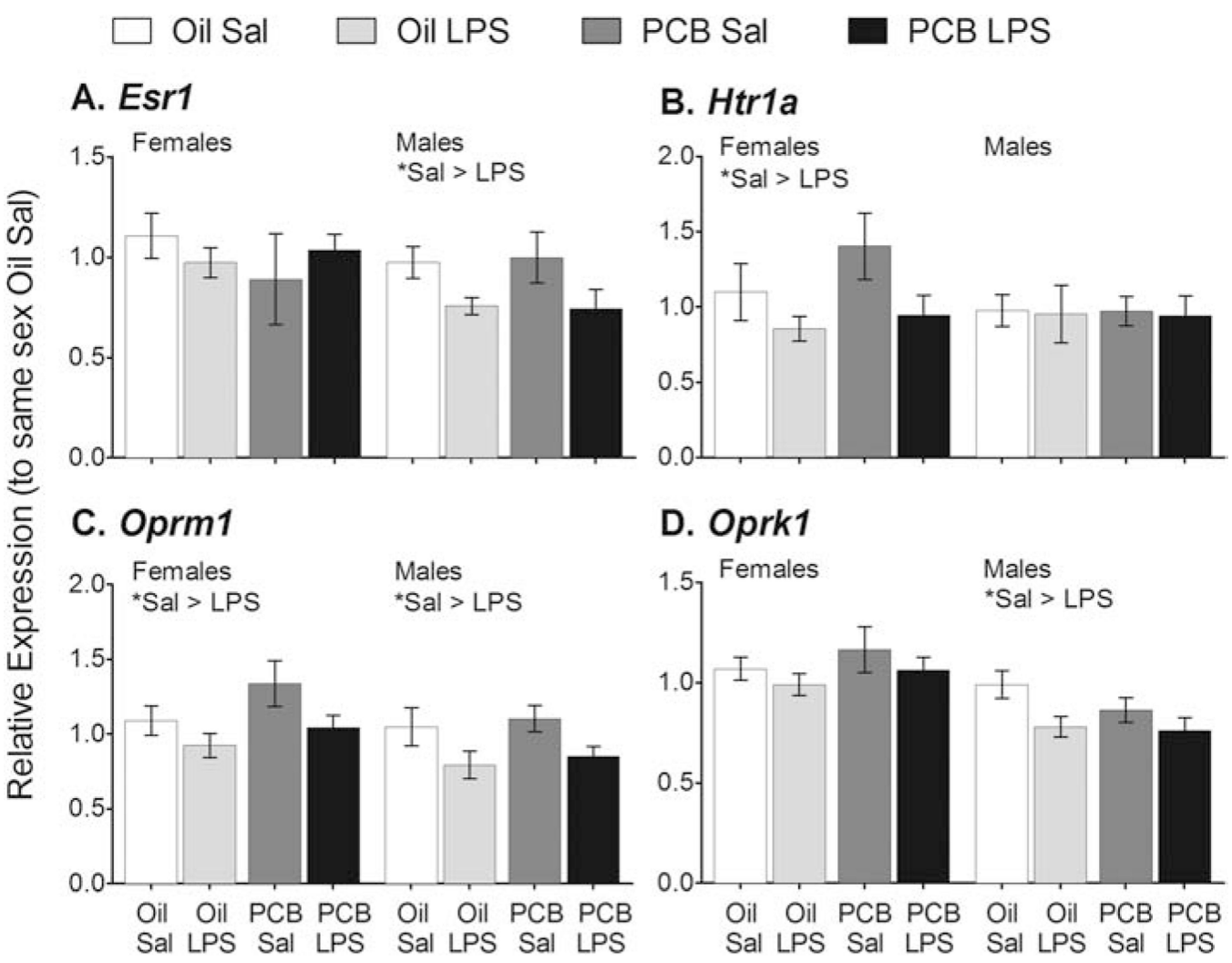


**Figure 5.**

LPS reduced gene expression of four neurotransmission modulators in the hypothalamus. Expression of *Esr1* in males (A); *Htr1a* in females (B); *Oprm1* in females and males (C); and *Oprk1* in males (D); were all lower in LPS than Sal counterparts. No effects of PCBs were detected on these targets. Data shown are mean +/− SEM. Within-sex main effects of LPS treatment are noted in each subtitle, *$p < 0.05$.

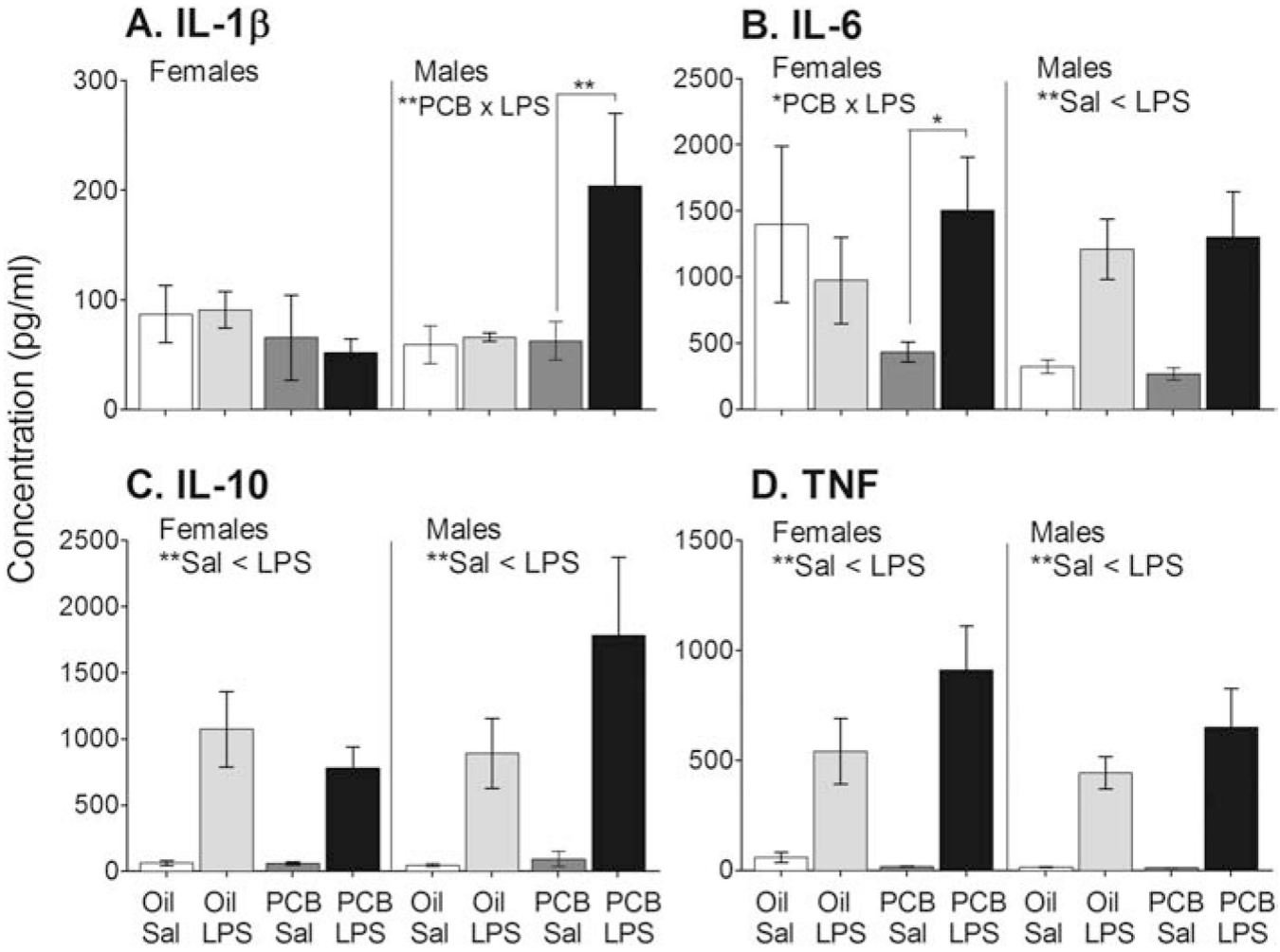


**Figure 6.**

Serum concentrations are shown for four cytokines significantly affected by treatments. LPS increased interleukin-1β in PCB exposed males only (A), and interleukin-6 in PCB exposed females only (B). A significant main effect of LPS (greater than Sal) was found for IL-6 in males (B), and for interleukin-10 (C) and tumor necrotic factor (D) in all groups. No main effects of PCBs were observed. Data shown are mean +/− SEM. Within-sex main effects of LPS treatment are noted in each subtitle, and sources of significant PCB × LPS interactions are shown by bars. *$p < 0.05$ and **$p < 0.01$.

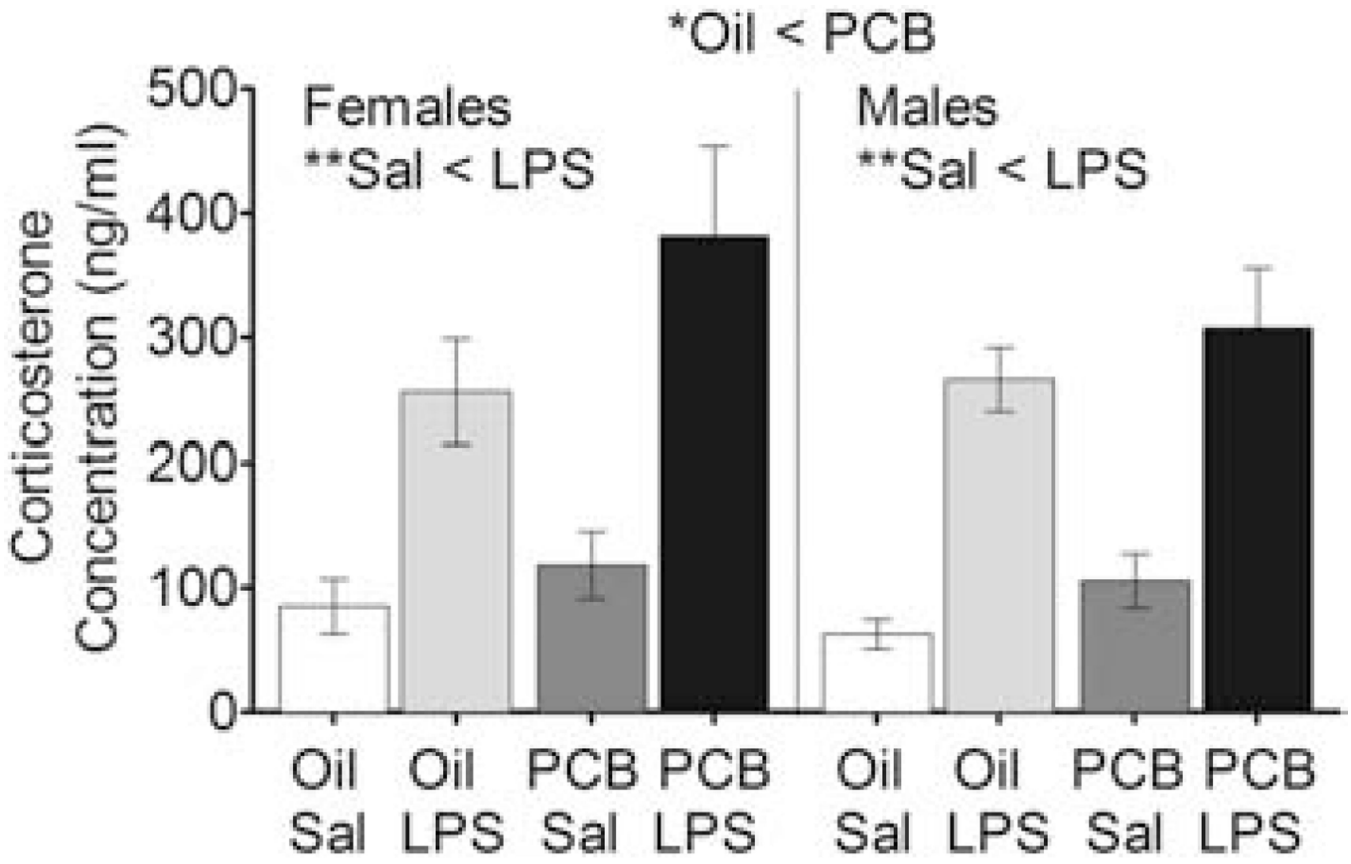


**Figure 7.**

Serum Hormone Results. A) Corticosterone was greater in LPS- than Sal-treated males and females, and in PCB- than Oil-exposed animals (but only when analyzed across sexes but not within). Data shown are mean (bar height or line) +/− SEM. Within sex main effects of PCB or LPS treatment are noted in each subtitle, *$p < 0.05$ and **$p < 0.01$.

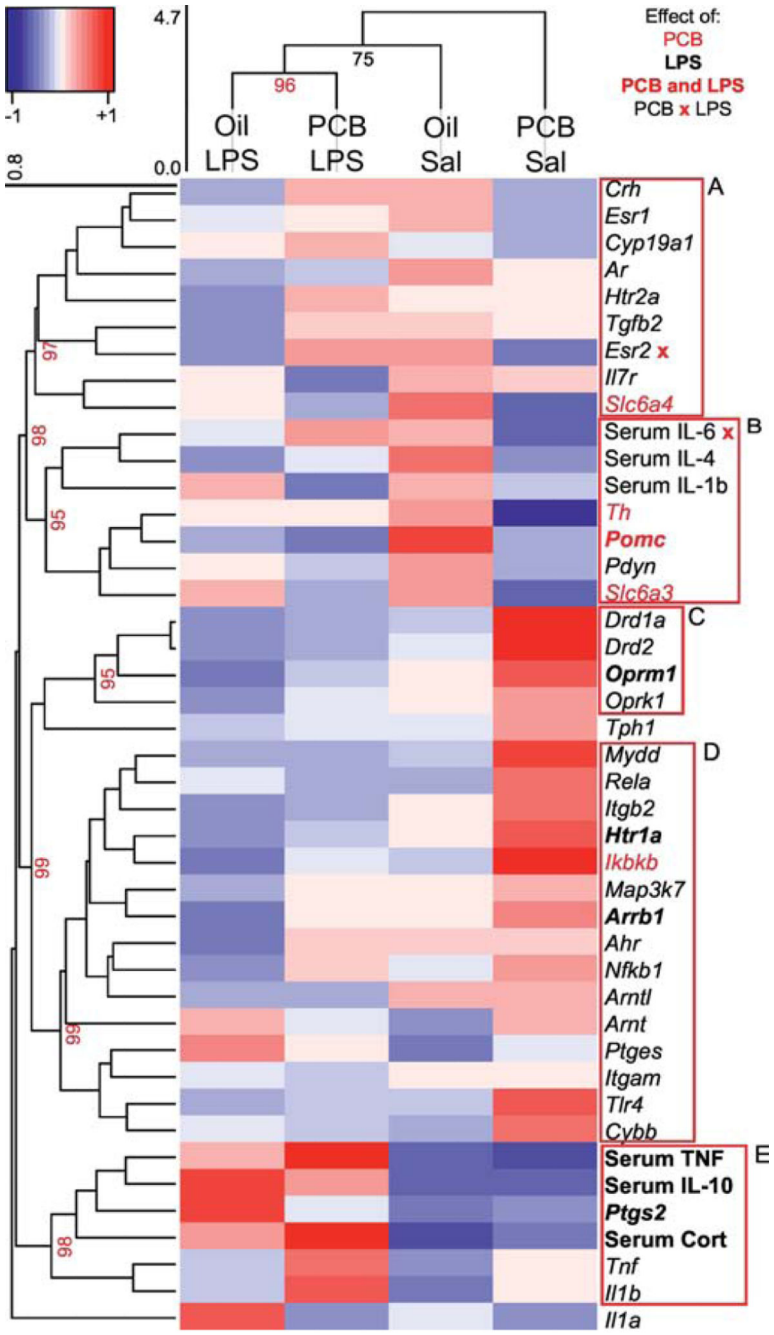


**Figure 8.**

Female Heatmap. Hierarchical cluster analysis using correlation coefficients of hypothalamic gene expression, serum hormones, and serum cytokines was used to create dendrograms showing relationships within the targets and groups. The height of the clusters indicates the distance between measures, and a p value (>95 is significant) and red box indicates validated clusters. For visual clarity, only highest tier significant target clusters are shown, and some large clusters are broken down into similarly significant and more functionally relevant groups. Dendrograms are linked to a heatmap showing z-scores of

target expression, with red indicating highest expression and blue indicating lowest. Target labels are red if targets are significantly affected by PCBs, bold if targets are significantly affected by LPS, and both red and bold if targets are affected by both PCBs and LPS. Interactions between PCB and LPS treatments on *Esr2* and Serum IL-6 are indicated by an x.

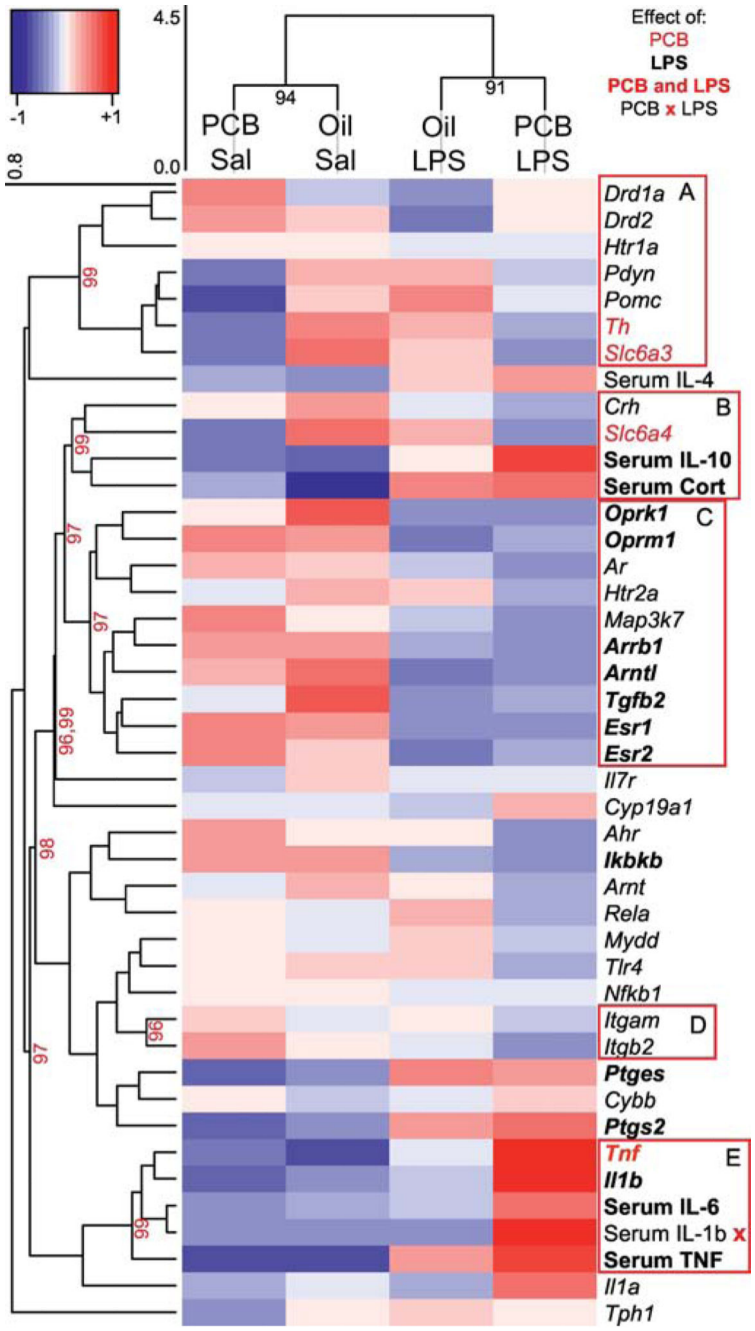


**Figure 9.**

Male Heatmap. Hierarchical cluster analysis using correlation coefficients of hypothalamic gene expression, serum hormones, and serum cytokines was used to create dendrograms showing relationships within the targets and groups. The height of the clusters indicates the distance between measures, and a p value (>95 is significant) and red box indicates validated clusters. For visual clarity, only highest tier significant target clusters are shown, and some large clusters are broken down into similarly significant and more functionally relevant groups. Dendrograms are linked to a heatmap showing z-scores of target expression, with

red indicating highest expression and blue indicating lowest. Target labels are red if targets are significantly affected by PCBs, bold if targets are significantly affected by LPS, and both red and bold if targets are affected by both PCBs and LPS. Interactions between PCB and LPS treatments on serum IL-1b are indicated by an x.

**Table 1.**

Effects of LPS or PCB exposure to increase (↑) or decrease (↓) expression of hypothalamic genes in females (left) and males (right).

| | | Female | | | Male | |
|---|---|---|---|---|---|---|
| **Gene** | **effect of:** | **LPS** | **PCB** | | **LPS** | **PCB** |
| Xenobiotic signaling | | | | | | |
| *AhR* | aryl hydrocarbon receptor | | | | | |
| *Arnt* | aryl hydrocarbon receptor nuclear translocator | | | | | |
| *Cyp1a1* | cytochrome P450, family 1, subfamily a, polypeptide 1 | | | | | |
| *Rela* | v-rel reticuloendotheliosis viral oncogene homolog A (avian) | | | | | |
| Inflammatory signaling | | | | | | |
| *Ccl22* | chemokine (C-C motif) ligand 22 | | PCB é ** | | | |
| *Cxcl9* | chemokine (C-X-C motif) ligand 9 | LPS é ** | | | LPS é ** | |
| *Cybb* | cytochrome b-245, beta polypeptide | | | | | |
| *Ifna1* | interferon-alpha 1 | | | | | |
| *Ikbkb* | inhibitor of kappa light polypeptide gene enhancer in B-cells, kinase beta | | PCB é * | | LPS ê * | |
| *Il1a* | interleukin 1 alpha | | | | | |
| *Il1b* | interleukin 1 beta | | | | LPS é ** | |
| *Il6* | interleukin 6 | | | | | |
| *Il7r* | interleukin 7 receptor | | | | | |
| *Itgam* | integrin, alpha M | | | | | |
| *Itgb2* | integrin, beta 2 | | | | | |
| *Myd88* | myeloid differentiation primary response gene 88 | | | | | |
| *Nfkb1* | nuclear factor of kappa light polypeptide gene enhancer in B-cells 1 | | | | | |
| *Ptgs2* | prostaglandin-endoperoxide synthase 2 | LPS é ** | | | LPS é ** | |
| *Ptges* | prostaglandin E synthase | | | | LPS é ** | |
| *Tlr4* | toll-like receptor 4 | | | | | |
| *Tnf* | tumor necrosis factor | | | | LPS é ** | PCB é * |
| Inflammatory modulators | | | | | | |
| *Anrtl* | aryl hydrocarbon receptor nuclear translocator-like | | | | LPS ê ** | |
| *Arrb1* | arrestin, beta 1 | | | | LPS ê * | |
| *Map3k7* | mitogen activated protein kinase kinase kinase 7 | | | | | |
| *Tgfb2* | transforming growth factor, beta 2 | | | | LPS ê * | |
| Hormones, enzyme, and receptors | | | | | | |
| *Ar* | androgen receptor | | | | | |
| *Crh* | corticotropin releasing hormone | | | | | |
| *Cyp19a1* | cytochrome P450, family 19, subfamily a, polypeptide 1 | | | | | |
| *Esr1* | estrogen receptor 1 | | | | LPS ê * | |

| | | Female | | | Male | |
|---|---|---|---|---|---|---|
| **Gene** | **effect of:** | **LPS** | **PCB** | | **LPS** | **PCB** |
| *Esr2* | estrogen receptor 2 | PCB × LPS * | | | LPS ↑ * | |
| Opioid precursors and receptors | | | | | | |
| *Oprk1* | opioid receptor, kappa 1 | | | | LPS ↑ * | |
| *Oprm1* | opioid receptor, mu 1 | LPS ↑ * | | | LPS ↑ * | |
| *Pdyn* | prodynorphin | | | | | |
| *Pomc* | proopiomelanocortin | LPS ↑ * | PCB ↑ * | | | |
| Dopamine enzymes, receptors, and transporter | | | | | | |
| *Drd1a* | dopamine receptor D1A | | | | | |
| *Drd2* | dopamine receptor D2 | | | | | |
| *Th* | tyrosine hydroxylase | | PCB ↑ * | | | PCB ↑ * |
| *Slc6a3* | solute carrier family 6, member 3 | | PCB ↑ * | | | PCB ↑ * |
| Serotonin enzymes, receptors, and transporter | | | | | | |
| *Htr1a* | 5-hydroxytryptamine (serotonin) receptor 1A | LPS ↑ * | | | | |
| *Htr2a* | 5-hydroxytryptamine (serotonin) receptor 2A | | | | | |
| *Ido1* | indoleamine 2,3-dioxygenase 1 | | | | | |
| *Tph1* | tryptophan hydroxylase 1 | | | | | |
| *Slc6a4* | solute carrier family 6, member 4 | | PCB ↑ * | | | PCB ↑ * |

* $p < 0.05$

** $p < 0.01$.