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Prenatal bisphenol A (BPA) exposure alters the transcriptome of the neonate rat amygdala in a sex-specific manner: a CLARITY-BPA consortium study

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Abstract

Bisphenol A (BPA) is a widely recognized endocrine disruptor prevalent in many household items. Because experimental and epidemiological data suggest links between prenatal BPA exposure and altered affective behaviors in children, even at levels below the current US FDA No Observed Adverse Effect Level (NOAEL) of 5 mg/kg body weight (bw)/day, there is concern that early life exposure may alter neurodevelopment. The current study was conducted as part of the CLARITY-BPA (Consortium Linking Academic and Regulatory Insights on BPA Toxicity) program and examined the full amygdalar transcriptome on postnatal day (PND) 1, with the hypothesis that prenatal BPA exposure would alter the expression of genes and pathways fundamental to sex-specific affective behaviors. NCTR Sprague-Dawley dams were gavaged from gestational day 6 until parturition with BPA (2.5, 25, 250, 2500, or 25000 µg/kg bw /day), a reference estrogen (0.05 or 0.5 µg ethinyl estradiol (EE₂)/kg bw/day), or vehicle. PND 1 amygdalae were microdissected and gene expression was assessed with qRT-PCR (all exposure groups) and RNAseq (vehicle, 25 and 250 µg BPA, and 0.5 µg EE₂ groups only). Our results demonstrate that that prenatal BPA exposure can disrupt the transcriptome of the neonate amygdala, at doses below the FDA NOAEL, in a sex-specific manner and indicate that the female amygdala may be more sensitive to BPA exposure during fetal development. We also provide additional evidence that developmental BPA exposure can interfere with estrogen, oxytocin, and vasopressin signaling pathways in the developing brain and alter signaling pathways critical for synaptic organization and transmission.

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Keywords

endocrine disruptor; EDC; brain; oxytocin; sex difference; estrogen; androgen

1. INTRODUCTION

Bisphenol A (BPA) is a widely recognized endocrine disruptor and ubiquitous environmental contaminant prevalent in many household items including food and beverage containers, medical equipment, plastic water pipes, and thermal receipt paper. In industrialized countries greater than 90% of individuals have detectable levels of BPA in their bodies, with exposure occurring primarily through diet [1–5]. Additionally, levels of BPA have been detected in placental tissue, amniotic fluid, and maternal and fetal plasma [6–8], which is of particular concern because it is well-established that exposure to chemicals during the critical period of fetal brain development can cause long-term impairments to brain function [9]. Moreover, throughout this period of rapid growth, the blood-brain barrier is immature and provides limited protection against neurotoxic and neuroendocrine disrupting agents [10, 11]. Here we extended on prior work in the hypothalamus and hippocampus [12], conducted as part of a uniquely constructed research consortium, to test the hypothesis that prenatal BPA exposure produces sex-specific transcriptomic changes in the neonatal rat amygdala.

Animal and human data suggest that early-life BPA exposure may disrupt neurodevelopmental processes and contribute to, at least in part, the increasing incidence of sex-biased neurobehavioral and mood disorders [13, 14]. Extensive experimental and epidemiological evidence supports associations between developmental BPA exposure and sex-specific socioemotional behavioral outcomes including hyperactivity, anxiety, aggression, and cognitive deficits, even at doses below the current US Food and Drug Administration No Observed Adverse Effect Level (NOAEL) of 5 mg/kg body weight (bw)/day [13, 15–21]. Furthermore, a published report by the Food and Agriculture Organization of the United Nations and the World Health Organization identified “changes in anxiety and convergence of anatomical brain sex differences” as a potential human-relevant health risk of developmental BPA exposure [22]. The mammalian amygdala plays an integral part in the regulation of socioemotional behaviors, particularly those related to anxiety and fear [23–27], and is thus a conceivable target of prenatal BPA exposure.

Previous work has revealed that early-life exposure to low doses of BPA can induce structural, molecular and functional changes in the amygdala that are associated with altered behaviors [28]. For example, perinatal BPA exposure was found to alter synaptic transmission and plasticity in the basolateral amygdala of juvenile rats [29]. An additional study found that prenatal and lactational exposure to BPA disrupted levels of the neurotransmitters GABA and glutamate in the amygdala of adult mice in a sex-specific manner [30]. Moreover, studies from our group and others have observed modified expression of genes encoding DNA methyltransferase 1 [31], estrogen receptors (ERs) [15], AMPA and NMDA receptor subunits [32], and vasopressin [33], in juvenile and adult rats and mice developmentally exposed to BPA.

Although available literature suggests the developing amygdala is vulnerable to BPA disruption, very little is known regarding the impact of exclusively prenatal exposure on gene expression in the amygdala and, to our knowledge, no one has assessed the effects on BPA on the amygdalar transcriptome. The present studies extend prior work by examining the full transcriptome in the amygdala on postnatal day (PND) 1, with the hypothesis that prenatal BPA exposure alters the expression of genes and pathways fundamental to sex-specific socioemotional behaviors including anxiety.

Because few published studies are designed for the specific purpose of informing human risk assessment and thus fail to meet the strict criteria for inclusion, recent reviews of the BPA literature by regulatory agencies exclude most studies from consideration and maintain the position that BPA is safe at current exposure levels (documents available for download here: <https://www.fda.gov/NewsEvents/PublicHealthFocus/ucm064437.htm>). As part of a collaborative research program known as the consortium linking academic and regulatory insights on BPA toxicity (CLARITY-BPA), the present study, and the other published and forthcoming studies encompassed in the program, fill a critical data communication gap because they were specifically designed to resolve controversies surrounding the design and interpretation of BPA toxicity studies and to be informative for risk assessment [34–37]. CLARITY-BPA studies incorporate research recommendations published by the WHO and others for enhancing robustness and reproducibility of endocrine disrupting chemical (EDC) studies [22, 38–41]. This includes strict use of blinding, controlling for potential litter effects, minimizing exogenous EDC exposures, oral dosing, use of a reference estrogen, and evaluation of multiple BPA doses, particularly levels at or below the FDA NOAEL. Additionally, we have ensured that our methodological and data reporting adhere to ARRIVE (Animal Research: Reporting of In Vivo Experiments) guidelines as published by the National Centre for the Replacement Refinement and Reduction of Animals in Research (NC3Rs) to maximize reproducibility and utility for systematic review.

Using tissues from a complementary, previously published study [12], a transcriptome-wide approach was used for the first time to identify genes and pathways targeted by low levels of BPA during fetal development in the amygdala. Pregnant NCTR Sprague Dawley rats (NCTR-SD) were exposed to a wide range of BPA doses (2.5, 25, 250, 2500, and 25,000 µg/kg bw/day), ethinyl estradiol (EE₂; 0.05- or 0.5-µg/kg bw/day), or vehicle from gestational day (GD) 6 to parturition through oral gavage. Quantitative real-time PCR (qRT-PCR) was used to evaluate the expression of six candidate genes preselected because of their (1) role in socioemotional behaviors, (2) sex-biased expression pattern in the amygdala, (3) sensitivity to BPA or estrogen and/or, (4) importance in sexual differentiation of the amygdala (Table 1). Additionally, RNA sequencing (RNAseq) and enrichment analysis were used to characterize the neonatal amygdala transcriptome of four exposure groups (vehicle, BPA 25, BPA 250, and 0.05 EE₂) and to probe for evidence of previously unidentified modes of action. Additional genes were also subsequently analyzed by qRT-PCR to validate the RNAseq analysis.

2. MATERIALS AND METHODS

2.1 Animal Care

Study animals were housed in an Association for Assessment and Accreditation of Laboratory Animal Care (AALAC) accredited facility. All procedures were approved in advance by the National Center for Toxicological Research Institutional Animal Care and Use Committee (NCTR-IACUC). PND 1 pups were obtained from litters produced for the CLARITY-BPA program [35, 37]. Methods for animal husbandry, diet, breeding, dose preparation and administration, and necropsy are described in detail elsewhere [34]; therefore, only relevant methods are reviewed below.

Sprague-Dawley rats from the NCTR colony (NCTR-SD strain code 23) were housed in solid-bottomed polysulfone caging with hardwood chip bedding at $23 \pm 3^{\circ}\text{C}$ with a relative humidity level of $50 \pm 20\%$ on a 12:12h light/dark cycle (0600–1800). Food (soy- and alfalfa-free diet verified casein diet 10 IF 5K96; Cat. 1810069; Purina Mills, Richmond, IN) and Millipore-filtered water in glass water bottles with silicone stoppers (#7721 clear, The Plasticoid Co., Elkton, MD) were provided for *ad libitum* consumption. Extracts of each diet lot were analyzed for BPA and myco/phytoestrogens (genistein, daidzein, zearalenone, and coumestrol) by liquid chromatography and mass spectrometry [42] and all had levels below the average analytical method blanks [34]. Drinking water, polysulfone cage leachates, and bedding extracts were also found to have BPA levels below the level of the average analytical method blanks [34].

2.2 Reagents and Dosing

The BPA (CAS # 80-05-7, catalog # B0494, TCI America, Portland, OR) and EE₂ (CAS # 57-63-6, catalog #E4876, Sigma-Aldrich, St. Louis, MO) were more than 99% pure and administered in 0.3% aqueous carboxymethyl cellulose (CMC; catalog # C5013, Sigma-Aldrich, St. Louis, MO). The EE₂ groups were included to serve as the “reference estrogen” and to determine if BPA-related effects were consistent with an estrogenic mode of action.

Two weeks before mating, dams were randomized to one of eight exposure groups stratified by body weight to produce approximately equal mean body weights in each group. Sires were randomly assigned subject to the constraint that no sibling or first cousin mating was permitted, as previously described [42]. Mating was confirmed by the presence of a sperm plug or sperm-positive vaginal cytology [defined as GD 0]. To model the exposure route used to establish the NOAEL, dams were gavaged daily with vehicle (0.3% CMC/kg bw/day), BPA (2.5, 25, 250, 2500, or 25000 µg BPA/kg bw/day), or EE₂ (0.05 or 0.5 µg EE₂/kg bw/day) from GD6 until the day of parturition [postnatal day (PND) 0]. Dams and pups were left undisturbed on PND0. On PND 1, pups (one per sex per litter) were weighed and euthanized by rapid decapitation. Heads were collected, snap frozen, and shipped coded (blinded) to the Patisaul lab where they were stored at -80°C until processing.

2.3 Tissue Collection and Preparation

Each whole head was coronally cryosectioned (Leica CM1900, Nussloch, Germany) from the caudal end until the caudal borders of the amygdala were identified. Two sequential

bilateral punches, each 1.00 mm in diameter and 1.00 mm in depth, were collected caudally to rostrally; this corresponded with plates 69–75 of the Atlas of the Developing Mouse Brain [43] (Supplemental Fig. 1). All four punches, which collectively comprised the entire amygdala, were combined and stored in BPA-free Eppendorf tubes at -80°C . These punches were collected at the same time we collected hypothalamic and hippocampal studies for a prior, published study [12] with the intention of performing the amygdalar assessment as a follow-up (secondary analysis) if any significant observations were found in the other brain regions. The outcomes of that prior study informed the selection of the dose groups and primary genes of interest for the present study.

2.4 Quantitative real-time PCR

Analysis was performed on eight exposure groups ($n = 5\text{--}7$ for the predetermined genes, $n = 3\text{--}7$ for the validation genes; sample size based on availability of cDNA): vehicle, BPA 2.5, 25, 250, 2500, and 25000 and EE₂ 0.05 and 0.5. Total RNA was extracted with the Qiagen RNEasy Miniprep kit. An Agilent 2100 Bioanalyzer with an RNA 6000 Nano Chip was used to determine RNA purity and concentration and each sample had a RIN of 10. Single-stranded cDNA synthesis was performed with 350 ng of RNA input using the high capacity RNA-to-cDNA kit (Applied Biosystems, Cat. 4387406) and samples were stored at -20°C until use. qRT-PCR was performed as previously published [12] using a TaqMan probe-based protocol and detected on a StepOnePlus™ Real-Time PCR System (Applied Biosystems, Life Technologies, Grand Island, NY) with the following cycling parameters: 50°C for 2 min, 95°C for 10 min, followed by 40 cycles of 95°C for 15 sec and 60°C for 1 min. Each sample was run in triplicates the sample maximization approach was followed to avoid technical and run-to-run variation [44]. Cycle threshold (Ct) values for the gene of interest were normalized to the Ct for 18s rRNA and relative data were determined by the Livak cycle threshold ($2^{-\Delta\text{Ct}}$) method [45].

2.5 RNAseq Data Analysis

The experimental design for transcriptome sequencing was developed in consultation with the NCSU Genomic Sciences Laboratory (GSL). Transcriptome sequencing was performed by the GSL on 24 amygdala samples ($n = 3$ per sex per group). Four experimental groups were examined: vehicle, 25 BPA, 250 BPA, and 0.5EE₂. RNA extraction was performed with the Qiagen RNEasy Miniprep kit according to the manufacturer protocol (Qiagen, Cat. 74134). Total RNA samples were submitted to the North Carolina State Genomic Sciences Laboratory for Illumina RNA library construction and sequencing. Prior to library construction, RNA integrity, purity, and concentration were assessed using an Agilent 2100 Bioanalyzer with an RNA 6000 Nano Chip (Agilent Technologies, USA). All samples had an RNA integrity number (RIN) of 10. To optimize library complexity, only samples that had greater than 300 ng of total RNA were used as input material for library preparation.

As previously described [12], messenger RNA (mRNA) was purified using the oligo-dT beads provided in the NEBNext Poly (A) mRNA Magnetic Isolation Module (New England Biolabs, Cat. E7490). Complementary DNA (cDNA) libraries for Illumina sequencing were prepared with the NEBNext Ultra Directional RNA Library Prep Kit and the NEBNext Multiplex Oligos (New England Biolabs, Cat. E7420 and E7335). Briefly, mRNA was

isolated, heat fragmented, and primed with random oligos for first strand cDNA synthesis. Second strand cDNA synthesis was performed with dUTPs to preserve strand orientation information. Next, the double-stranded cDNA fragments were purified using AMPure XP beads (Beckman Coulter Genomics, Cat. A63881), end-repaired, and ligated onto adaptors specifically designed for the Illumina platform. Following ligation, the samples were size-selected to a final library size of 400–550 bp (adapters included) using sequential AMPure XP bead isolation. Protocol-specified PCR amplification was performed to enrich adaptor-ligated cDNA and add specific indexes for each sample. The amplified library fragments were purified and quality and final concentration was assessed using an Agilent 2200 TapeStation. The final quantified cDNA libraries were pooled into equimolar amounts for clustering and sequencing on an Illumina HiSeq 2500 DNA sequencer (4 lanes), utilizing a 125 bp single end sequencing reagent kit (Illumina, USA). Approximately 37.5 million reads were generated per sample. The software package Real Time Analysis (RTA), was used to generate raw bcl, or base call files, which were then de-multiplexed by sample into fastq files for data submission.

Data analysis for RNAseq was performed in consultation with the Bioinformatics Core of the NCSU Center for Human Health and Environment. Sequence data was evaluated with FastQC and 12 poor quality bases were trimmed from the 5'-end. The good quality reads were aligned to the *Rattus norvegicus* (rn6) reference genome (downloaded from UCSC) using the STAR software package [46]. For each replicate, per-gene counts of uniquely mapped reads were calculated using htseq-count script from the HTSeq python package [47]. Count data were normalized for sequencing depth and distortion, and dispersion was estimated using the DESeq2 Bioconductor [48, 49] package in the R statistical computing environment. We fit a leaner model using treatment levels and differentially expressed genes were identified after applying multiple testing corrections using the Benjamini-Hochberg procedure ($\text{padj} < 0.05$) [46]. Lastly, canonical pathway-based functional analyses of transcriptomic datasets, with an adjusted p-value (padj) less than 0.05, were performed with Ingenuity Pathway Analysis (IPA; QIAGEN). To identify relevant pathways, IPA core analysis was initially filtered to only include annotations made in neurons, astrocytes, or the amygdala. Then the analysis was then re-run to evaluate the robustness and relevance of the main findings in the context of a more global analysis by including annotations made in all tissues types and cell-lines. Associated canonical pathways were generated with the negative log probability of a particular network being enriched due to random chance [$-\log(\text{p-value})$] and the p-values were calculated using a right-tailed Fisher's exact tests.

2.6 Data Blinding and Statistical Analysis

All dosing and related work conducted at NCTR was conducted by investigators blinded to exposure as described in detail in Heindel et al. (2015). The brains were then given a unique identifier and grouped by letter (group A, B, C etc.) and sent to NCSU so that analysis could be done blinded to exposure group and sex. All tissue micropunching was conducted blinded and the samples stored at -80°C until the prior, related study was completed and published [12] (which necessitated unblinding). Because the present study was considered a secondary, follow-up study, data from the prior study was critically necessary to inform the present one but the experimental design was conceived and developed under unblinded conditions. Once

the groups for RNAseq were selected, the individual samples were selected at random by an investigator blinded to individual and group ID, and all RNAseq work and bioinformatics was conducted blinded. Because qRT-PCR used all remaining samples (not all samples had sufficient cDNA for analysis), that work could not be done fully blinded, but to minimize risk of bias all of the individual samples were randomized across plates.

The statistical approach was developed to be consistent with previously published transcriptome projects of similar scale (equivalent sample size or smaller) in rat brain [12, 47] and guidelines for low dose endocrine disrupting chemical (EDC) studies [48]. Within each exposure group, no same-sex littermates were included, so potential litter effects did not need to be statistically accounted for.

qRT-PCR—Statistical analysis for all of the data was performed and graphed using Prism version 7 (GraphPad Software, Inc., La Jolla, CA). For each gene of interest, a Grubb's test for outliers ($\alpha = 0.05$) was conducted and up to one outlier per group was removed. In total, only two outliers were removed from all of the qRT-PCR data: a 2.5 BPA male and a vehicle female from the vasopressin receptor (*Avpr1a*) analysis. Next, a two-tailed Mann-Whitney U test was used to determine if any sex differences in gene expression were detected in the unexposed controls. Finally, a two-tailed Mann-Whitney U test was used to compare each exposure group to the same-sex vehicle control. When BPA-related effects were found, qualitative comparisons to the EE₂ groups were made to see if directionality was consistent with an “estrogenic” effect. In all cases, effects were considered significant at $p \leq 0.05$.

RNAseq—The DESeq2 package was used to fit a negative binomial model for each gene using an extended model matrix. To identify baseline sex differences in gene expression, the male and female controls were compared independent of other exposure groups. With that exception, all other data were compared within sex. For each comparison, the p-value was adjusted for multiple testing using the Benjamini-Hochberg method at a false discovery rate of 5% [46]. A cutoff of $\text{padj} \leq 0.05$ was used to select differentially expressed transcripts and genes.

3. RESULTS

3.1 Impact of prenatal BPA or EE₂ exposure on specific genes of interest in the PND 1 amygdala

Based on our a priori hypotheses and prior publications [12], specific genes of interest were selected for analysis by qRT-PCR because of their (1) importance in socioemotional behaviors; (2) role in sexual differentiation; (3) sensitivity to BPA or estrogen; and/or (4) sex-biased expression pattern in the PND 1 amygdala (Table 1). Five of the six genes were significantly altered by prenatal BPA or EE₂ exposure and a summary of the descriptive statistics for significant outcomes is listed in Table 2.

ER α (*Esr1*) expression levels in the amygdala were not affected by BPA or EE₂ in either sex (Fig. 1A). In males, ER β (*Esr2*) expression was significantly upregulated in the BPA 25 groups, whereas in females, *Esr2* expression was upregulated in BPA 250 group (Fig. 1B). In males, oxytocin receptor (*Oxtr*) expression levels were significantly increased in the BPA 25

and BPA 250 groups. In females, higher *Oxtr* expression was observed in the BPA 2.5, BPA 25, BPA 250, BPA 25000, and EE₂ 0.5 groups (Fig. 1C). Prenatal BPA exposure had sex-specific effects on *Avpr1a*. In males, expression was downregulated in the BPA 2.5 groups and upregulated in the BPA 25 and BPA 250 groups. Male *Avpr1a* expression was also increased in the EE₂ 0.5 group. Conversely, no significant effects of prenatal BPA or EE₂ were observed on *Avpr1a* among females (Fig. 1D). Androgen receptor (*Ar*) expression was significantly upregulated in the BPA 25 groups in males, whereas in females, *Ar* expression in the BPA 250 and EE₂ 0.5 groups was upregulated (Fig. 1E). There were no significant effects of BPA on growth arrest and DNA damage-inducible beta (*Gadd45b*) expression in either males or females. In females, *Gadd45b* expression was upregulated in the EE₂ 0.5 group (Fig. 1F).

3.2 Impact of prenatal BPA or EE₂ exposure on the PND 1 amygdalar transcriptome

RNAseq transcriptome profiling was evaluated in animals exposed to vehicle, 25 or 250 µg BPA/kg bw/day, and 0.5 µg EE₂/kg bw/day. These doses were selected because they cover the lower range of exposures at which BPA-induced effects have been reported in the scientific literature, including other CLARITY-BPA studies, and are below the current FDA NOAEL of 5 mg/kg bw/day. Unsupervised principal component analysis of all gene expression profiles did not reveal a clear separation between exposure groups (Supplementary Fig. 2A). When PCA was performed within sex, female exposure groups clustered more distinctly than males (Supplementary Fig. 2B). Thus all subsequent analyses were conducted within sex. With the exception of one female 0.5 EE₂ replicate, samples treated with BPA and EE₂ clustered together and were clearly distinct from the female vehicle control samples (Supplementary Fig. 2C).

When compared to the same-sex vehicle group, males exposed to 25 µg BPA showed more significant transcript changes than males exposed to 250 µg BPA (89 genes and 1 gene, respectively; Supplementary Table 1A–B). IPA identified (1) STAT activation (3 genes, $p = 0.01$); (2) Wnt/β-catenin signaling (4 genes, $p = 0.01$); (3) RAR activation (4 genes, $p = 0.01$); (4) corticotrophin releasing hormone (CRH) signaling (3 genes, $p = 0.01$); and PTEN signaling (3 genes, $p = 0.01$) as the top five most significantly enriched canonical pathways by the male 25 BPA dataset (Fig. 2A). Only 52 genes were significantly altered by 0.5 µg EE₂ in the male amygdala (Supplementary Table 1C), however a large portion of these genes (35/52) overlapped with the set of genes significantly altered in the male BPA 25 group (Fig. 3A). (1) GABA receptor signaling (2 genes, $p = 0.01$); (2) protein ubiquitination pathway (3 genes, $p = 0.02$); (3) CRH signaling (2 genes, $p = 0.04$); and (4) DNA methylation and transcriptional repression signaling (1 gene, $p = 0.045$) were the only pathways significantly enriched by the differentially expressed genes in the male 0.05 EE₂ dataset (Fig. 2A).

BPA- and EE₂- related transcriptional changes were more numerous in females than males. In total 251 and 341 genes were differentially expressed in the 25 BPA and 250 BPA groups, respectively, with robust overlap between the two dose groups (174 genes) (Supplementary Table 2A–B and Fig. 3B). The top scoring canonical pathways enriched with genes perturbed by 25 µg BPA were: (1) G protein coupled receptor (GPCR) signaling (17 genes, $p = 0.001$); (2) GABA signaling (8 genes, $p = 0.001$); (3) CREB signaling in neurons (12

genes, $p = 0.001$); (4) dopamine-DARPP32 feedback in cAMP signaling (11 genes, $p = 0.001$); and (5) CRH signaling (9 genes, $p = 0.001$). IPA identified (1) GPCR signaling (17 genes, $p = 0.001$); (2) neuregulin signaling (10 genes, $p = 0.001$); (3) synaptic long term potentiation (11 genes, $p = 0.001$); (4) GABA receptor signaling (8 genes, $p = 0.001$); and (5) bone morphogenetic protein (BMP) signaling (9 genes, $p = 0.001$) as the top scoring pathways enriched by the differentially expressed genes in the female 250 BPA dataset (Fig. 2B). Finally, the most robust effects of prenatal exposure were observed in the 0.5 EE₂ group in which 629 genes were significantly altered (Fig. 3B and Supplementary Table 2C). The five top scoring results from the IPA pathway analysis were (1) GPCR signaling (24 genes, $p = 0.001$); (2) synaptic long term potentiation (15 genes, $p = 0.001$); (3) dopamine-DARPP32 feedback in cAMP signaling (17 genes, $p = 0.001$); (4) neuregulin signaling (12 genes, $p = 0.001$); and (5) CRH signaling (13 genes, $p = 0.001$) (Fig. 2B).

3.3 Validation of differentially expressed genes of interest identified by transcriptomics in the PND 1 amygdala

qRT-PCR was used to follow up, in all exposure groups, on two differentially expressed genes identified by RNAseq. We focused on metabotropic glutamate receptor mGluR 5 (*Grm5*) and calmodulin-dependent protein kinase type IV (*Camk4*) because they modulate neurodevelopment [70], synaptic transmission, glutamate receptor signaling, CREB signaling, cAMP-mediated signaling, and other canonical pathways identified as significantly enriched in the female 25 and 250 BPA groups (Table 3). In males, *Camk4* was upregulated in the 25 BPA group. In females, higher *Camk4* expression was observed in the BPA 2.5, BPA 25, BPA 250, BPA 25000, EE₂ 0.05 and EE₂ 0.5 groups and there was a trend for upregulation in the BPA 2500 group (Fig. 1G and Table 2). *Grm5* expression was significantly increased in the male BPA 25 BPA. In females, *Grm5* expression was increased in the BPA 250, BPA 2500, BPA 25000, EE₂ 0.05, and EE₂ 0.05 groups and there was a trend for upregulation observed in the BPA 25 group (Fig. 1H and Table 2).

3.4 Sex differences in PND 1 amygdala gene expression

RNAseq identified relatively few sex differences in overall gene expression in the PND 1 amygdala and no genes were expressed exclusively in one sex or the other (Supplementary Table 3). Only nine genes had sex differences in expression, with four being more highly expressed in males and five being more highly expressed in females. Three of the four genes that were more highly expressed in males were Y-linked: eukaryotic translation initiation factor 2 subunit 3 (*Eif2s3y*), lysine demethylase 5D (*Kdm5d*), and DEAD (Asp-Glu-Ala-Asp) box polypeptide 3 (*Ddx3*). Among all of the genes identified as differentially expressed between unexposed males and females, *Eif2s3y* exhibited the largest fold change (−11.27). As shown in Fig. 4A–F, qRT-PCR identified no sex differences in genes of interest selected a priori (*Esr1*, *Esr2*, *Oxtr*, *Avpr1a*, *Ar*, and *Gadd45b*) or *Grm5* (Fig. 4H), although there was a nonsignificant trend for lower female expression of *Oxtr* ($p = 0.08$; Table 2). Expression of *Camk4*, however, was significantly higher in males than females (Fig. 4G and Table 2).

4. DISCUSSION

The results from the present study demonstrate for the first time that prenatal BPA exposure, at doses below the current FDA NOAEL, can alter the transcriptome of the neonate amygdala. Additionally, these data are consistent with, and provide further evidence that developmental BPA exposure can interfere with estrogen, oxytocin and vasopressin signaling pathways in the developing brain. Transcriptome profiling revealed sex-specific effects of prenatal BPA exposure with evidence of altered GPCR-signaling, CREB-signaling, synaptic plasticity, and pathways related to nervous system growth and development in the female amygdala. While the functional and physiological significance of these gene expression changes within the neonate amygdala remain unclear, the present data yield further insight into the mechanisms by which BPA may influence socioemotional behaviors in a sex-specific manner. In addition, the present data contribute important fundamental information regarding sex-specific gene expression patterns in the developing brain as, to our knowledge, this is the first report of sex differences in the full amygdala transcriptome at PND 1.

In the PND 1 amygdala, expression of *Oxtr* and *Avpr1a* was disrupted by prenatal BPA exposure in a sex- and dose- dependent manner. In females, *Oxtr* expression was significantly upregulated by prenatal BPA exposure (2.5, 25, 250, and 25,000 µg) and there was a trend ($p = 0.08$) for upregulation at the 2,500 µg dose. In males, *Oxtr* was also significantly upregulated by prenatal exposure to 25 and 250 µg BPA. Amygdalar *Avpr1a* expression was downregulated by the lowest dose of BPA (2.5 µg) and upregulated by 25 and 250 µg BPA in males but female levels were unaffected. These data are consistent with prior studies reporting BPA-related disruption of oxytocin (OXT) and vasopressin (AVP) systems in the brains of juvenile and adult rodents ([12, 15, 20, 71, 72]; reviewed in [58]). For example, perinatal BPA exposure to BPA via drinking water (1 mg/L) resulted in heightened anxiety-related behaviors in juvenile Wistar rats that were concomitant with decreased amygdalar expression of genes, including *Esr2*, crucial for the production and release of OXT and AVP in the paraventricular nucleus of the hypothalamus [20]. The neuropeptides OXT and AVP are powerful mediators of social behaviors including affiliation, anxiety, and stress regulation [56, 73–75]. In adult rodents, *Oxtr* and *Avpr1a*, are robustly expressed throughout the brain and have been found in several amygdalar subnuclei, including the central nucleus and medial amygdala [57]. Their receptors are also present during development and both can be detected in the fetal rat brain as early as embryonic day 12 [76]. Numerous studies in rodents and non-human primates clearly demonstrate that changes in the OXT or AVP system during early life can permanently alter the brain and behavior [69, 77–79]. This converging evidence points to a possible organizational role of OXT and AVP, where their activity during critical periods of brain development may program later social behavior. Overall, our data adds to the growing body of literature indicating BPA-related disruption of the organization of OXT and AVP systems in the brain. While the functional significance of the transcriptional data reported here remains to be established, sex-specific disruption of *Oxtr* and *Avpr1a* in the developing amygdala likely contribute to the sex-specific behavioral changes attributed to early-life BPA exposure, including anxiety-related behaviors.

A surprising outcome of this study was the minimal effect of prenatal BPA exposure on ER expression. *Esr2* expression was increased in males and females prenatally exposed to 25 and 250 µg of BPA, respectively, but *Esr1* expression was unaffected by prenatal BPA exposure in both sexes. Early-life BPA exposure has repeatedly been shown to disrupt levels of ER expression in the hypothalamus and limbic nuclei of neonatal and older rodents [12, 21, 61, 62, 65]. To our knowledge, however, only two studies to date have explored the impact of early-life BPA exposure on ER mRNA in the developing amygdala and both found sex-specific increases in *Esr1* and *Esr2* expression, with effects on *Esr2* being more robust [21, 65]. Notably, the magnitude of effects varied among amygdalar subnuclei, suggesting that discrepancies between these prior studies and the present one are likely due to methodological differences. The prior studies used in situ hybridization, which has finer spatial resolution for mRNA quantification than the micropunch technique used in the current experiment. By examining the entire amygdala as a whole, it is plausible that subnuclei specific differences in ER expression (between exposure groups and/or sexes) were homogenized and, consequently, not fully detectable. However, a critical goal of the present study was to maximize anatomical specificity while simultaneously ensuring we obtained enough tissue to extract sufficient quantities of RNA for sequencing. Additionally, in a prior study conducted in collaboration with NCTR we found differences in amygdalar ER expression, particularly *Esr2* expression, between gavaged vehicle and ungavaged naïve controls suggesting a possible effect of gavage itself [21]. Orogastric gavage was selected as the exposure route in CLARITY-BPA experiments because (1) oral administration is considered to be human relevant and (2) compared to other methods of oral dosing (such as delivering it via a food treat or similar), gavage is considered more precise for studies intended for use in risk assessment. A caveat of the CLARITY-BPA program was the lack of a naïve control group, which would have helped distinguish between BPA- and gavage-related effects on neuronal gene expression, and other endpoints [21].

Prenatal exposure to 25 and 250 µg of BPA enhanced *Ar* expression in males and females, respectively. Information regarding the impact of developmental BPA exposure on *Ar* expression is sparse but one study reported increased levels of *Ar* in the cerebrum of PND 2 ICR mice exposed to 50 mg/kg of BPA on gestational days 6 and 15 [80]. In addition, embryonic exposure of zebrafish to BPA (1.6 µg/L) resulted in precocious hypothalamic neurogenesis that was dependent on androgen receptor (AR) mediated upregulation of aromatase [18]. Studies using rats and mice that lack ARs (i.e., rodents with the testicular feminization mutation) demonstrate that ARs are required for the full masculinization of many brain regions, especially the amygdala [81]. For example, the volume of the posterodorsal portion of the medial amygdala (MePD) is larger in males than females and the male MePD has more neurons with larger soma, more astrocytes, greater dendritic length, and higher spine density [52, 82–84]. All of these sex differences are heavily dependent on the presence of ARs during perinatal development. *Ar* expression in the rat forebrain is regulated by estrogen, not androgen, during the early postnatal period [53]. Therefore, upregulation of *Ar* by BPA in the neonate amygdala is consistent with an estrogenic mode of action that may disrupt sexual differentiation of the developing amygdala. In a prior CLARITY-BPA study, we reported that perinatal exposure to 2,500 µg BPA/kg bw/day enlarged the volume of the right MePD in juvenile males [28]. While this singular effect

may be due to disruption of *Ar* expression, causality cannot be established because the exposure paradigms and the doses at which BPA-related effects were observed differed from the current study. Thus, further investigation would be required to confirm this putative mechanism and characterize the functional implications of heightened *Ar* expression. This finding of AR disruption is particularly significant when considering possible human risk because AR plays such a fundamental role in brain masculinization as opposed to ERs in rats and mice.

We found no effect of prenatal BPA exposure on *Gadd45b* expression in the developing amygdala, and also failed to find the expected sex difference in *Gadd45b* expression. A prior study, also in the Sprague-Dawley rat and examining the amygdala as a whole, observed a female biased expression pattern of *Gadd45b* at PND 1 [67]. Diet may be one factor contributing to our inability to replicate the finding. In the previous experiment, the diet was not described in detail, but rather described as “standard.” Prior literature has demonstrated that “standard laboratory chow” is typically soy-based and thus contains significant amounts of hormonally active phytoestrogens [85–87]. We and others have shown that this source of background of EDC exposure can obfuscate or alter sex differences [15, 88]. Amygdalar *Gadd45b* expression was of interest because it is implicated in the regulation of “rough and tumble play,” a juvenile social play behavior that occurs more frequently in males than females [68], but also because *Gadd45b* facilitates activity-induced DNA demethylation [89, 90]. While a number of studies provide evidence of BPA-related effects on epigenetic marks [13, 60, 91–93], no studies have examined whether prenatal BPA exposure can perturb epigenetic ‘erasers’ in the developing brain. Our observation does not rule out the possibility that early-life BPA exposure may be interfering with other epigenetic regulators. For instance, prenatal BPA exposure has been shown to induce sex-specific effects on social and anxiety-like behaviors in adult BALB/c mice that were associated with changes in DNA methylation and mRNA levels of ER α in the hypothalamus and hippocampus [60].

In addition to targeted qRT-PCR, we used an unbiased discovery-based approach to profile differential gene expression changes within the neonate amygdalar transcriptome to further investigate potential mechanisms underlying the sex-specific effects of early-life BPA exposure on the brain. One caveat of this approach is that prior work by us and others has demonstrated that RNAseq may not be capable of sufficient transcript sampling to adequately resolve group differences in expression because most genes of interest have low abundance and effect size between differentially expressed genes is low compared to other organs [12, 94, 95]. Amygdalar transcription changes identified by RNAseq were overwhelmingly female specific, with substantial overlap between the two BPA groups examined (25 and 250 μ g). In contrast, extremely few differentially expressed genes were detected in males exposed to 25 and 250 μ g BPA (89 genes and 1 gene, respectively) and there was no overlap between these genes suggesting they are not biologically meaningful. We conclude that the amygdala transcriptome is sex-specifically vulnerable to gestational BPA exposure with females more sensitive than males. By contrast, transcriptional effects in the PND 1 hypothalamus and hippocampus of these same animals was more pronounced in males than females, with disruption more robust in the hypothalamus [12]. Collectively, these data indicate that early-life BPA exposure can have sex-specific and region-specific

effects on the transcriptome of the developing brain and highlight the importance of sex and region as biological variables in neurodevelopmental EDC studies.

qRT-PCR was used to assess expression levels of two differentially expressed genes of interest identified by RNAseq in all exposure groups: *Camk4* and *Grm5*. The magnitude and directionality of the results generally confirmed the RNAseq data. A sex differences in *Camk4* expression was also detected in the unexposed controls by RNAseq but the statistical significance of that difference did not survive FDR. Females prenatally exposed to BPA and EE₂ displayed robust enhancement of *Camk4* at every dose with the exception of 2500 µg of BPA, although there was a modest trend for enhancement ($p = 0.08$). In the nervous system, Ca²⁺/CaM dependent kinase IV (CaMKIV) is a well-known mediator of calcium dependent gene expression, and emerging evidence indicates that CaMKIV is essential for neurodevelopment, synaptic plasticity, and the consolidation of behavioral memory [70, 96, 97]. Furthermore, consistent with our pathway analyses, overexpression of CAMKIV in transgenic C57BL/6 mice was shown to significantly increase long-term potentiation in the juvenile anterior cingulate cortex [98]. *Grm5* expression was also increased by BPA (250, 2500, and 25000 µg) and EE₂ (0.05 µg) exposure in females and a trend towards upregulation was observed for 25 µg BPA and 0.05 µg EE₂ ($p = 0.07$ and 0.06 , respectively). Group I metabotropic glutamate receptors (mGluRs), which include glutamate metabotropic receptor 5 (GRM5), mediate a diverse variety of neuronal functions and are critical modulators of activity-dependent synaptic plasticity in a variety of brain regions, including the amygdala [99, 100]. Notably, dysregulation of group I mGluRs mediated signaling has been implicated in a range of childhood neurodevelopmental disorders [101–103].

IPA analyses of the female transcriptomic data revealed a number of canonical pathways significantly activated by both 25 and 250 µg of BPA, many of which are associated with neuronal development and synaptic transmission. Deeper analysis of specific genes within each of these pathways revealed BPA and EE₂ had a number of overlapping and directionally similar effects on expression (Table 3). Available data regarding the impact of BPA exposure on neuronal development and synaptic transmission is sparse but effects on juvenile and adult synaptogenesis, neuronal differentiation/migration, and synaptic plasticity have been reported following prenatal and neonatal exposure to BPA [29, 104–106]. The impact of BPA on the developing brain, however, remains largely unknown because research to date has focused almost exclusively on juvenile or adult animals. In the current study, pathways involved in synaptic plasticity, CREB signaling in neurons, and cAMP-mediated signaling displayed predicted increases in the amygdala of females exposed to 25 and 250 µg of BPA prenatally. Consistent with these findings, accelerated neuronal differentiation and migration was found in the neocortex of ICR/Jcl mice on embryonic days 12.5 and 16.5 following daily exposure to 20 µg/kg of BPA via maternal injection; however, sex was not considered a factor in this study [105]. In vivo, doses of BPA ranging from 1 to 100 nM of BPA induced rapid effects on LTP and increased spine density in the CA1 and CA3 regions of hippocampal slice cultures of rats [107]. In rodents, perinatal brain development is associated with activity-dependent synaptic refinement and extensive remodeling that contributes to growth and stabilization of connections [108, 109]. Although many of these processes are hormonally modulated by estradiol during development [110, 111], our current understanding of amygdala physiology is limited and based almost exclusively on research

conducted in adult animals [112, 113]. Numerous experimental studies on early-life stress, however, have demonstrated that environmental insult can alter synaptic patterning in the amygdala and lead to impaired affective behavior later in life, including anxiety and social behavior [114–118]. Although beyond the scope of this paper, these studies suggest that disruption of neuronal development and synaptic transmission may potentially underlie the behavioral effects associated with early-life BPA exposure in females. However, this is a purely speculative and further studies are needed to definitively demonstrate that relationship.

5. CONCLUSIONS

These data reveal that prenatal BPA exposure disrupts the transcriptome of the neonate amygdala at doses below the FDA NOAEL, with females appearing to be more sensitive than males. Within females, BPA-related transcriptional changes were reasonably concordant with EE₂-related changes suggesting that at least some outcomes were consistent with an “estrogenic” mode of action. However, the pathway analysis supports the hypothesis that other mechanisms are also likely involved. That conclusion is consistent with our prior CLARITY-BPA studies, which also reported discordant effects of EE₂ and BPA on the volume of sexually dimorphic brain regions and the PND 1 hippocampal and hypothalamic transcriptome [12, 28]. We also provide additional evidence that BPA can disrupt the organization of AVP and OT pathways in the developing brain and alter signaling pathways critical for synaptic organization and transmission. That some responses appear to be non-linear is consistent with a wealth of prior literature showing that BPA and other EDCs can have non-monotonic dose response curves [62, 119–121], the mechanism(s) by which these occur remains unresolved. The observation of effects at the lower end of the dose curve is also consistent with prior CLARITY-BPA studies by us on behavioral endpoints and also in brain, ovary and heart [12, 28, 36, 122–124].

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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HIGHLIGHTS

- BPA is hypothesized to sex-specifically impair neurodevelopment.
- We examined the impact of prenatal BPA exposure on the PND 1 amygdalar transcriptome.
- Female amygdala appears more sensitive to BPA during fetal development.
- *Oxt*, *Avpr1a*, *Esr2*, *Ar*, *Camk4*, and *Grm5* were altered in sex-specific manner.
- Prenatal BPA may alter pathways for synaptic transmission and neurodevelopment.

Fig. 1. Effects of gestational BPA or EE₂ on neonatal amygdalar expression of selected genes
Esr1 was unaffected by BPA or EE₂ (A). *Esr2* was increased by 2.5 µg BPA in males and 250 µg BPA in females (B). *Oxt* was increased by 250 and 250 µg BPA in males and 2.5, 25, 250, and 25,000 µg BPA and 0.5 µg EE₂ in females (C). Male *Avpr1a* was decreased by 2.5 µg BPA and increased by 25 and 250 µg BPA (D). *Ar* was increased by 25 µg BPA in males and 250 µg BPA and 0.5 µg EE₂ in females (E). Female *Gadd45b* was increased by 0.5 µg EE₂ (F). *Camk4* was upregulated in males by 25 µg BPA and in females by 2.5, 25, 250, and 25,000 µg BPA and 0.05 and 0.5 µg EE₂ (G). *Grm5* was increased by 25 µg BPA in males and 250, 2500, 25,000 µg BPA and 0.05 µg EE₂ in females (H). Graphs depict mean ± SEM (*p < 0.05, **p < 0.01, and §p < 0.08).

Fig. 2. Top canonical pathways enriched by differentially expressed genes

The x-axis represents negative log p values based on the probability that molecules in the uploaded dataset were included in the predefined IPA canonical pathways by true association as opposed to inclusion of molecules based on chance alone. For each male (A) and female (B) exposure group, only the top 10 pathways with the largest negative log p values are shown. The dashed line indicates the threshold of significance for a p -adjusted value of 0.05.

Fig. 3. Prenatal exposure to BPA and EE₂ result in common and unique differentially expressed genes

Differentially expressed genes ($p_{adj} \leq 0.05$) were identified in males (A) and females (B) prenatally exposed to 25 and 250 µg BPA and 0.5 µg EE₂. Venn diagrams were created using Venny (<http://bioinfogp.cnb.csic.es/tools/venny/>).

Fig. 4. Sex differences in amygdala expression of selected genes

Relative differences in gene expression between male and female control (unexposed) groups with male gene expression set as baseline. No a priori selected genes were sexually dimorphic in the neonate amygdala. (A–F). Expression of *Camk4* was significantly higher in males than females (G). No sex difference in *Grm5* was detected (H). Graphs depict mean $\pm$ SEM (** p 0.01 and § p 0.08).

Table 1

Rationale for genes of interest selected a priori

Preselected Genes of Interest	Rationale			Selected References
Androgen receptor (<i>Ar</i>)	A	C	D	[49–55]
Arginine vasopressin (<i>Avpr1a</i>)	A	C		[56–59]
Estrogen receptor α (<i>Esr1</i>)	A	B	D	[12, 21, 49, 50, 60–64]
Estrogen receptor β (<i>Esr2</i>)	A	B	D	[12, 21, 49, 50, 61–66]
Growth arrest and DNA damage inducible β (<i>Gadd4b</i>)		B		[67, 68]
Oxytocin receptor (<i>Oxt</i>)	A	C		[56–58, 69]

Legend

- A Previously shown to be influenced by BPA
- B Sex biased expression in amygdala
- C Estrogen-dependent expression
- D Important for sexual differentiation of the amygdala

Table 2

qRT-PCR outcomes and descriptive statistics for genes found to be significantly altered by BPA or EE2 exposure. Sample sizes for each group are in parentheses.

Gene Symbol	Comparison (n)	Relative Abundance	U	p- value
<i>Preselected genes of interest</i>				
<i>Esr2</i>	♂ 25 BPA (7) to ♂ Veh (6)	2.38	3	0.01
	♀ 250 BPA (6) to ♀ Veh (7)	1.67	6	0.04
<i>Oxtr</i>	♂ Veh (7) to ♂ Veh (6)	0.70	8	0.08
	♂ 25 BPA (7) to ♂ Veh (6)	1.95	2	0.01
	♂ 250 BPA (7) to ♂ Veh (6)	1.37	6	0.04
	♀ 2.5 BPA (6) to ♀ Veh (7)	1.37	8	0.04
	♀ 25 BPA (6) to ♀ Veh (7)	1.90	4	0.01
	♀ 250 BPA (6) to ♀ Veh (7)	1.97	5	0.02
	♀ 25000 BPA (6) to ♀ Veh (7)	1.58	4	0.01
	♀ 0.5 EE2 (5) to ♀ Veh (7)	2.01	0	0.01
<i>Avpr1a</i>	♂ 2.5 BPA (4) to ♂ Veh (6)	0.75	2	0.04
	♂ 25 BPA (7) to ♂ Veh (6)	2.27	0	0.01
	♂ 250 BPA (7) to ♂ Veh (6)	1.56	4	0.01
	♂ 0.5 EE2 (7) to ♂ Veh (6)	1.41	6	0.04
<i>Ar</i>	♂ 25 BPA (7) to ♂ Veh (6)	2.43	3	0.01
	♀ 250 BPA (6) to ♀ Veh (7)	1.84	6	0.04
<i>Gadd45b</i>	♀ 0.5 EE2 to (5) ♀ Veh (7)	1.77	5	0.05
<i>Genes selected from RNAseq analysis</i>				
<i>Camk4</i>	♀ Veh (5) to ♂ Veh (5)	0.54	0	0.01
	♂ 25 BPA (7) to ♂ Veh (5)	1.75	3	0.01
	♀ 2.5 BPA (6) to ♀ Veh (5)	2.54	0	0.01
	♀ 25 BPA (6) to ♀ Veh (5)	2.02	0	0.01
	♀ 250 BPA (6) to ♀ Veh (5)	2.24	0	0.01
	♀ 2500 BPA (6) to ♀ Veh (5)	1.68	5	0.08
	♀ 25000 BPA (6) to ♀ Veh (5)	2.48	0	0.01
	♀ 00.5 EE2 (6) to ♀ Veh (5)	1.83	0	0.01
	♀ 0.5 EE2 (5) to ♀ Veh (5)	2.52	0	0.01
<i>Grm5</i>	♂ 25 BPA (7) to ♂ Veh (6)	1.54	4	0.01
	♀ 25 BPA (6) to ♀ Veh (4)	1.62	3	0.07
	♀ 250 BPA (6) to ♀ Veh (4)	1.89	0	0.01
	♀ 2500 BPA (5) to ♀ Veh (4)	1.71	0	0.02
	♀ 25000 BPA (6) to ♀ Veh (4)	2.01	2	0.04
	♀ 0.05 EE2 (6) to ♀ Veh (4)	1.46	1	0.02
	♀ 0.5 EE2 (3) to ♀ Veh (4)	2.14	0	0.06

Differentially expressed genes identified by RNAseq within selected canonical pathways identified by IPA analysis in exposed females (normalized to ♀ vehicle; adjusted p-value 0.05).

Table 3

Gene Symbol	Gene Name	♀ 25 µg BPA		♀ 250 µg BPA		♀ 0.5 µg EE ₂		IPA Canonical Pathway(s)
		Padj	FC	Padj	FC	Padj	FC	
<i>Adcy1</i>	Adenylate Cyclase 1	2.34E-08	1.91	5.92E-09	1.94	8.09E-10	2.01	cAMP-Mediated Signaling CREB Signaling in Neurons GABA Receptor Signaling Synaptic Long Term Potentiation
<i>Adcy5</i>	Adenylate Cyclase 5	3.97E-02	1.30	NS	NS	NS	NS	cAMP-Mediated Signaling CREB Signaling in Neurons GABA Receptor Signaling
<i>Adcy9</i>	Adenylate Cyclase 9	4.81E-02	1.43	NS	NS	3.94E-02	1.41	cAMP-Mediated Signaling CREB Signaling in Neurons GABA Receptor Signaling
<i>Akap11</i>	A-Kinase Anchoring Protein 11	5.97E-03	1.30	2.11E-03	1.31	NS	NS	cAMP-Mediated Signaling
<i>Atf2</i>	Activating Transcription Factor 2	NS	NS	NS	NS	2.62E-04	1.35	cAMP-Mediated Signaling CREB Signaling in Neurons Synaptic Long Term Potentiation
<i>Braf</i>	B-Raf Proto-Oncogene, Serine/Threonine Kinase	NS	NS	NS	NS	4.13E-03	1.44	cAMP-Mediated Signaling
<i>Camk2A</i>	Calcium/Calmodulin-Dependent Protein Kinase II Alpha	3.99E-03	1.33	2.62E-03	1.33	NS	NS	cAMP-Mediated Signaling CREB Signaling in Neurons Synaptic Long Term Potentiation
<i>Camk4</i>	Calcium/Calmodulin-Dependent Protein Kinase IV	NS	NS	1.72E-02	1.41	NS	NS	cAMP-Mediated Signaling CREB Signaling in Neurons Glutamate Receptor Signaling Synaptic Long Term Potentiation
<i>Chrm3</i>	Cholinergic Receptor, Muscarinic 3	9.57E-03	1.41	4.56E-02	1.32	9.64E-03	1.37	cAMP-Mediated Signaling
<i>Creb1</i>	Camp Responsive Element Binding Protein 1	NS	NS	NS	NS	2.82E-02	1.43	cAMP-Mediated Signaling CREB Signaling in Neurons Synaptic Long Term Potentiation
<i>Gabrb1</i>	Gamma-Aminobutyric Acid Type A Receptor Beta 1 Subunit	NS	NS	2.16E-02	1.35	3.31E-03	1.40	GABA Receptor Signaling
<i>Gabrb2</i>	Gamma-Aminobutyric Acid Type A Receptor Beta 2 Subunit	4.22E-03	1.51	6.11E-05	1.67	9.03E-05	1.64	GABA Receptor Signaling
<i>Gabrb3</i>	Gamma-Aminobutyric Acid Type A Receptor Beta 3 Subunit	NS	NS	1.07E-02	1.37	1.64E-02	1.33	GABA Receptor Signaling
<i>Gabrg1</i>	Gamma-Aminobutyric Acid Type A Receptor Gamma 1 Subunit	4.96E-02	1.26	1.26E-04	1.42	3.66E-03	1.32	GABA Receptor Signaling
<i>Gabrg2</i>	Gamma-Aminobutyric Acid Type A Receptor Gamma 2 Subunit	2.40E-02	1.32	4.16E-03	1.37	1.45E-02	1.32	GABA Receptor Signaling
<i>Griaq</i>	G Protein Subunit Alpha Q	1.57E-02	1.30	4.56E-04	1.40	8.22E-04	1.37	CREB Signaling in Neurons Synaptic Long Term Depression

Gene Symbol	Gene Name	9 25 µg BPA		9 250 µg BPA		9 0.5 µg EE ₂		IPA Canonical Pathway(s)
		Padj	FC	Padj	FC	Padj	FC	
								Synaptic Long Term Potentiation
<i>Grik3</i>	Glutamate Ionotropic Receptor Kainate Type Subunit 3	1.43E+00	0.04	4.45E-02	1.41	NS	NS	CREB Signaling in Neurons Glutamate Receptor Signaling
<i>Gria2B</i>	Glutamate Ionotropic Receptor Nmda Type Subunit 2B	NS	NS	NS	NS	2.71E-02	1.38	CREB Signaling in Neurons Glutamate Receptor Signaling Synaptic Long Term Potentiation
<i>Gria3A</i>	Glutamate Ionotropic Receptor Nmda Type Subunit 3A	NS	NS	NS	NS	2.34E-02	1.37	Glutamate Receptor Signaling Synaptic Long Term Potentiation
<i>Gria3</i>	Glutamate Metabotropic Receptor 3	NS	NS	2.83E-02	1.43	2.34E-02	1.42	CREB Signaling in Neurons Glutamate Receptor Signaling Synaptic Long Term Depression Synaptic Long Term Potentiation
<i>Gria5</i>	Glutamate Metabotropic Receptor 5	1.11E-02	1.41	2.66E-03	1.44	2.83E-04	1.51	CREB Signaling in Neurons Glutamate Receptor Signaling Synaptic Long Term Depression Synaptic Long Term Potentiation
<i>Gucyl1a2</i>	Guanylate Cyclase 1 Soluble Subunit Alpha 2	NS	NS	3.25E-02	1.41	3.15E-02	1.39	Synaptic Long Term Depression
<i>Htr1A</i>	5-Hydroxytryptamine Receptor 1A	NS	NS	NS	NS	2.34E-02	1.44	cAMP-Mediated Signaling
<i>Htr1B</i>	5-Hydroxytryptamine Receptor 1B	1.29E-02	1.44	1.13E-02	1.43	2.14E-04	1.58	cAMP-Mediated Signaling
<i>Irs1</i>	Insulin Receptor Substrate 1	NS	NS	NS	NS	1.45E-03	1.54	CREB Signaling in Neurons
<i>Kcnaq2</i>	Potassium Voltage-Gated Channel Subfamily Q Member 2	2.88E-02	1.35	2.16E-02	1.35	2.84E-02	1.32	GABA Receptor Signaling
<i>Kcnaq3</i>	Potassium Voltage-Gated Channel Subfamily Q Member 3	2.98E-02	1.40	4.48E-03	1.48	2.66E-03	1.49	GABA Receptor Signaling
<i>Kl</i>	Klotho	2.58E-02	-1.34	NS	NS	3.54E-02	-1.30	CREB Signaling in Neurons
<i>Kras</i>	Kras Proto-Oncogene, Gtpase	NS	NS	8.34E-03	1.33	2.18E-03	1.36	CREB Signaling in Neurons Synaptic Long Term Depression Synaptic Long Term Potentiation
<i>Mapk1</i>	Mitogen Activated Protein Kinase 1	NS	NS	NS	NS	2.24E-02	1.26	cAMP-Mediated Signaling CREB Signaling in Neurons Synaptic Long Term Potentiation
<i>Opri1</i>	Opioid Related Nociceptin Receptor 1	NS	NS	2.28E-02	1.32	9.63E-05	1.48	cAMP-Mediated Signaling
<i>Pde4D</i>	Phosphodiesterase 4D	NS	NS	NS	NS	3.16E-02	1.36	cAMP-Mediated Signaling
<i>Pde9A</i>	Phosphodiesterase 9A	2.46E-02	-1.39	4.20E-02	-1.35	4.20E-03	-1.44	cAMP-Mediated Signaling
<i>Pde10A</i>	Phosphodiesterase 10A	2.80E-02	1.39	1.18E-02	1.42	3.22E-03	1.46	cAMP-Mediated Signaling
<i>Plcl1</i>	Phospholipase C-Like 1	NS	NS	NS	NS	2.23E-03	1.41	CREB Signaling in Neurons Synaptic Long Term Potentiation

Gene Symbol	Gene Name	♀ 25 µg BPA		♀ 250 µg BPA		♀ 0.5 µg EE ₂		IPA Canonical Pathway(s)
		Padj	FC	Padj	FC	Padj	FC	
<i>Ppm1l</i>	Protein Phosphatase, Mg ²⁺ /Mn ²⁺ Dependent, 1L	1.75E-02	1.27	1.18E-02	1.27	3.80E-02	1.22	Synaptic Long Term Depression
<i>Prkacb</i>	Protein Kinase Camp-Activated Catalytic Subunit Beta	2.55E-02	1.36	5.61E-04	1.50	2.24E-03	1.43	cAMP-Mediated Signaling CREB Signaling in Neurons Synaptic Long Term Potentiation
<i>Prkar2a</i>	Protein Kinase Camp-Dependent Type 2 Regulatory Subunit Alpha	NS	NS	3.12E-03	1.49	1.48E-02	1.40	cAMP-Mediated Signaling CREB Signaling in Neurons Synaptic Long Term Potentiation
<i>Prkca</i>	Protein Kinase C, Alpha	9.91E-03	1.49	1.12E-02	1.46	2.08E-03	1.52	Synaptic Long Term Depression Synaptic Long Term Potentiation
<i>Prkce</i>	Protein Kinase C, Epsilon	1.26E-03	1.51	8.43E-04	1.50	5.35E-03	1.41	CREB Signaling in Neurons Synaptic Long Term Depression Synaptic Long Term Potentiation
<i>Slc1a2</i>	Solute Carrier Family 1 Member 2	3.20E-05	1.74	2.73E-05	1.72	4.72E-06	1.76	Glutamate Receptor Signaling
<i>Slc38a1</i>	Solute Carrier Family 38, Member 1	2.92E-03	1.40	1.35E-04	1.47	2.48E-04	1.44	Glutamate Receptor Signaling