**Postnatal maternal care impacts hypothalamic *Esrrg* gene expression, co-expression profiles, and the DNA methylome in prenatal bisphenol-exposed rats**

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**Abstract**
Environmental exposures co-occurring during early life have a profound influence on neurodevelopment. Our previous work in rats suggests that postnatal maternal care modulates the effects of prenatal exposure to bisphenols, an estrogenic endocrine disrupting chemical, on offspring neurodevelopment. Elevated postnatal maternal licking/grooming and prenatal bisphenol exposure have known opposing effects on estrogen receptor alpha (*Esr1*) expression in the medial preoptic area (MPOA) of the hypothalamus, which could impact expression of estrogen-responsive genes. Opposing effects on DNA methylation of Esr1 have also been observed as a consequence of these exposures, with potentially broad transcriptional effects. Based on this previous work, we hypothesized that postnatal maternal licking/grooming would mitigate the effects of prenatal bisphenol exposure on *Esr1* expression and estrogen-responsive genes in the developing MPOA. In addition, we hypothesized that there would be interactive effects of prenatal bisphenol exposure and postnatal maternal licking/grooming on DNA methylation, particularly nearby estrogen responsive elements. We administered either a bisphenol mixture (BPA, BPF, BPS), BPA only, or a corn oil control to pregnant rat dams. Postnatal maternal care was measured using home-cage videos. Brains from male and female pups were collected at postnatal day 10 and the transcriptome and DNA methylome were assessed in the MPOA. Our results indicated a significant interaction between prenatal bisphenol exposure and maternal postnatal licking/grooming on estrogen-related receptor gamma (*Esrrg*) expression, exclusively in female pups. These interactions were also evident in co-expression gene profiles in female pups; the majority of which were enriched for estrogen-responsive genes. Finally, DNA methylation analyses indicated that adding postnatal maternal licking/grooming as a covariate influenced the number of differentially methylated regions for prenatal bisphenol-exposed male and female pups. While these differentially methylated regions were not enriched for estrogen responsive elements, there were binding motifs for transcription factors that are known to interact with estrogen receptors, suggesting some secondary effects on postnatal gene regulation. These results suggest a novel biological mechanism in which postnatal maternal care can mitigate the negative neurodevelopmental impacts of prenatal bisphenol exposure.

**Keywords:** Bisphenols, maternal care, estrogen receptor signaling, transcriptome, DNA methylation, estrogen-related receptor gamma

## Introduction

Early-life environmental exposures have a profound influence on neurodevelopmental trajectories and can lead to stable changes in behavior throughout life. Outside of controlled laboratory conditions, individuals are exposed to a mixture of risk and protective factors that shape neurodevelopmental trajectories via distinct and common biological pathways. In particular, interactions between early-life environmental and social experiences appear to be prevalent in predicting risk and resilience to neurodevelopmental and mental health conditions (1,2). Some examples include early-life foster care for institutionalized children (3,4), perinatal nurse-family visitation programs for families in low socioeconomic conditions (5,6), and postnatal kangaroo care/skin-to-skin contact for preterm infants (7,8). The biological mechanisms underlying these interactions are largely unknown but are critical for developing effective intervention programs to mitigate the impacts of early-life adversity.

Prenatal exposure to endocrine disrupting chemicals has been shown in human and nonhuman animal studies to alter neurodevelopmental trajectories and increase the risk of neurodevelopmental and mental health conditions (9–11). Bisphenols (BP) are commonly used plasticizers that are present in many plastics, such as food storage containers and reusable water bottles, as well as the lining of food cans. Bisphenols such as BPA and other "BPA-free" structural analogs, including BPF and BPS, have also been shown to act as endocrine disruptors that primarily affect estrogen receptor signaling (12,13). Previous studies have demonstrated that bisphenols can bind to both estrogen receptor alpha (ESR1) and estrogen receptor beta (ESR2) to exert effects on gene transcription (12,14,15). Prenatal exposure to bisphenols has been associated with negative neurodevelopmental and behavior outcomes for children in human epidemiological studies (16–22) as well as in rodent models of prenatal bisphenol exposure (23–28).

Bisphenol exposure during pregnancy has also been linked to impairments in postnatal maternal care (25,29–32), though previous work in our lab has also suggested that the impacts of prenatal bisphenol exposure on offspring neurodevelopment and some aspects of postnatal maternal care might be dissociable (33,34). Regardless of the impact of prenatal bisphenols on postnatal maternal care, variations in caregiving experienced during postnatal development can have a profound impact on molecular, physiological and behavioral trajectories (35–38). In our previous work, we found that postnatal maternal care (licking/grooming and nest attendance) interacts with prenatal bisphenol exposure to predict offspring phenotype, including neurodevelopmental milestones (eye opening timing), adult behavior (attentional set shifting performance and anxiety-like behavior in the open field), transcriptional profiles in the developing brain, and DNA methylation in the adult brain (34,39). These effects were observed to be sex-specific and dose-dependent. Interestingly, we also found that higher postnatal maternal care can mitigate the impacts of prenatal bisphenol exposure on a subset of offspring phenotypes (34), suggesting that higher postnatal maternal care received and prenatal bisphenol exposure impact similar biological pathways in opposing directions. Understanding these biological pathways are

important to identify and propose more targeted and effective interventions to mitigate the deleterious neurodevelopmental impacts of prenatal bisphenol exposure.

Prenatal bisphenol exposure (25,27,40,41) and variations in postnatal maternal care (42–45) are associated with changes in DNA methylation levels across the genome which can lead to stable changes in gene expression. However, the underlying mechanisms linking prenatal bisphenol exposure as well as postnatal maternal care with DNA methylation modifications at specific loci are not well-understood. There is accumulating evidence that steroid hormone receptor signaling links environmental exposures to DNA methylation modifications proximal to their respective transcription factor binding sites (46–50). This includes estrogen receptor signaling, where estrogen receptor binding at estrogen responsive elements (EREs) has been associated with downstream changes in DNA methylation levels proximal to EREs (51–54). Rats with perinatal BPA exposure have decreased transcript abundance of *Esr1* in the medial preoptic area (MPOA) of the hypothalamus later in life (55,56), particularly in females. Prenatal bisphenol exposure has also been associated with altered expression of estrogen-responsive genes with corresponding changes in DNA methylation levels within these genes (40,57). Therefore, the effects of bisphenols on *Esr1* expression and estrogen receptor signaling at EREs may confer changes in DNA methylation levels at proximal CpG sites. Concurrently, high levels of postnatal maternal care received (licking/grooming or licking-like stimulation) has been associated with increased gene expression and decreased DNA methylation of *Esr1* throughout life in the MPOA (44,45,58), an effect also occurring predominantly in females. Therefore, higher maternal licking/grooming received might mitigate the effects of prenatal bisphenol exposure on DNA methylation proximal to EREs later in life by normalizing *Esr1* transcript abundance and ESR1 binding to DNA in the developing MPOA, particularly in female offspring. Consistent with this hypothesis, our prior transcriptome analyses of the developing prefrontal cortex and amygdala found significant interactive effects of prenatal bisphenol exposure and postnatal maternal care on co-expressed gene modules that are related to estrogen receptor signaling (34), further suggesting that the interactions between prenatal bisphenol exposure and postnatal maternal care converge on estrogen receptor signaling changes.

The primary objective of the current study was to examine the interactive effects of prenatal bisphenol exposure and postnatal maternal licking/grooming on the transcriptome and DNA methylation modifications at specific transcription factor binding sites, notably EREs, in the developing MPOA. The MPOA may be especially vulnerable to prenatal bisphenol exposure because there is a high concentration of estrogen receptors in this brain region. The MPOA is also highly sexually dimorphic and might contribute to the sex-specific effects observed in prenatal bisphenol exposure studies in humans and rodents. In addition, we adapted a correlative method to identify DNA motifs and potential transcription factor binding sites at differentially methylated regions. Suffix Array Kernel Smoothing (SArKS) has been previously implemented in RNA-seq datasets and allows the user to correlate a continuous range of gene expression changes to proximal transcription factors binding sites (59). We hypothesized that postnatal maternal licking/grooming would

mitigate the effects of prenatal bisphenol exposure on *Esr1* expression and estrogen-responsive genes. In addition, we hypothesized that there would be interactions between prenatal bisphenol exposure and postnatal maternal licking/grooming on DNA methylation modifications, particularly nearby EREs and other transcription factor binding sites. Finally, we hypothesized that there would be sex-specific effects, specifically that there would be larger impacts in female offspring compared to male offspring.

## Methods

### Husbandry and breeding

All animal procedures were approved by the Institutional Animal Care and Use Committee at the University of Texas at Austin and conformed to the guidelines of the American Association for Laboratory Animal Science. Seven-week-old female (n = 108) and nine-week-old male (n = 26) Long-Evans rats were obtained from Charles River Laboratories and acclimated to the animal facility for two weeks. Rats were housed in same-sex pairs on a 12:12 hour inverse light-dark cycle (lights-off at 10:00 during standard time, 11:00 during daylight savings time) with ad libitum access to standard chow diet (#5LL2, Lab Diet) and water. All rats were provided glass water bottles, polysulfone cages, and aspen wood shavings as bedding to limit additional external exposure to bisphenols and other xenoestrogens.

### Gestational bisphenol administration

Male and female rats were screened for breeding receptivity by pairing one male with one or two females. The rats were left to breed overnight (gestational day 0) if mounting for the male and lordosis for the female(s) were observed. From gestation day 8 until parturition (gestational day 21-22), cage-pairs of dams were randomly assigned to receive control Corn Oil (n = 22), 50 µg/kg BPA (n = 30), 50 µg/kg Mixed Bisphenols (BP) (n = 23), or 150 µg/kg Mixed BP (n = 22). This time period includes the start of brain sexual differentiation and when estrogen receptors begin to appear in the fetal brain (60). Bisphenol stocks were created by dissolving 0.1 g of either bisphenol A (BPA; #B0494, TCI, ≥ 99 %), bisphenol S (BPS; #A17342, Alpha Aesar, ≥ 99 %), or bisphenol F (BPF; #A11471, Alfa Aesar, ≥ 98 %) in 10 mL of corn oil (#405435000, Acros Organics). Equal parts BPA, BPS, and BPF were used for both Mixed BP treatment groups. Experimenters involved with the administration of treatments were blinded to the treatment groups by using color codes for each group throughout the administration period. After parturition (postnatal day 0; PND0), offspring were counted and sex-determined using relative anogenital distance. Litters were culled to six male and six female pups and kept for subsequent testing. Following the maternal care video recordings at PND10, the brains from one male pup and one female pup per litter were collected and flash-frozen in hexanes on dry ice.

### Postnatal maternal care observations and quantification

Home cage maternal behavior was recorded using Raspberry Pi 3B+ minicomputers that were programmed to record for one hour starting one hour after lights-off from PND1-10. Maternal behavior was scored by either manual coding (when the dam was on the nest for

2 minutes or less during the video) or through the AMBER pipeline (61). All postnatal maternal care measures were normalized to seconds of observed behavior per day to account for missing observations. Based on prior analyses (34,39) we used licking/grooming and nest attendance from PND1-5 as independent variables or covariates for the current study. Maternal behavior measures used in the current study did not vary between prenatal bisphenol exposure groups (34).

**RNA extraction and 3' tag sequencing (Tag-seq)**

All brains were stored at -80°C until cryosectioning. PND10 brains (n = 6 per sex per prenatal treatment group) were cryosectioned in 50 µm slices using a ThermoFisher Scientific CryoStar NX50 cryostat or a Leica CM3050S cryostat. The medial preoptic area (MPOA; 0.20 to -0.60 mm Bregma) was microdissected using an atlas for the developing rat brain (62) and a supplementary atlas for the PND10 rat brain (63). RNA was extracted using the MagMAX FFPE DNA/RNA Ultra Kit (#A31881, ThermoFisher Scientific). For unfixed frozen tissue, 210 µl of protease solution was pipetted onto each MPOA sample and allowed to incubate at 55°C at 900 rpm for at least one hour. The homogenate was immediately processed for RNA and DNA extraction using the Kingfisher Flex System following the manufacturer's instructions. RNA quantity was assessed with the Quant-iT RNA Assay kit (#Q33140, ThermoFisher Scientific) and RNA quality was assessed using the RNA 6000 Pico Assay kit (#5067-1513, Agilent Technologies). All RNA samples were diluted to 20 ng/µl before submission for library preparation and tag-seq (64,65) at the Genome Sequence and Analysis Facility at the University of Texas at Austin. Reads were sequenced on the NovaSeq S1 (100 bp single-end reads).

**DNA extraction and oxidative reduced representation bisulfite sequencing (oxRRBS)**

DNA was extracted using the MagMAX FFPE DNA/RNA Ultra Kit (#A31881, ThermoFisher Scientific). A subset of samples (two 150 µg/kg Mixed BP females, one 150 µg/kg Mixed BP male) did not contain sufficient DNA for library preparation for oxRRBS. Another subset of samples (one 50 µg/kg BPA male, one 150 µg/kg Mixed BP female, one Corn Oil female) likely had substantial anterior pituitary gland contamination based on the tag-seq results (described in the tag-seq preprocessing section) and were not processed for oxRRBS. To maintain a sample size of 6 per sex per prenatal treatment group, DNA was extracted from additional MPOA samples using the MagMAX DNA Multi-Sample Ultra 2.0 Kit following the manufacturer's instructions for tissue samples (#A45721, ThermoFisher Scientific). DNA quantity was assessed with the Quantifluor dsDNA system (#E2670, Promega).

Library preparation for oxRRBS was done using the Ovation RRBS Methyl-Seq System with the TrueMethyl® oxBS module (Tecan) using 55 ng of input DNA. Between 12-20 samples were processed per batch of library preparation with a total of 5 batches, balanced by prenatal treatment group and sex. The oxidation of DNA prior to bisulfite conversion converts 5hmC to 5fC and allows for discrimination between 5mC and 5hmC at single base-pair resolution. For this study, oxidative bisulfite conversion was used to remove 5hmC and detect only 5mC in the samples. Final libraries were quality-checked using the Agilent Technologies High Sensitivity DNA Kit. Five libraries (one 50 µg/kg BPA female, one

50 µg/kg Mixed BP male, one 50 µg/kg Mixed BP female, and two 150 µg/kg Mixed BP females) were removed due to insufficient final yield. The remaining libraries (n = 4-6 per sex per prenatal treatment group) were pooled with 8-9 libraries per pool (balanced by prenatal treatment group and sex) and submitted for an additional Ampure XP bead clean-up and sequencing to the Genome Sequence and Analysis Facility at the University of Texas at Austin. Reads were sequenced on the NovaSeq SP (150 bp paired-end reads).

**Pipeline and Analyses: Tag-seq**

**Preprocessing** The tag-seq pipeline overview can be found in **Figure 1** and all data and code for the differential gene expression and weighted gene co-expression network analysis can be found at https://github.com/SLauby/bisphenols_maternalcare_epigenome. Adaptor sequences, poly-A tails (> 8 base-pairs long), and low-quality reads were trimmed using a custom script for tag-seq processing (https://github.com/z0on/tag-based_RNAseq) followed by cutadapt (version 1.18). Trimmed reads were mapped to the rat genome (mRatBN7.2; RefSeq) using STAR (version 2.7.3a) with the default settings (output files were unsorted BAM) and sorted using samtools (version 1.10). About 90% of reads were uniquely mapped to the rat genome for all samples. Gene counts were calculated for each sample using the bedtools (version 2.27.1) multicov function. Three samples (one 50 µg/kg BPA male, one 150 µg/kg Mixed BP female, one Corn Oil female) had unusually high counts (over 100x of the other samples) for anterior pituitary gland-specific genes, including *Pomc, Gnrhr,* and *Prl*. These samples were removed from the dataset prior to all downstream analyses.

**Differential gene expression analysis** To examine differential expression of individual genes, including all estrogen and estrogen-related receptors, gene count data was normalized and analyzed for differential expression using the DESeq2 R package (version 1.34.0), separated by sex. Genes with more than 5 counts for more than 6 samples were analyzed. Prenatal treatment and the postnatal maternal care measures (licking/grooming, nest attendance) from PND1-5 were used as independent variables. Each postnatal maternal care measure was scaled and centered where the average is 0 and standard deviation is 1. Comparisons between prenatal treatment groups and interactions with prenatal treatment group and postnatal maternal care were always done relative to the Corn Oil group. The estrogen and estrogen-related receptors (*Esr1, Esr2, Esrra, Esrrb, Esrrg*) were specifically examined for significant interactions between each prenatal treatment group and licking/grooming. Other potential differentially expressed genes (DEGs) were identified with a false discovery rate (FDR) alpha $\leq 0.10$ and $\leq 0.05$ with Benjamani-Hochburg correction. All effects were reported as statistically significant if $p \leq 0.05$ and marginally significant if $p \leq 0.10$.

**Weighted gene co-expression network analysis (WGCNA)** To examine differential expression of co-expressing gene sets, gene networks were created with the WGCNA R package (version 1.72-1) using normalized and log-transformed gene count data, separated by sex. Genes with very low counts and variation were filtered prior to network construction using the goodSamplesGenes function in R with verbosity set to 3. WGCNA constructs matrices with Pearson correlations between every gene and creates clusters of highly correlated genes into eigengene modules (minimum size of 30 genes per module) using a

soft power threshold of 8. A portion of modules had eigengene values that were characterized by one extreme outlier (> 0.90) and low variation following outlier removal (standard deviation < 0.10) and were therefore excluded from further analyses. The eigengene values for the remaining modules were analyzed using the lm function in R. Prenatal treatment and the postnatal maternal care measures (licking/grooming, nest attendance) from PND1-5 were used as independent variables. Each postnatal maternal care measure was scaled and centered where the average is 0 and standard deviation is 1. Comparisons between prenatal treatment groups and interactions with prenatal treatment group and postnatal maternal care were always done relative to the Corn Oil group. For any eigengene module that showed statistically significant main effects or interactions, the gene sets were extracted and analyzed using Enrichr (66,67) using the full gene list used for network construction as the background (https://maayanlab.cloud/Enrichr/). To examine if the gene sets were enriched in estrogen-responsive genes, ESR1 was examined in the ENCODE and ChEA Consensus TFs from ChIP-X module. To examine if the gene sets were enriched in genes that are known to interact with estrogen receptors, ESR1 was examined in the Transcription Factor Protein-Protein Interaction module. To examine the degree of connectivity with the co-expressed gene modules with specific estrogen receptors, the kME was calculated for all estrogen and estrogen-related receptors in the WGCNA package. The kME determines how closely a gene of interest is related to a particular module and is represented as a positive or negative percentage of all genes that have highly correlated expression to the gene of interest. Negative values indicate inverse relationships. All effects were reported as statistically significant if $p \leq 0.05$ and marginally significant if $p \leq 0.10$.

**Pipeline and Analyses: oxRRBS**

**Preprocessing** The oxRRBS pipeline overview can be found in **Figure 1** and all data and code for the differentially methylated region analysis can be found at https://github.com/SLauby/bisphenols_maternalcare_epigenome. Adaptor sequences and low-quality reads (q < 30) were trimmed using trim galore (version 0.6.10) and cutadapt (version 4.6). Diversity sequences and reads without the MspI site (CGG) were removed using a custom python script provided by the manufacturer of the oxRRBS kit (https://github.com/nugentechnologies/NuMetRRBS). Paired-end reads were mapped to the rat genome (mRatBN7.2; RefSeq) using bismark (version 0.22.3) and bowtie2 (version 2.5.2) with the default settings. About 75% of reads were uniquely mapped to the rat genome for all samples. PCR duplicates were removed based on the genomic coordinates of the reads and a 6-bp unique molecular identifier found in the read index using a custom python script provided by the manufacturer of the oxRRBS kit (https://github.com/tecangenomics/nudup). An average of about 8.5 million uniquely aligned, de-duplicated reads per sample were analyzed for DNA methylation levels using the bismark methylation extractor. Bisulfite conversion rate was > 99% for all samples.

**Differentially methylated region (DMR) analysis** Differentially methylated regions (500 bp) between prenatal treatment groups were examined using Methylkit (version 1.26.0), separated by sex. Regions were included in the analysis if there were over 10x reads for at least 4 samples per prenatal treatment group. Batch of library preparation was used as a

covariate in all analyses. DMRs were identified with an FDR q-value ≤ 0.05 using the SLIM method and had > 5% overall methylation difference between groups. Each prenatal treatment group was compared to the Corn Oil group. DMR analyses are currently limited to pairwise comparisons, so an alternative approach was undertaken to examine potential interactions between prenatal bisphenol exposure and postnatal maternal licking/grooming. Each comparison between the prenatal treatments groups and Corn Oil group was done including and excluding postnatal maternal licking/grooming (scaled and centered) as a covariate and the degree of overlap in DMRs was identified. DMRs that were lost or added when including postnatal maternal licking/grooming as a covariate in the analysis were considered to be influenced by postnatal maternal licking/grooming. More specifically, DMRs that were added were assumed to be due to interactions between prenatal bisphenol exposure and postnatal maternal licking/grooming.

**Differentially methylated motif and transcription factor binding site analysis**
All code for the SArKS and MEME Suite analyses can be found at https://github.com/denniscwylie/prenatal_bp_x_lg_epigenome_analysis.
**Suffix Array Kernel Smoothing (SArKS)** For each individual sample, observed methylation sites were partitioned into disjoint genomic ranges separated by at least 51 base pairs. The bedtools merge command was then applied (using the bedtoolsr::bt.merge function in a custom R script) to combine the resulting partitioned range sets from each individual sample into a single set of partitioned genomic ranges in which every individual sample range is contained within one of the merged range intervals. The merged set of genomic ranges were then trimmed using the bedtools coverage command together with a custom R script, only retaining positions which were covered by one of the individual sample-partitioned ranges for at least 25 (out of 43 total) of the individual samples analyzed. For each sample, a local methylation rate was assigned to each genomic position within the merged set of (trimmed) genomic ranges defined above. Each individual base position within each of these partitioned regions is assigned a local methylation rate equal to the estimated methylation percentage for the nearest site observed methylation site (within 50 bp). If the nearest site observed methylation site is > 50 bp, the local methylation rate is considered undefined for the sample. Finally, a single average methylation level for each sample was then computed for each of the merged (and trimmed) genomic ranges as the mean of the assigned individual base methylation rates over all positions within the range (omitting any positions with undefined local methylation rates).

For each genomic range R in the merged (and trimmed) set, a linear model was incorporated to model the arcsin-transformed range-averaged methylation rates as the dependent variable and the library preparation batch covariate and prenatal treatment group as independent predictor variables. Each comparison was done including and excluding postnatal maternal licking/grooming (scaled and centered) as a covariate. In this way, an estimated model coefficient was obtained for each combination of genomic range R and prenatal bisphenol treatment group to the Corn Oil group with and without postnatal maternal licking/grooming as a covariate. For each prenatal bisphenol treatment group comparison, SArKS (59; https://github.com/denniscwylie/sarks) was used to identify

overrepresented sequence motifs found within the various merged genomic ranges whose presence within genomic range R correlates with more extreme (either higher or lower, depending on which direction the analysis was run) estimated model coefficients for each prenatal treatment group comparison associated with the region R.
**MEME Suite** DMRs that were found using Methylkit were used to identify overrepresented sequence motifs with MEME Suite (Version 5.5.7; (68)) using previously published parameters (69) with some modifications. To make the analysis more comparable to SArKS, we included regions that had differential methylation with a nominal p-value ≤ 0.05 and had > 0% overall methylation difference between groups. In addition, MEME was used in discriminative mode using a set of sequences that were not differentially methylated between the prenatal treatment groups and Corn Oil group.

For both SArKS and MEME Suite methods, overrepresented sequence motifs were examined for potential transcription factor binding sites using the JASPAR 2024 CORE (non-redundant) database for vertebrates (70). E-values ≤ 0.05 were considered statistically significant.

**Multi-omic factor analysis (MOFA) of tag-seq and oxRRBS datasets**
Directly comparing gene expression differences and DNA methylation modifications can be challenging when the analysis pipeline for each measure is not integrated. To examine the interactions between prenatal BP exposure and maternal care on the epigenome in a more holistic manner, a MOFA was performed using the MOFA2 package in R (version 1.10.0), separated by sex. Multi-omic analyses can only be performed if the tag-seq and oxRRBS datasets were derived from the same MPOA samples; therefore, the sample sizes were smaller than either the tag-seq or oxRRBS analyses (n = 3-6 per sex per prenatal treatment group). Gene count data were normalized with DESeq2 and log-transformed. DNA methylome data were restricted to one 500 bp region per 2000 bp window to reduce the influence of the high covariance structure of DNA methylation in proximal regions. Proportion of DNA methylation (p) was then log-transformed, similar to M-values in microarray datasets:

$$y = \mathrm{Log2}\left(\left(\frac{p}{1.025} - p\right) + 0.025\right)$$

The gene count dataset was filtered to only include the top 5,000 most variable genes and the DNA methylome dataset was filtered to only include the top 10,000 most variable regions. MOFA2 was performed using the default options, except that the scale views option was set to TRUE, convergence mode was set to slow, and the number of factors was reduced to 5. The weights from each sample and factor were extracted analyzed using the lm function in R. Prenatal treatment and the postnatal maternal care measures (licking/grooming, nest attendance) from PND1-5 (scaled and centered) were used as independent variables. Comparisons between prenatal treatment groups and interactions with prenatal treatment group and postnatal maternal care were always done relative to the Corn Oil group. For any factor that showed statistically significant main effects or interactions, the top genes and regions by weight (250 positive weights and 250 negative

weights) were extracted. The most proximal gene was determined from each region (if within the promoter or gene body) from the oxRRBS dataset and examined for any overlap with the top genes from the tag-seq dataset. The top genes (removing the uncharacterized genes) from the tag-seq and oxRRBS datasets were separately analyzed with Enrichr. Enrichment for ESR1 was examined in the list of ENCODE and ChEA Consensus TFs from ChIP-X and Transcription Factor Protein-Protein Interaction modules. All effects were reported as statistically significant if $p \leq 0.05$ and marginally significant if $p \leq 0.10$.

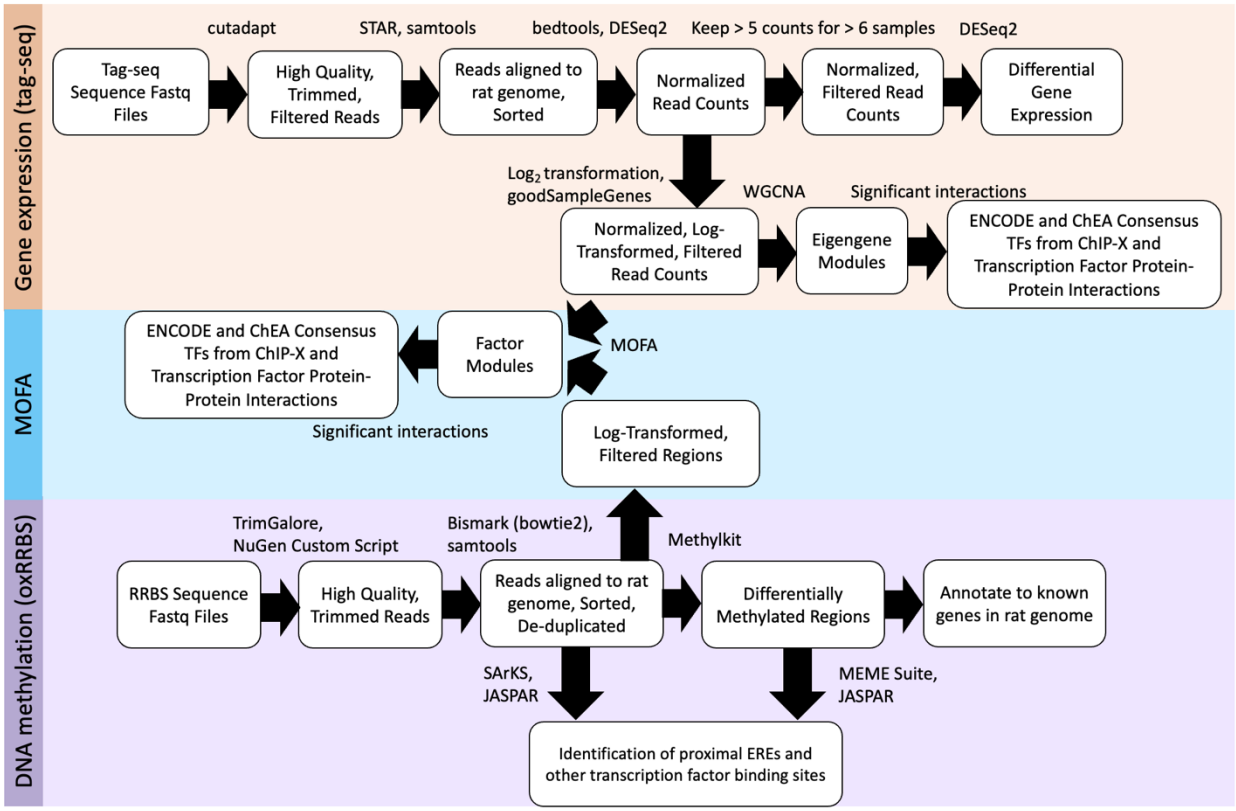


**Figure 1.** Preprocessing and analysis pipelines for gene expression, DNA methylation, and multi-omic analyses.

## Results

### *Interactive effects of prenatal bisphenol exposure and postnatal maternal licking/grooming on Esrrg expression in the MPOA of female pups*

We found a significant interaction between the 150 µg/kg Mixed BP exposure group and maternal licking/grooming on estrogen-related receptor gamma expression (*Esrrg*; $p = 0.0004$) in the female pups (**Figure 2A**) but not the male pups (**Figure 2B**). There were no other significant interactions with the other estrogen and estrogen-related receptors or in the male pups. There were also no significant interactions found between prenatal bisphenol exposure and nest attendance.

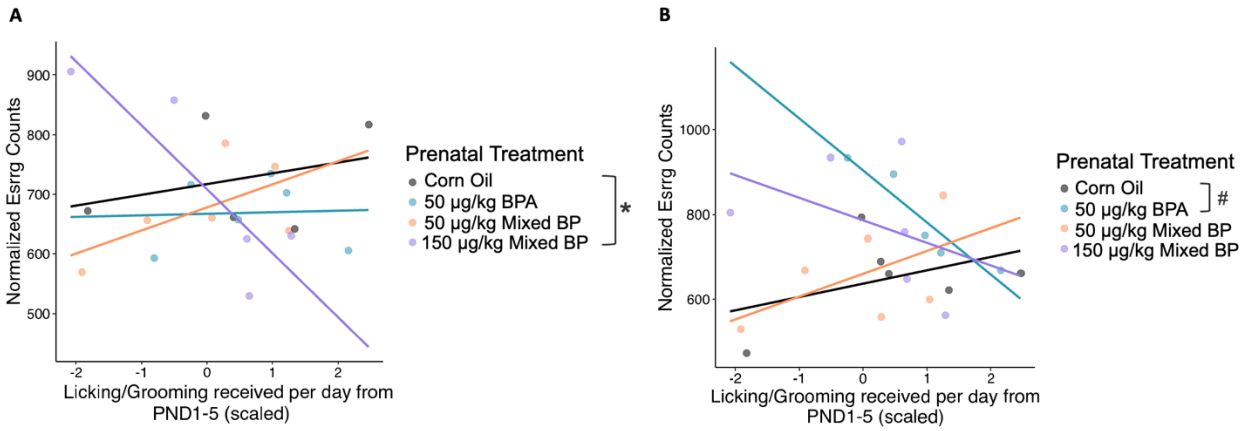


**Figure 2**. Sex-specific interaction between prenatal bisphenol exposure and postnatal maternal licking/grooming on *Esrrg* expression in the medial preoptic area. A significant interaction between the 150 µg/kg Mixed BP exposure group and maternal licking/grooming was found in female pups (**A**) but not male pups (**B**). Scatterplots are displayed with linear regression lines for each prenatal treatment group. * $p < 0.05$ interaction between prenatal treatment and postnatal maternal care; # $p < 0.10$ interaction between prenatal treatment and postnatal maternal care

***Limited interactive effects of prenatal bisphenol exposure and postnatal maternal licking/grooming on differential expressed genes (DEGs)***

We examined the effects of prenatal bisphenol exposure and its interactions with postnatal maternal care on DEGs. **Supplementary Table 1** summarizes the DESeq2 output. Overall, there were very few DEGs found in all comparisons. The only exception was that there were 63 DEGs (FDR $\leq 0.10$) found when examining the interactions between the 150 µg/kg Mixed BP exposure group and maternal licking/grooming in female pups (including *Esrrg*); however, none of these DEGs passed FDR correction of $\leq 0.05$.

***WCGNA gene expression modules associated with interactive effects of prenatal bisphenol exposure and postnatal maternal licking/grooming***

For the WGCNA analyses, a total of 7 modules for male pups and 13 modules for female pups were analyzed. For the male pups, there was only a significant main effect of prenatal treatment group found for one module, where the eigenvalues for the 50 µg/kg Mixed BP exposure group were lower than the Corn Oil group. For the female pups, there were significant or marginal interactions between the 150 µg/kg Mixed BP exposure group and maternal licking/grooming on five modules (Blue, Brown, Magenta, Turquoise, Yellow). More specifically, there was a marginal interaction ($t = -2.179$, $p = 0.0544$) for the Blue module (4,022 genes; **Figure 3A**), a significant interaction ($t = -2.544$, $p = 0.0292$) for the Brown module (2,481 genes; **Figure 3B**), a marginal interaction ($t = -2.205$, $p = 0.0520$) for the Magenta module (980 genes; **Figure 3C**), a significant interaction ($t = 2.291$, $p = 0.0449$) for the Turquoise module (5,229 genes; **Figure 3D**), and a significant interaction ($t = 2.061$, $p = 0.0264$) for the Yellow module (1,445 genes; **Figure 3E**). Top GO terms for each of the modules in which there significant or marginal interactions in female offspring are presented in **Figure 3F**. There were no significant interactions found between prenatal

bisphenol exposure and nest attendance; however, including nest attendance and the interaction between prenatal bisphenol exposure and nest attendance in the linear model was required to reveal the interactions between the 150 µg/kg Mixed BP exposure group and maternal licking/grooming.

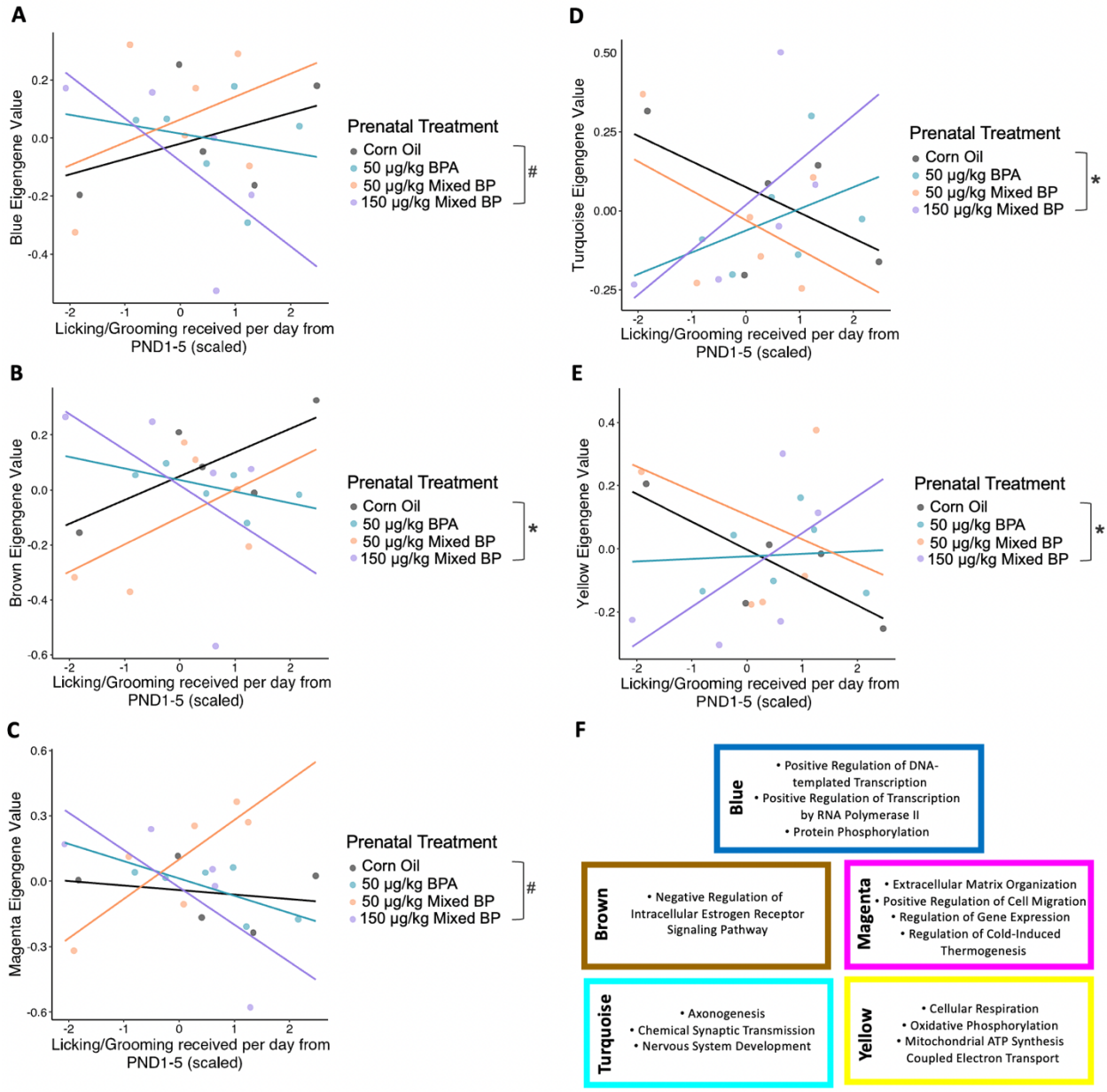


**Figure 3.** Interactions between prenatal bisphenol exposure and postnatal maternal licking/grooming on co-expressed gene modules in the medial preoptic area of female pups. Significant or marginal interactions between the 150 µg/kg Mixed BP exposure group and maternal licking/grooming were found in the Blue (**A**), Brown (**B**), Magenta (**C**), Turquoise (**D**), and Yellow (**E**) eigengene modules. Top GO terms (**F**) from these modules were related to estrogen receptor signaling, regulation of gene expression, neurodevelopment, neurotransmission, and cellular metabolism. Scatterplots are

displayed with linear regression lines for each prenatal treatment group. * $p < 0.05$ interaction between prenatal treatment and postnatal maternal care; # $p < 0.10$ interaction between prenatal treatment and postnatal maternal care

***Gene expression module enrichment for estrogen-responsive genes and genes that interact with estrogen receptors***

Among the modules in which there were significant or marginal interactions between prenatal BP and postnatal maternal care in female offspring (**Figure 3**), we examined enrichment for estrogen-related and -responsive genes. In the Blue module, there was a significant enrichment of ESR1 found in the lists of ENCODE and ChEA Consensus TFs from ChIP-X (FDR = 0.003) and Transcription Factor Protein-Protein Interactions (FDR < 0.001). In the Brown module, there was no enrichment of ESR1 found in the list of ENCODE and ChEA Consensus TFs from ChIP-X or Transcription Factor Protein-Protein Interactions. Notably, this module contains *Esr1*, *Esr2*, and *Esrrg*. In the Magenta module, there was a significant enrichment of ESR1 found in the list of ENCODE and ChEA Consensus TFs from ChIP-X (FDR = 0.043) but not Transcription Factor Protein-Protein Interactions. In the was a marginal enrichment of ESR1 found in the list of ENCODE and ChEA Consensus TFs from ChIP-X (FDR = 0.054) and a significant enrichment of ESR1 found in the list of Transcription Factor Protein-Protein Interactions (FDR = 0.003). In the Yellow module, there was a significant enrichment of ESR1 found in the list of ENCODE and ChEA Consensus TFs from ChIP-X (FDR = 0.038) and Transcription Factor Protein-Protein Interactions (FDR < 0.001).

***Connectivity between Esrrg and all gene expression modules with interactions between prenatal bisphenol exposure and postnatal maternal licking/grooming***

We examined the connectivity of each of the estrogen and estrogen-related receptors for each module in which there were significant or marginal interactions between prenatal BP and postnatal maternal care in female offspring. **Table 1** summarizes these results. Most notably, *Esrrg* had moderate-high positive connectivity with the Blue, Brown, and Magenta modules (68%, 73%, and 50% respectively) and moderate-high negative connectivity with the Turquoise and Yellow modules (-69% and -54%, respectively).

**Table 1.** Connectivity (kME) of estrogen receptors with genes within the co-expressed gene modules generated by the WGCNA analysis. Values are represented as percentages of genes within each module that have significant correlations. Negative values indicate inverse correlations.

| | kME Blue | kME Brown | kME Magenta | kME Turquoise | kME Yellow |
|---|---|---|---|---|---|
| ***Esr1*** | 9.98 | 32.93 | -30.90 | -13.40 | -1.53 |
| ***Esr2*** | 25.06 | 41.06 | 16.37 | -34.39 | -48.27 |
| ***Esrra*** | 13.74 | 18.76 | -9.93 | -20.91 | -14.89 |
| ***Esrrb*** | 62.23 | 25.80 | 81.92 | -54.04 | -17.94 |
| ***Esrrg*** | 68.27 | 73.13 | 50.20 | -68.89 | -53.58 |

***Impact of postnatal maternal licking/grooming on differentially methylated regions in prenatal bisphenol-exposed pups***

We examined the influence of postnatal maternal licking/grooming on differentially methylated regions (DMRs) in the developing MPOA among the prenatal treatment groups. To do so, we assessed the degree of overlap in DMRs when we include or exclude maternal licking/grooming as a covariate while comparing DNA methylation levels between each prenatal bisphenol treatment group to the Corn Oil group. Overall, while many DMRs were unchanged when including maternal licking/grooming as a covariate, there were some discrepancies found for all comparisons between prenatal bisphenol treatment groups in male and female pups. Notably, in most comparisons there were more DMRs that were added than lost when including maternal licking/grooming as a covariate.

**DMRs influenced by maternal licking/grooming in male pups** For male pups with prenatal 50 µg/kg BPA exposure, there were 43 DMRs (corresponding to 32 genes) that were unchanged, 49 DMRs (corresponding to 31 genes) that were lost, and 54 DMRs (corresponding to 38 genes) that were added when including maternal licking/grooming as a covariate (**Figure 4A**; **Supplementary Table 2**). In the prenatal 50 µg/kg Mixed BP group, there were 31 DMRs (corresponding to 16 genes) that were unchanged, 7 DMRs (corresponding to one gene) that were lost, and 47 DMRs (corresponding to 35 genes) that were added when including maternal licking/grooming as a covariate (**Figure 4B**; **Supplementary Table 3**). In the prenatal 150 µg/kg Mixed BP group, there were 32 DMRs (corresponding to 21 genes) that were unchanged, 18 DMRs (corresponding to 10 genes) that were lost, and 64 DMRs (corresponding to 36 genes) that were added when including maternal licking/grooming as a covariate (**Figure 4C**; **Supplementary Table 4**).

**DMRs influenced by maternal licking/grooming in female pups** For female pups with prenatal 50 µg/kg BPA exposure, there were 20 DMRs (corresponding to nine genes) that were unchanged, 23 DMRs (corresponding to 14 genes) that were lost, and 29 DMRs (corresponding to 15 genes) that were added when including maternal licking/grooming as a covariate (**Figure 4D; Supplementary Table 5**). In the prenatal 50 µg/kg Mixed BP group, there were 15 DMRs (corresponding to 12 genes) that were unchanged, five DMRs (corresponding to three genes) that were lost, and two DMRs (both in intergenic regions) that were added when including maternal licking/grooming as a covariate (**Figure 4E; Supplementary Table 6**). In the prenatal 150 µg/kg Mixed BP group, there were 60 DMRs (corresponding to 48 genes) that were unchanged, 10 DMRs (corresponding to eight genes) that were lost, and 25 DMRs (corresponding to 16 genes) that were added when including maternal licking/grooming as a covariate (**Figure 4F; Supplementary Table 7**).

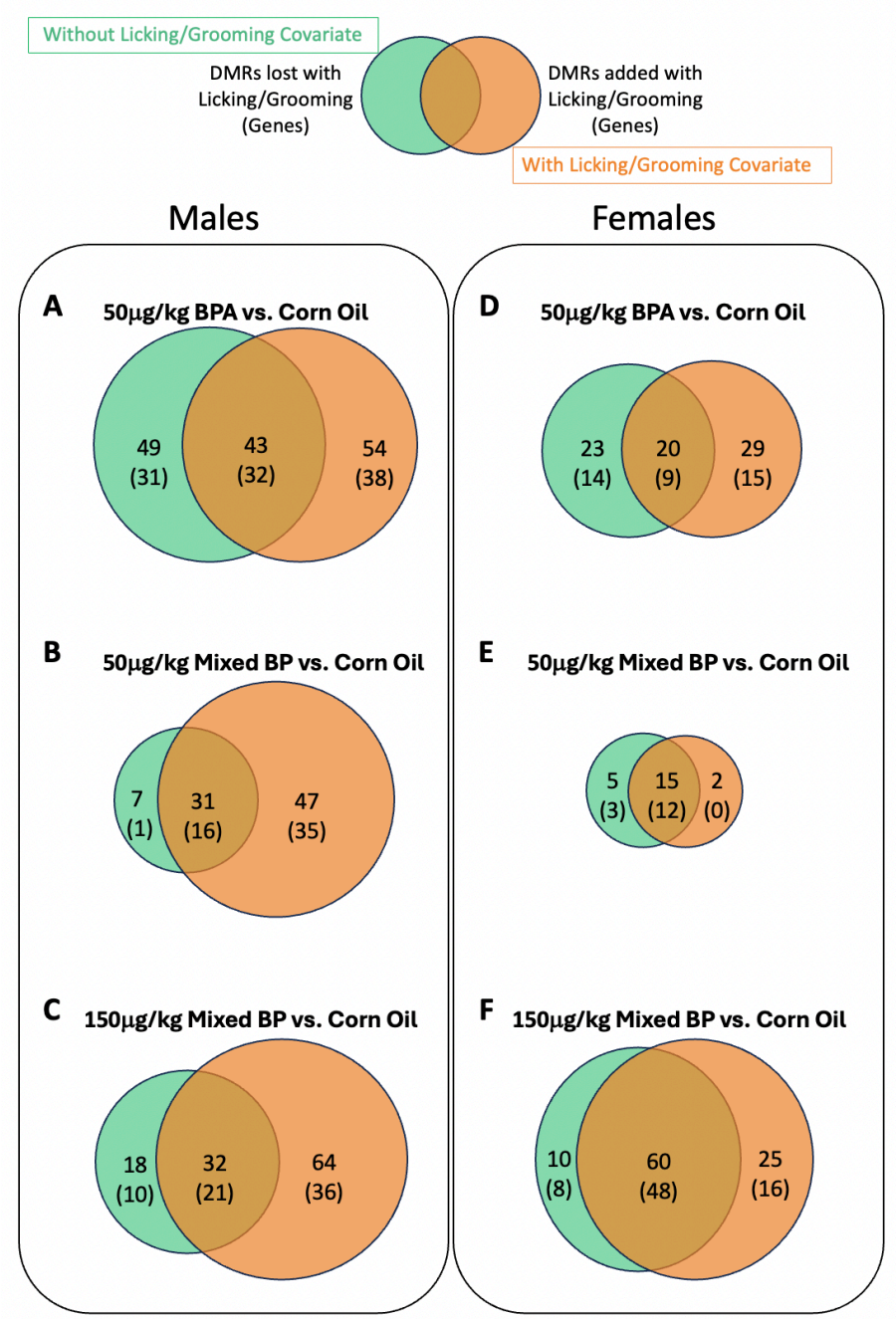


**Figure 4.** Influence of postnatal maternal licking/grooming on differentially methylated regions (DMRs) of prenatal bisphenol-exposed male and female pups. Including maternal licking/grooming as a covariate altered DMRs in all prenatal treatment groups in both male (**A-C**) and female (**D-F**) pups. Venn diagrams are displayed with the number of DMRs and annotated genes when excluding (green) and including (orange) maternal licking/grooming as a covariate. DMRs that do not overlap are considered to be influenced by postnatal maternal licking/grooming.

While the gene lists for each comparison were too small to run any functional enrichment analyses (e.g., GO), there were genes related to neurotransmission (e.g., Grik1, Kcnip1,

Hcn2), neurodevelopment (e.g., Epha6, Nlgn3, Sash1), gene regulation (e.g., Arx, Zfp521, Mir135b), and mitochondrial function (e.g., Mtfr2, Gpam, Mthfd2) throughout all of the comparisons between prenatal bisphenol treatment and Corn Oil groups, similar to the gene modules of interest in the WGCNA analysis. There were also a few notable neuroendocrine-related genes (Ddc, Maoa, Kiss1, Glpr1, Igf2bp2) found throughout all comparisons between prenatal bisphenol treatment and Corn Oil groups.

***Impact of postnatal maternal licking/grooming on differentially methylated transcription factor binding sites in prenatal bisphenol-exposed pups***

We next examined the influence of postnatal maternal licking/grooming on differential methylation of proximal transcription factor binding sites and EREs. To assess this outcome, we performed a motif analysis using two different methods (SArKS and MEME Suite) to identify overrepresented sequence motifs and JASPAR to match the motifs to known transcription factor binding sites.

**Differentially methylated transcription factor binding sites in male pups** When excluding licking/grooming as a covariate in male pups, SArKS identified four significant motifs associated with four unique transcription factors in hypomethylated regions with prenatal 50 µg/kg BPA exposure, 23 significant motifs associated with 17 unique transcription factors in hypermethylated regions with prenatal 50 µg/kg Mixed BP exposure, and six significant motifs associated with five unique transcription factors in hypomethylated regions with prenatal 150 µg/kg Mixed BP exposure (**Supplementary Table 8**). When including licking/grooming as a covariate, SArKS identified 18 significant motifs associated with seven unique transcription factors in hypomethylated regions with prenatal 50 µg/kg BPA exposure, 16 significant motifs associated with 13 unique transcription factors in hypermethylated regions with prenatal 50 µg/kg Mixed BP exposure, and four significant motifs associated with four unique transcription factors in hypomethylated regions with prenatal 150 µg/kg Mixed BP exposure (**Supplementary Table 9**). Overall, when including maternal licking/grooming as a covariate, three new transcription factor binding sites (EBF2, GLI3, TFAP2C) were identified in hypomethylated regions with prenatal 50 µg/kg BPA exposure, three new transcription factor binding sites were identified (NRF1, E2F3, EGR3) in hypermethylated regions with prenatal 50 µg/kg Mixed BP exposure, and two new transcription factor binding sites (EGR3, E2F7) were identified in hypomethylated regions with prenatal 150 µg/kg Mixed BP exposure (**Figure 5A**).

**Differentially methylated transcription factor binding sites in female pups** When excluding licking/grooming as a covariate in female pups, SArKS identified 34 significant motifs associated with 10 unique transcription factors in hypomethylated regions and two significant motifs associated with two transcription factors in hypermethylated regions with prenatal 50 µg/kg Mixed BP exposure only (**Supplementary Table 10**). When including licking/grooming as a covariate, SArKS identified three significant motifs associated with two unique transcription factors in hypermethylated regions with prenatal 50 µg/kg BPA exposure as well as two significant motifs associated with two unique transcription factors in hypomethylated regions and four significant motifs associated with four transcription

factors in hypermethylated regions with prenatal 50 µg/kg Mixed BP exposure (**Supplementary Table 11**). Overall, when including maternal licking/grooming as a covariate, two new transcription factor binding sites (SP5, ZNF610) were identified with prenatal 50 µg/kg BPA exposure and two new transcription factor binding sites (HAND2, IKZF2) were identified with prenatal 50 µg/kg Mixed BP exposure, all within hypermethylated regions (**Figure 5B**).

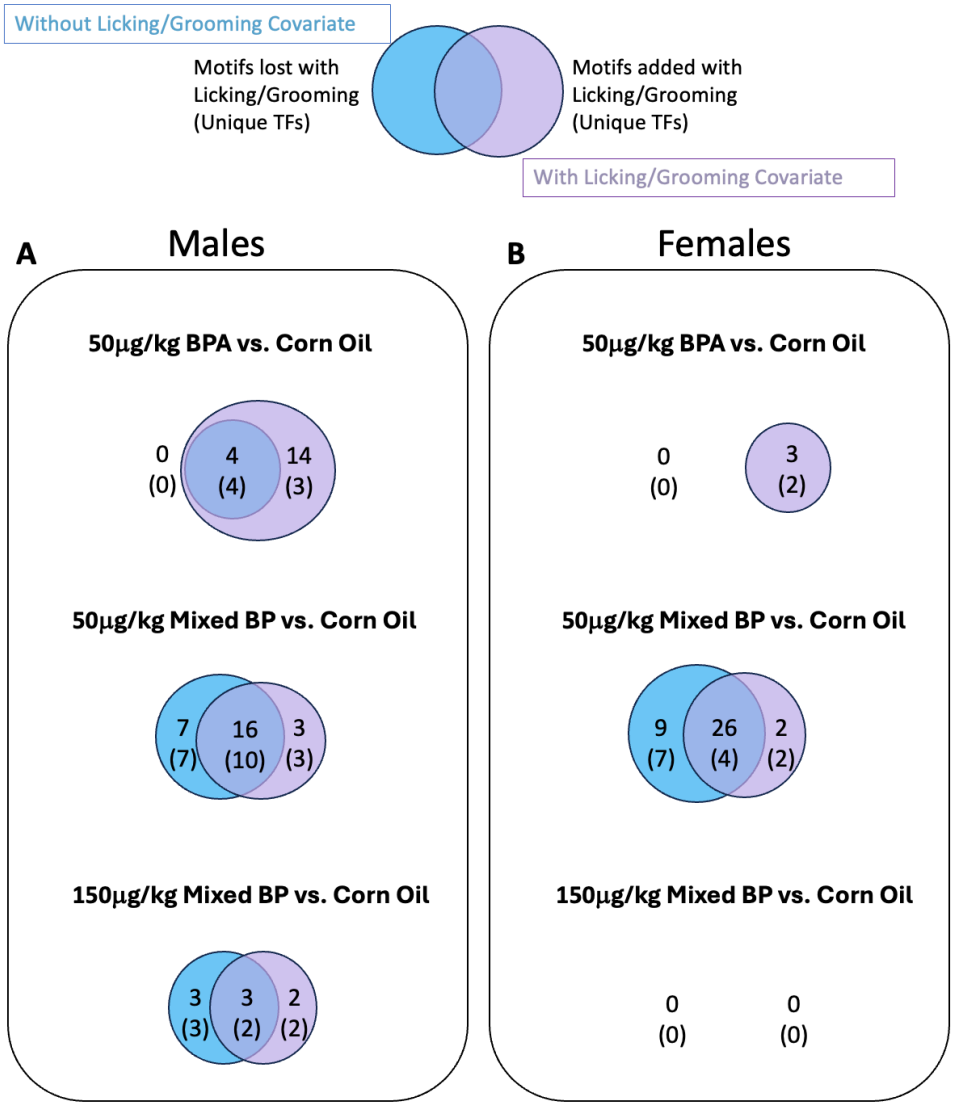


**Figure 5.** Influence of postnatal maternal licking/grooming on differentially methylated transcription factor binding sites of prenatal bisphenol-exposed male and female pups using the SArKS method. Including maternal licking/grooming as a covariate altered overrepresented sequence motifs and predicted transcription factor binding sites in most prenatal treatment groups in male (**A**) and female (**B**) pups. Venn diagrams are displayed with the number of motifs and unique transcription factors when excluding (blue) and including (purple) maternal licking/grooming as a covariate. Motifs that do not overlap are considered to be influenced by postnatal maternal licking/grooming.

There were several predicted transcription factor binding sites that reappeared using the MEME Suite method in male offspring (ZEB1, SP5, HAND2, RBPJ) and female offspring (ELK1, IKZF2, SP5, RBPJ, ZNF610, HAND2). There were also changes in motifs and predicted transcription factor binding sites when adding postnatal maternal licking/grooming as a covariate with MEME Suite (**Supplementary Figure 1**). In many comparisons, there were a larger number of motifs and predicted transcription factor binding sites that were altered with postnatal maternal licking/grooming and less overlap using the MEME Suite method than the SArKS method.

While no estrogen receptors were found in the list of predicted transcription factor binding sites, many of the predicted transcription factors have known interactions with estrogen receptors. This includes transcription factors that are known to dimerize with ESR1, block estrogen receptors from binding to DNA, facilitate transcription of *Esr1*, or are estrogen-responsive genes themselves (**Table 2**).

**Table 2.** List of unique transcription factors associated with prenatal bisphenol exposure and/or postnatal maternal licking/grooming in male and female pups and their known interactions with estrogen receptors.

| Analysis | Methylation comparison | Transcription factor name | Related to estrogen receptors? |
|---|---|---|---|
| **Males** | | | |
| **Unchanged with Licking/Grooming** | | | |
| 50 μg/kg BPA vs. Corn Oil | Hypomethylated | **ZEB1** | (71,72) |
| 50 μg/kg BPA vs. Corn Oil | Hypomethylated | **RBPJ** | (73) |
| 50 μg/kg BPA vs. Corn Oil | Hypomethylated | SP5 | |
| 50 μg/kg BPA vs. Corn Oil | Hypomethylated | **HAND2** | (74,75) |
| 50 μg/kg Mixed BP vs. Corn Oil | Hypermethylated | ZNF610 | |
| 50 μg/kg Mixed BP vs. Corn Oil | Hypermethylated | E2F7 | |
| 50 μg/kg Mixed BP vs. Corn Oil | Hypermethylated | **NHLH2** | (76) |
| 50 μg/kg Mixed BP vs. Corn Oil | Hypermethylated | ZNF417 | |
| 50 μg/kg Mixed BP vs. Corn Oil | Hypermethylated | ZFP57 | |
| 50 μg/kg Mixed BP vs. Corn Oil | Hypermethylated | **SP3** | (77) |
| 50 μg/kg Mixed BP vs. Corn Oil | Hypermethylated | **KLF15** | (78–80) |
| 50 μg/kg Mixed BP vs. Corn Oil | Hypermethylated | **SP1** | (77,81,82) |
| 50 μg/kg Mixed BP vs. Corn Oil | Hypermethylated | YY2 | |
| 50 μg/kg Mixed BP vs. Corn Oil | Hypermethylated | ZBED1 | |
| 150 μg/kg Mixed BP vs. Corn Oil | Hypomethylated | ZNF417 | |
| 150 μg/kg Mixed BP vs. Corn Oil | Hypomethylated | SP5 | |
| **Lost with Licking/Grooming** | | | |
| 50 μg/kg Mixed BP vs. Corn Oil | Hypermethylated | GCM2 | |
| 50 μg/kg Mixed BP vs. Corn Oil | Hypermethylated | SP5 | |

| | | | |
|---|---|---|---|
| 50 μg/kg Mixed BP vs. Corn Oil | Hypermethylated | **GLI3** | (83) |
| 50 μg/kg Mixed BP vs. Corn Oil | Hypermethylated | **KLF13** | (84) |
| 50 μg/kg Mixed BP vs. Corn Oil | Hypermethylated | **EBF1** | (85,86) |
| 50 μg/kg Mixed BP vs. Corn Oil | Hypermethylated | EBF4 | |
| 150 μg/kg Mixed BP vs. Corn Oil | Hypomethylated | EBF2 | |
| 150 μg/kg Mixed BP vs. Corn Oil | Hypomethylated | **HAND2** | (74,75) |
| 150 μg/kg Mixed BP vs. Corn Oil | Hypomethylated | **RBPJ** | (73) |
| **Added with Licking/Grooming** | | | |
| 50 μg/kg BPA vs. Corn Oil | Hypomethylated | EBF2 | |
| 50 μg/kg BPA vs. Corn Oil | Hypomethylated | **GLI3** | (83) |
| 50 μg/kg BPA vs. Corn Oil | Hypomethylated | **TFAP2C** | (87,88) |
| 50 μg/kg Mixed BP vs. Corn Oil | Hypermethylated | **NRF1** | (89–91) |
| 50 μg/kg Mixed BP vs. Corn Oil | Hypermethylated | **EGR3** | (92) |
| 50 μg/kg Mixed BP vs. Corn Oil | Hypermethylated | E2F3 | |
| 150 μg/kg Mixed BP vs. Corn Oil | Hypomethylated | **EGR3** | (92) |
| 150 μg/kg Mixed BP vs. Corn Oil | Hypomethylated | E2F7 | |
| **Females** | | | |
| **Unchanged with Licking/Grooming** | | | |
| 50 μg/kg Mixed BP vs. Corn Oil | Hypomethylated | LHX1 | |
| 50 μg/kg Mixed BP vs. Corn Oil | Hypomethylated | BHLHE22 | |
| 50 μg/kg Mixed BP vs. Corn Oil | Hypermethylated | **RBPJ** | (73) |
| 50 μg/kg Mixed BP vs. Corn Oil | Hypermethylated | SP5 | |
| **Lost with Licking/Grooming** | | | |
| 50 μg/kg Mixed BP vs. Corn Oil | Hypomethylated | **KLF15** | (78–80) |
| 50 μg/kg Mixed BP vs. Corn Oil | Hypomethylated | ERF::NHLH1 | |
| 50 μg/kg Mixed BP vs. Corn Oil | Hypomethylated | **ELK1** | (93) |
| 50 μg/kg Mixed BP vs. Corn Oil | Hypomethylated | LHX5 | |
| 50 μg/kg Mixed BP vs. Corn Oil | Hypomethylated | ARGFX | |
| 50 μg/kg Mixed BP vs. Corn Oil | Hypomethylated | POU6F1 | |
| 50 μg/kg Mixed BP vs. Corn Oil | Hypomethylated | E2F7 | |
| **Added with Licking/Grooming** | | | |
| 50 μg/kg BPA vs. Corn Oil | Hypermethylated | ZNF610 | |
| 50 μg/kg BPA vs. Corn Oil | Hypermethylated | SP5 | |
| 50 μg/kg Mixed BP vs. Corn Oil | Hypermethylated | **HAND2** | (74,75) |
| 50 μg/kg Mixed BP vs. Corn Oil | Hypermethylated | IKZF2 | |

**Interactive effects of prenatal bisphenol exposure and postnatal maternal licking/grooming on the multi-omic epigenome in the MPOA of female pups**

We examined the influence of postnatal maternal licking/grooming on the combined DNA methylome and transcriptome in the developing MPOA using MOFA. For male pups, there were two factors that showed significant interactions of prenatal BP exposure and maternal

licking/grooming; however, upon further inspection these interactions appear to be solely driven by one outlier in both factors (data not shown). For female pups, there was one factor (Factor 4) that showed significant interactions between the 150 µg/kg Mixed BP exposure group and maternal licking/grooming (t = 4.456, p = 0.0043; **Figure 6A**) as well as nest attendance (t = 3.182, p = 0.0190; **Figure 6B**). Similar to the WGCNA analysis, including nest attendance and the interaction between prenatal bisphenol exposure and nest attendance in the linear model was required to reveal the significant interaction between the 150 µg/kg Mixed BP exposure group and maternal licking/grooming.

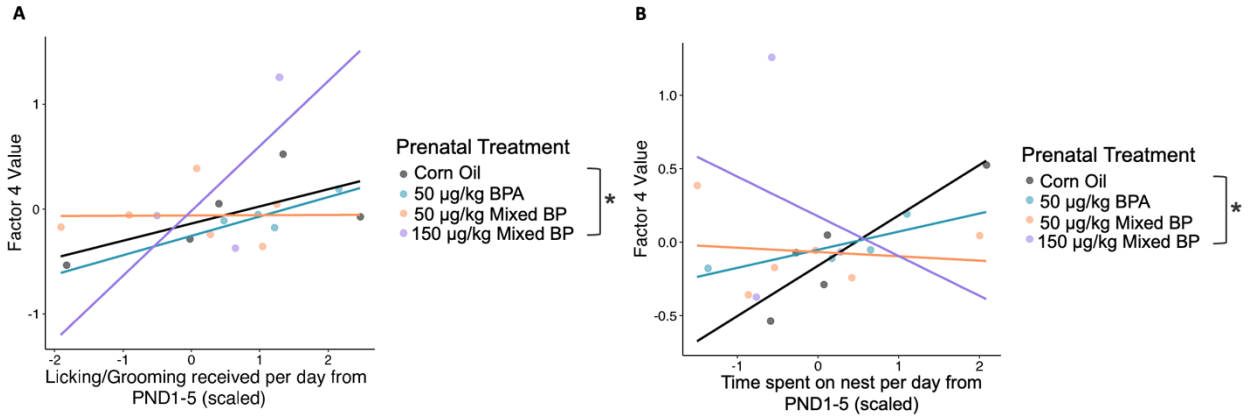


**Figure 6.** Interactions between prenatal bisphenol exposure and postnatal maternal licking/grooming on Factor 4 generated from the multi-omic factor analysis (MOFA) in female pups. Significant interactions between the 150 µg/kg Mixed BP exposure group and maternal licking/grooming (**A**) and nest attendance (**B**) were found when combining the transcriptome and DNA methylome datasets. Scatterplots are displayed with linear regression lines for each prenatal treatment group. * $p < 0.05$ interaction between prenatal treatment and postnatal maternal care

***Factor module enrichment for estrogen-responsive genes and genes that interact with estrogen receptors*** For Factor 4, there were only 14 genes that overlapped between the top genes in the tag-seq and oxRRBS datasets. For the top genes in the tag-seq dataset (467 genes analyzed) there was a significant enrichment of ESR1 found in the list of Transcription Factor Protein-Protein Interactions (FDR < 0.001). For the top genes in the oxRRBS dataset (423 genes analyzed) there was a significant enrichment of ESR1 found in the list of ENCODE and ChEA Consensus TFs from ChIP-X (FDR = 0.031). There was also a nominal enrichment of ESR1 found in the list of Transcription Factor Protein-Protein Interactions (p = 0.0178), but it did not pass FDR correction (FDR > 0.10).

## Discussion

In this study, we examined the interacting and possibly mitigating impacts of postnatal maternal licking/grooming in prenatal bisphenol-exposed pups on the transcriptome and the DNA methylome in the developing MPOA. We also investigated the role of estrogen receptor signaling changes as a potential biological mechanism underlying these

interactions. Overall, we found that postnatal maternal licking/grooming has a substantial influence on gene expression and DNA methylation changes, particularly in female pups. This might be driven by the mitigating effect of maternal licking/grooming on *Esrrg* expression rather than *Esr1*. Given that ESRRG has a high affinity for bisphenols (94,95) and can facilitate gene expression by binding to estrogen responsive elements or its own specific binding motif, these results suggest a novel biological mechanism in which postnatal maternal care can mitigate the negative neurodevelopmental impacts of prenatal bisphenol exposure.

We hypothesized that the interactions between prenatal bisphenol exposure and postnatal maternal licking/grooming would primarily involve changes in *Esr1* expression in the MPOA and that the effects would be stronger in female pups than male pups. However, we found sex-specific interactions in *Esrrg* expression that appear to be associated with multiple co-expressed gene modules in the WGCNA analysis. While it is known that BPA exposure can stimulate expression of *Esrrg* in a sex-specific manner (25,96) and has a high binding affinity to ESRRG (94,95), less is known about the effects of postnatal maternal care on *Esrrg* expression or signaling changes. A prior study examining prenatal BPA exposure and postnatal maternal care conducted in our lab found brain region and sex-specific effects of maternal care on *Esrrg* expression, where there was an increase in expression in the prefrontal cortex in female offspring only and in the hypothalamus in male and female offspring (25). There were no interactions between prenatal BPA and postnatal maternal care on *Esrrg* expression noted in the study. In our current study, we found postnatal maternal licking/grooming decreased and normalized *Esrrg* expression in the female pups with prenatal 150 µg/kg Mixed BP exposure. The discrepancies may be primarily due to the different ages assessed (weanlings vs. PND10 pups), the bisphenol doses used, and the bisphenol mixture used in our study may have a different effect than BPA alone. While the sex-specific interactions in gene expression were consistent in our hypothesis, we also found that postnatal maternal licking/grooming has a substantial influence on DNA methylation changes in prenatal bisphenol-exposed male offspring. We also found in prior studies that postnatal maternal care can interact and mitigate the impacts of prenatal bisphenol exposure on later-life phenotypes and gene expression in the prefrontal cortex and amygdala at PND10 in male offspring (34). Therefore, it is possible that postnatal maternal licking/grooming can mitigate the impacts of prenatal bisphenol exposure in male offspring, but the underlying biological mechanisms are currently unclear and/or are not strongly related to estrogen receptor signaling. As an example, previous work using another estrogenic endocrine disrupting chemical (Aroclor 1221) found estradiol levels predicted behavioral phenotypes in prenatal Aroclor 1221-exposed females while dopamine-related gene expression predicted behavioral phenotypes in prenatal Aroclor 1221-exposed male offspring (97). These sex-specific impacts are worth exploring in future studies.

Our WGCNA analysis indicated that including postnatal nest attendance in our statistical models was required to unveil the significant interactions between the 150 µg/kg Mixed BP exposure group and maternal licking/grooming but was not significantly associated with these modules by itself. Postnatal nest attendance includes licking/grooming and other

forms of pup contact, including thermotactile contact. This suggests that postnatal licking/grooming is the primary driver of these interactions and overall pup contact plays a minor but possibly important role (47) in gene expression changes for prenatal bisphenol-exposed female pups. Directly manipulating licking-like tactile stimulation while keeping other forms of pup contact consistent would confirm these findings and is an ongoing area of investigation in our lab.

Our DMR analysis indicates postnatal maternal licking/grooming has a substantial influence on DNA methylation changes in prenatal bisphenol-exposed pups. The finding that more DMRs were added than lost when including postnatal maternal licking/grooming as a covariate suggests that postnatal maternal licking/grooming plays more of a moderating role in shaping the DNA methylome in prenatal bisphenol-exposed pups, consistent with our prior findings (34,39). However, it was apparent that the differential methylation findings did not map consistently onto differential gene expression. While it is assumed that changes in DNA methylation and gene expression would be strongly linked, there are several reasons why there may be a discordance. First, we did not implement any substantial environmental challenges to the pups immediately prior to brain collection. While DNA methylation changes are relatively stable, gene expression changes are dependent on both the availability of chromatin to allow transcription and transcriptional activity itself. Therefore, the associations between DNA methylation and gene expression changes may be stimulus-dependent. The second reason is that there are inherent differences in the approach in which these changes are assessed. While we assessed the whole transcriptome, we only probed a small portion of CpG sites in the genome. In addition, many of these CpG sites are intergenic and we have an incomplete understanding of the role of DNA methylation in distal CpG sites on gene expression. Finally, in any particular cell, DNA methylation is mainly binary (methylated or unmethylated with rare instances of hemi-methylation) while gene expression changes can have a large range. This could complicate analyses when using bulk tissue and would need to be resolved at the single-cell level. Nevertheless, when we integrated the DNA methylation and gene expression data using MOFA, we generally replicate the gene expression findings. This confirms that the significant interactions between the 150 μg/kg Mixed BP exposure group and maternal licking/grooming in female pups apply to both gene expression and DNA methylation.

We explored potential transcription factor binding sites associated with DNA methylation changes with prenatal bisphenol exposure and postnatal maternal licking/grooming. To do so, we adapted the SArKS method to RRBS data so we can correlate DNA methylation changes in prenatal bisphenol-exposed pups with and without postnatal maternal licking/grooming as a covariate to motifs and possible transcription factor binding sites. As a comparison, we also used MEME Suite to identify motifs based on the changes in DMRs with and without postnatal maternal licking/grooming as a covariate. The main difference with using a correlative-based method is that it accounts for the continuous range of DNA methylation changes (0-100%) instead of a binary representation (i.e., it is differentially methylated or not differentially methylated). A correlative-based method can be

particularly advantageous with more complex study designs where multiple independent variables are assessed. When a binary representation is applied to DMR data with and without covariates, there is an assumption that all DMRs that change will also have the same effect size changes and would be equally important for motif discovery. In real-world datasets, accounting for the continuous range of differential methylation and effect size changes with covariates can provide a more accurate representation of the potential transcription factors involved with differential methylation at specific loci. With the SArKS method, we found numerous motifs and transcription factor binding site predictions that change when including postnatal maternal licking/grooming as a covariate, as we would have expected based on the DMR analysis. This was also the case with the MEME Suite method and the changes were sometimes even more pronounced, possibly due to the binary representation of the input sequences as described above.

A consistent theme throughout our analyses was that the genes and transcription factor binding site predictions associated with prenatal bisphenol exposure and postnatal maternal licking/grooming as well as their interactions were either related to estrogen receptor signaling or were estrogen-responsive genes. In prior transcriptome analyses in the PND10 prefrontal cortex and amygdala, we found consistently strong and significant enrichment of ESR1 in the Transcription Factor Protein-Protein Interactions module in Enrichr (34). This was also the case in the PND10 MPOA, but we also found significant (though weaker) enrichment of ESR1 in the ENCODE and ChEA Consensus TFs from ChIP-X module. These findings are consistent with our hypothesis that the interactions between prenatal bisphenol exposure and postnatal maternal licking/grooming converge on estrogen receptor signaling, and that we are primarily finding secondary effects of estrogen receptor signaling changes by PND10.

One notable limitation of this study was while we found consistent interactions between postnatal licking/grooming and the 150 µg/kg Mixed BP dose, this dosage is not considered environmentally relevant and is generally higher than human exposure levels. However, bisphenol exposure follows a non-monotonic dose response curve (98,99), meaning that very high levels and very low levels will exert biological effects. In our study, the 50 µg/kg Mixed BP and BPA-only dose did not facilitate any increases of *Esrrg* expression in female pups; however, *Esrrg* expression may change with doses lower than 50 µg/kg, which would be environmentally relevant. Consistent with this logic, our lab's previous work on prenatal BPA exposure found that the 2 µg/kg and 20 µg/kg doses alter *Esrrg* expression in the prefrontal cortex and hypothalamus (25). Additional studies using lower doses are needed to ensure the translational potential of our current findings.

Overall, we found that postnatal maternal licking/grooming can mitigate the impacts of prenatal bisphenol exposure on *Esrrg* expression and co-expressed gene modules in the developing MPOA in a dose-dependent and sex-specific manner. This appears to correspond to changes in DNA methylation, which can persist beyond both transient environmental exposures and impact neurodevelopmental trajectories and behavioral phenotype. Whether licking-like tactile stimulation can be implemented as an effective

intervention against the adverse neurodevelopmental impacts of prenatal bisphenol exposure in female offspring remains an open question but will be important to examine in the future. Exploring this issue will allow us to infer causality and confirm that prenatal bisphenol exposure and postnatal maternal licking/grooming are acting on the same biological pathways in opposing directions. This approach will be essential to identifying potential avenues to mitigate the effects of prenatal bisphenol exposure and improve health and well-being in human populations.

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