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

Short title: Postnatal maternal care impacts 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. 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. Our results suggest that maternal postnatal licking/grooming normalized prenatal bisphenol-induced upregulation of estrogen-related receptor gamma (*Esrrg*) expression in female pups. These mitigating impacts 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. These differentially methylated regions were enriched for binding sites 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. These results also suggest that postnatal tactile stimulation might be a potential intervention strategy to mitigate the neurodevelopmental risks from prenatal endocrine disrupting chemical exposure.

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

Author Summary

Neurodevelopment can be shaped by both aversive and positive experiences early in life, in part due to 'epigenetic' mechanisms such as DNA methylation. Here, we follow up on our previous studies that suggest high levels of postnatal maternal care could mitigate the negative impacts of prenatal bisphenol exposure, an estrogenic endocrine disrupting chemical. We focused on gene expression and DNA methylation changes in the developing medial preoptic area, a brain area that is enriched in estrogen receptors and important for sex-specific social behaviors. We found that maternal postnatal licking/grooming normalized prenatal bisphenol-induced upregulation of estrogen-related receptor gamma (*Esrrg*) expression in female pups only. We find similar patterns in multiple co-expressed gene networks that are enriched in estrogen-responsive genes. Finally, postnatal maternal licking/grooming influenced DNA methylation patterns for prenatal bisphenol-exposed male and female pups. These results suggest a novel biological mechanism in which postnatal maternal care can mitigate the negative neurodevelopmental impacts of prenatal bisphenol exposure. This is important because it suggests that postnatal tactile stimulation could be an effective intervention against the negative neurodevelopmental impacts from prenatal endocrine disrupting chemical exposure.

1. 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. However, we have also found that the impacts of prenatal bisphenol exposure and postnatal maternal care on DNA methylation in adult rats can be highly brain region-specific (39), so it is important to examine the MPOA directly to confirm that these interactive effects also extend to this brain region.

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. We chose to examine PND10 brains because this age shortly follows the sensitive period for both when exogenous estrogens can permanently alter brain sexual maturation in

163 females (59,60) and when variations in maternal care impact MPOA ESR1 levels (61). In
164 addition, we adapted a correlative method to identify DNA motifs and potential
165 transcription factor binding sites at differentially methylated regions. Suffix Array Kernel
166 Smoothing (SArKS) has been previously implemented in RNA-seq datasets and allows the
167 user to correlate a continuous range of gene expression changes to proximal transcription
168 factors binding sites (62). We hypothesized that postnatal maternal licking/grooming would
169 mitigate the effects of prenatal bisphenol exposure on *Esr1* expression and estrogen-
170 responsive genes. In addition, we hypothesized that there would be interactions between
171 prenatal bisphenol exposure and postnatal maternal licking/grooming on DNA methylation
172 modifications, particularly nearby EREs and other transcription factor binding sites. Finally,
173 we hypothesized that there would be sex-specific effects, specifically that there would be
174 larger impacts in female offspring compared to male offspring. Overall, we found that
175 postnatal maternal licking/grooming mitigated the impacts of prenatal bisphenol exposure
176 on *Esrrg* expression rather than *Esr1* in female pups only. This corresponded to significant
177 interactions between postnatal maternal licking/grooming and prenatal bisphenol
178 exposure in genome-wide gene expression and DNA methylation changes, with some
179 enrichment in estrogen-responsive genes and even higher enrichment in genes that are
180 known to interact with estrogen receptors.
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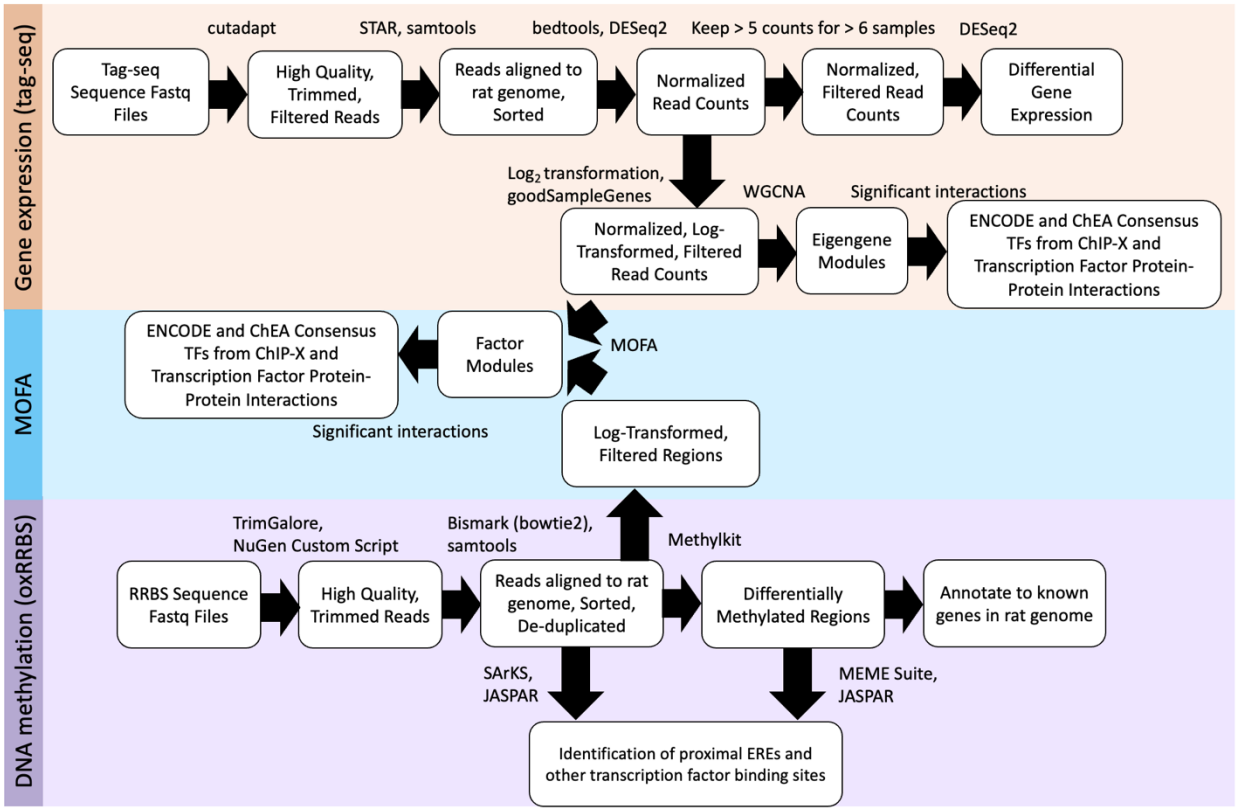

183
184 **Fig 1.** Preprocessing and analysis pipelines for gene expression, DNA methylation, and
185 multi-omic analyses.
186

2. Results

The preprocessing and analysis pipelines for 3' tag sequencing (tag-seq), oxidative reduced representation bisulfite sequencing (oxRRBS), and multi-omic factor analyses (MOFA) are summarized in **Fig 1.**

2.1 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 (**Fig 2A**) but not the male pups (**Fig 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.

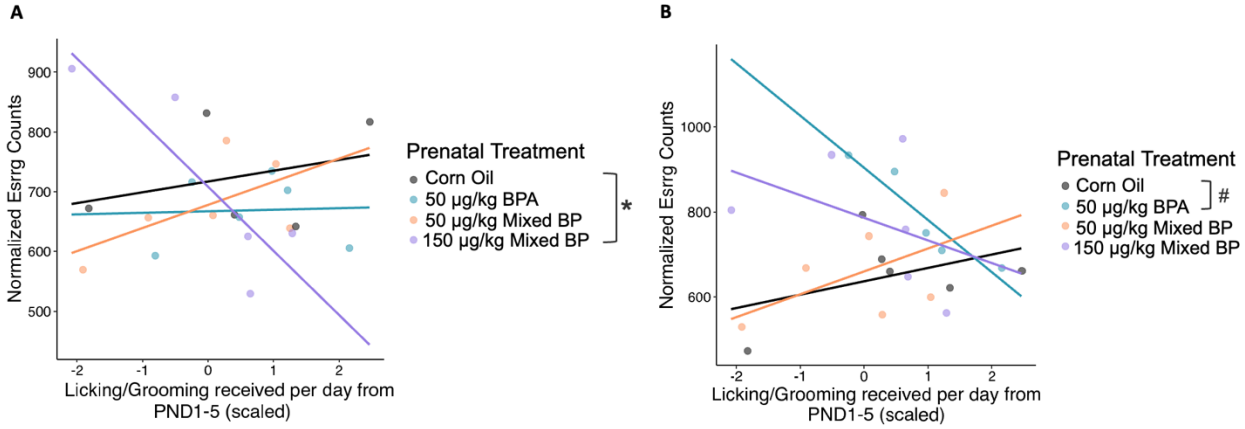

Fig 2. Sex-specific interaction between prenatal bisphenol exposure and postnatal maternal licking/grooming on *Esrrg* expression in the medial preoptic area. A significant interaction between the 150 µg/kg Mixed BP exposure group and maternal licking/grooming was found in female pups (**A**) but not male pups (**B**). In females from the 150 µg/kg Mixed BP group, maternal licking/grooming was negatively correlated with Esrrg expression. 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

2.2 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 S1** summarizes the DESeq2 output. Overall, there were very few DEGs found in all comparisons. The only exception was that there were 63 DEGs (FDR ≤ 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 ≤ 0.05.

2.3 WGCNA 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 were significant interactions for the Brown module (t = -2.544, p = 0.0292; 2,481 genes; **Fig 3A**), the Turquoise module (t = 2.291, p = 0.0449; 5,229 genes; **Fig 3B**), and the Yellow module (t = 2.061, p = 0.0264;1,445 genes; **Fig 3C**). There were also marginal interactions for the Blue module (t = -2.179, p = 0.0544; 4,022 genes; **Supplemental Fig S1A**) and the Magenta module (t = -2.205, p = 0.0520; 980 genes; **Supplemental Fig S1B**), Top GO terms for each of the modules with significant interactions in female offspring are presented in **Fig 3D;** the top GO terms for the modules with marginal interactions are presented in **Supplemental Fig S1C**. 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.

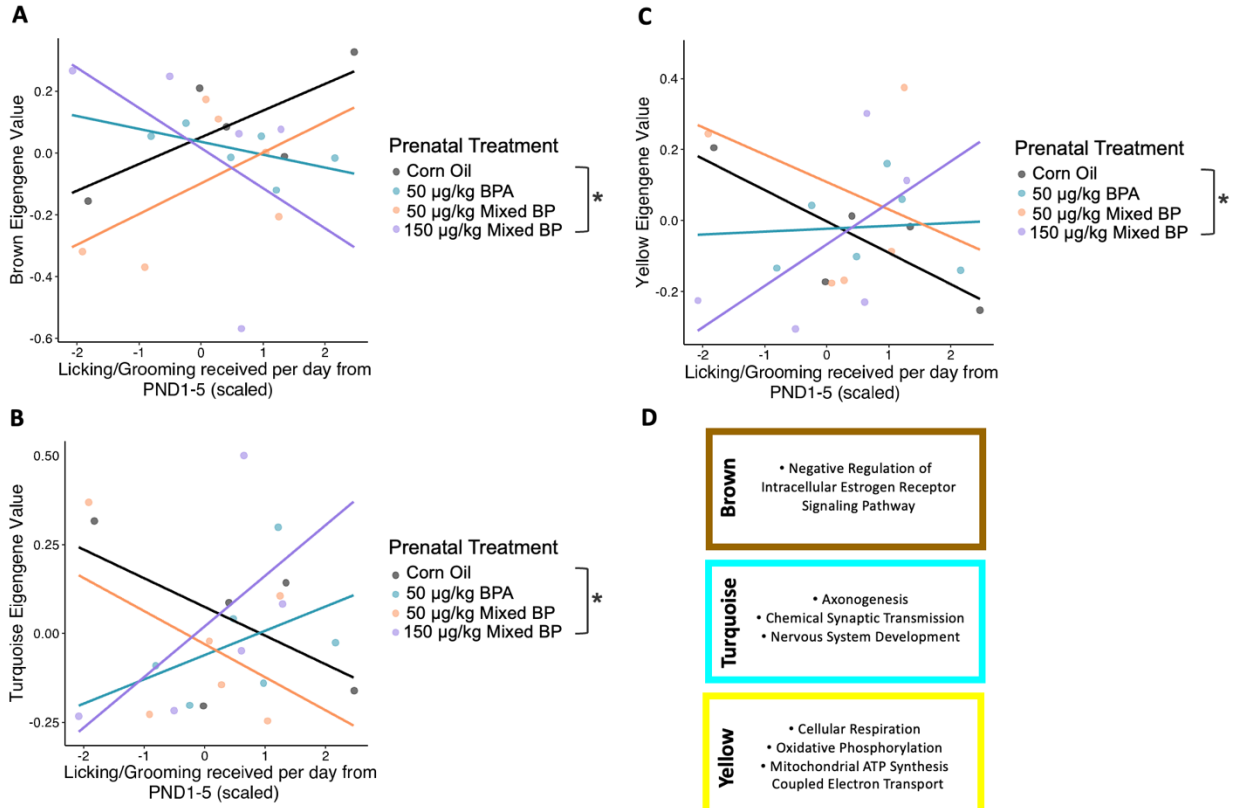

Fig 3. Interactions between prenatal bisphenol exposure and postnatal maternal licking/grooming on co-expressed gene modules in the medial preoptic area of female pups. Significant interactions between the 150 μg/kg Mixed BP exposure group and maternal licking/grooming were found in the Brown (**A**), Turquoise (**B**), and Yellow (**C**) eigengene modules. In females from the 150 μg/kg Mixed BP group, maternal licking/grooming was negatively correlated with the Brown eigengene values and positively correlated with the Turquoise and Yellow eigengene values. Top GO terms (**D**) from these modules were related to estrogen receptor signaling, 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

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

Among the modules in which there were significant interactions between prenatal BP and postnatal maternal care in female offspring (**Fig 3**), we examined enrichment for estrogen-related and -responsive genes. 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 Turquoise module, there 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).

2.5 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 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 Brown module (73%) 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 Brown	kME Turquoise	kME Yellow
Esr1	32.93	-13.40	-1.53
Esr2	41.06	-34.39	-48.27

Esrra	18.76	-20.91	-14.89
Esrrb	25.80	-54.04	-17.94
Esrrg	73.13	-68.89	-53.58

2.6 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.

2.6.1 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 (**Fig 4A**; **Supplementary Table S2**). 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 (**Fig 4B**; **Supplementary Table S3**). In the prenatal 150 µg/kg Mixed BP group, there were 44 DMRs (corresponding to 27 genes) that were unchanged, 6 DMRs (corresponding to four genes) that were lost, and 38 DMRs (corresponding to 21 genes) that were added when including maternal licking/grooming as a covariate (**Fig 4C**; **Supplementary Table S4**).

2.6.2 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 28 DMRs (corresponding to 15 genes) that were added when including maternal licking/grooming as a covariate (**Fig 4D; Supplementary Table S5**). In the prenatal 50 µg/kg Mixed BP group, there were 46 DMRs (corresponding to 27 genes) that were unchanged, 25 DMRs (corresponding to 19 genes) that were lost, and three DMRs (corresponding to two genes) that were added when including maternal licking/grooming as a covariate (**Fig 4E; Supplementary Table S6**). 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 (**Fig 4F; Supplementary Table S7**).

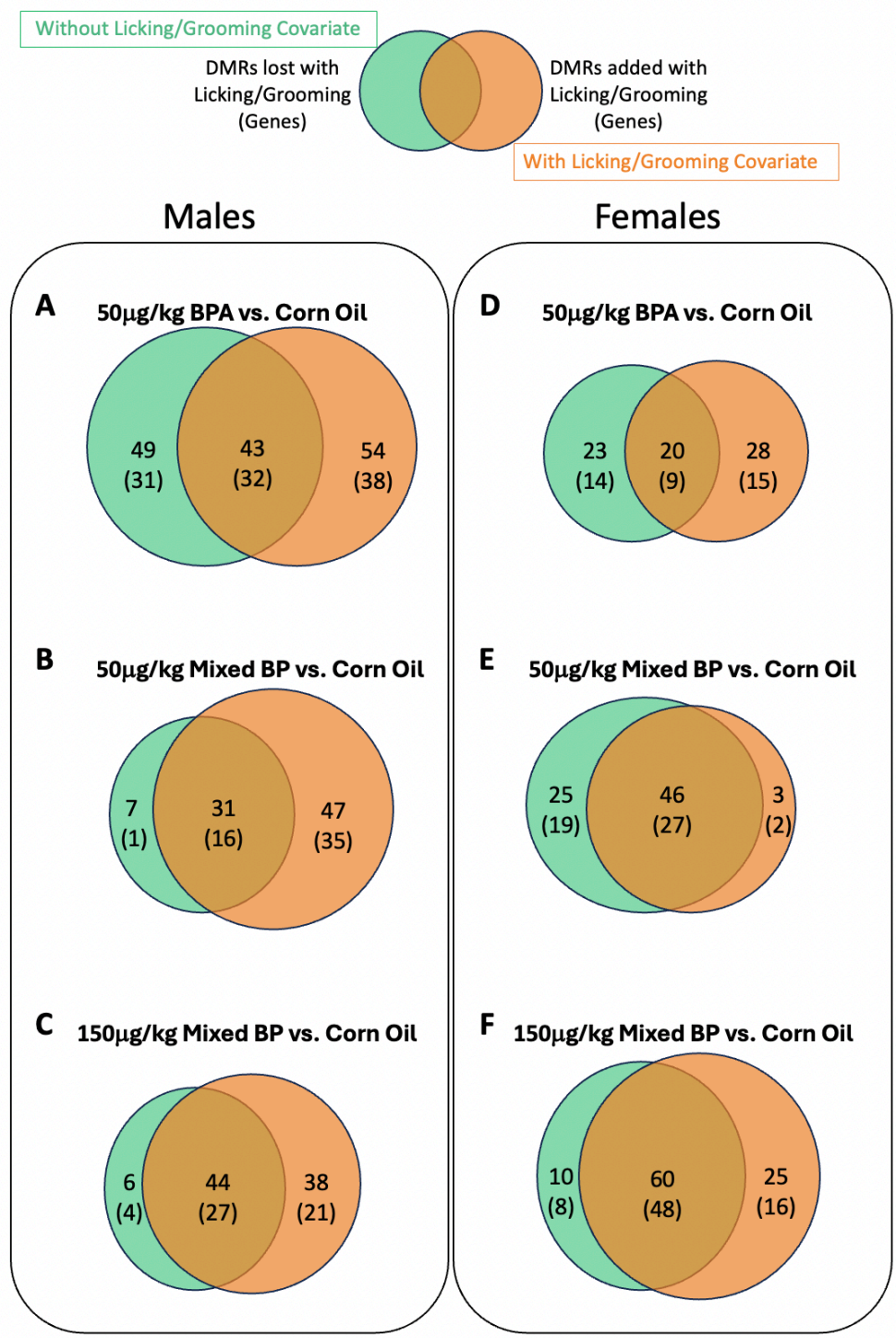

Fig 4. Influence of postnatal maternal licking/grooming on differentially methylated regions (DMRs) of prenatal bisphenol-exposed male and female pups. Including maternal licking/grooming as a covariate altered DMRs in all prenatal treatment groups in both male (**A-C**) and female (**D-F**) pups. For all comparisons except for the females in the 50 µg/kg Mixed BP group, more DMRs were added than lost when adding licking/grooming as a covariate. 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 several notable neuroendocrine-related genes (Ddc, Maoa, Kiss1, Glpr1, Igf2bp2, Drd5, Gper1, Hsd17b2) found throughout all comparisons between prenatal bisphenol treatment and Corn Oil groups.

2.7 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.

2.7.1 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 S8**). 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 S9**). 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 (NRF1, E2F3, EGR3) were identified 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 (**Fig 5A**).

2.7.2 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 S10**). 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 S11**). 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 (**Fig 5B**).

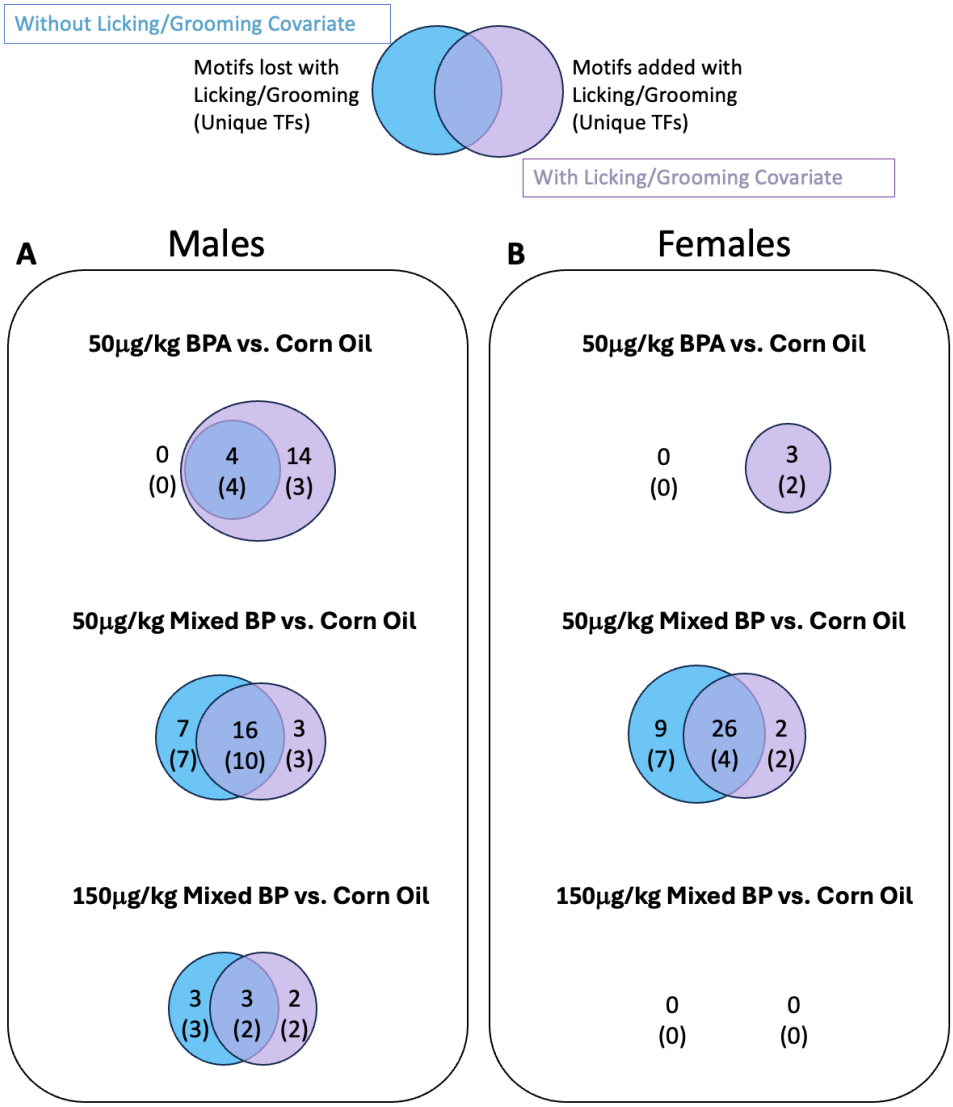

Fig 5. Influence of postnatal maternal licking/grooming on differentially methylated transcription factor binding sites of prenatal bisphenol-exposed male and female pups

using the SArKS method. Including maternal licking/grooming as a covariate altered overrepresented sequence motifs and predicted transcription factor binding sites in all prenatal treatment groups in male (**A**) and female (**B**) pups (except for females from the 150 µg/kg Mixed BP group). 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 (SP5, HAND2, RBPJ) and female offspring (ELK1, IKZF2, SP5, RBPJ, 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 Fig S2**). 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	Relationship to estrogen receptors
Males			
Unchanged with Licking/Grooming			
50 µg/kg BPA vs. Corn Oil	Hypomethylated	**ZEB1**	(63,64)
50 µg/kg BPA vs. Corn Oil	Hypomethylated	**RBPJ**	(65)
50 µg/kg BPA vs. Corn Oil	Hypomethylated	SP5	
50 µg/kg BPA vs. Corn Oil	Hypomethylated	**HAND2**	(66,67)
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**	(68)
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**	(69)
50 µg/kg Mixed BP vs. Corn Oil	Hypermethylated	**KLF15**	(70–72)

50 μg/kg Mixed BP vs. Corn Oil	Hypermethylated	**SP1**	(69,73,74)
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**	(75)
50 μg/kg Mixed BP vs. Corn Oil	Hypermethylated	**KLF13**	(76)
50 μg/kg Mixed BP vs. Corn Oil	Hypermethylated	**EBF1**	(77,78)
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**	(66,67)
150 μg/kg Mixed BP vs. Corn Oil	Hypomethylated	**RBPJ**	(65)
Added with Licking/Grooming			
50 μg/kg BPA vs. Corn Oil	Hypomethylated	EBF2	
50 μg/kg BPA vs. Corn Oil	Hypomethylated	**GLI3**	(75)
50 μg/kg BPA vs. Corn Oil	Hypomethylated	**TFAP2C**	(79,80)
50 μg/kg Mixed BP vs. Corn Oil	Hypermethylated	**NRF1**	(81–83)
50 μg/kg Mixed BP vs. Corn Oil	Hypermethylated	**EGR3**	(84)
50 μg/kg Mixed BP vs. Corn Oil	Hypermethylated	E2F3	
150 μg/kg Mixed BP vs. Corn Oil	Hypomethylated	**EGR3**	(84)
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**	(65)
50 μg/kg Mixed BP vs. Corn Oil	Hypermethylated	SP5	
Lost with Licking/Grooming			
50 μg/kg Mixed BP vs. Corn Oil	Hypomethylated	**KLF15**	(70–72)
50 μg/kg Mixed BP vs. Corn Oil	Hypomethylated	ERF::NHLH1	
50 μg/kg Mixed BP vs. Corn Oil	Hypomethylated	**ELK1**	(85)
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**	(66,67)
50 µg/kg Mixed BP vs. Corn Oil	Hypermethylated	IKZF2	

2.8 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 ($t = 4.456$, $p = 0.0043$; **Fig 6A**) as well as nest attendance ($t = 3.182$, $p = 0.0190$; **Fig 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.

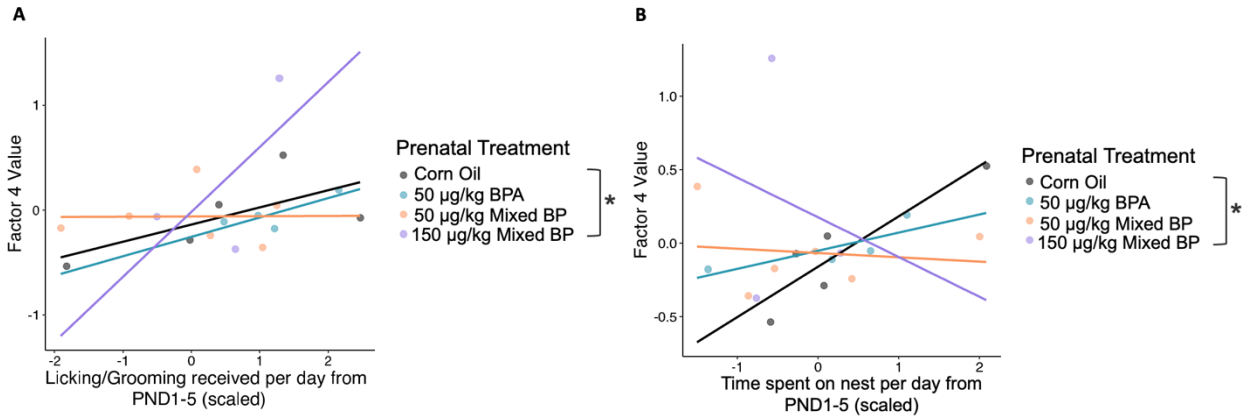

Fig 6. Interactions between prenatal bisphenol exposure and postnatal maternal licking/grooming on Factor 4 generated from the multi-omic factor analysis (MOFA) in female pups. Significant interactions between the 150 µg/kg Mixed BP exposure group and maternal licking/grooming (**A**) and nest attendance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

<u>2.8.1 Factor module enrichment for estrogen-responsive genes and genes that interact with estrogen receptors</u> 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).

3. 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 (86,87) 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. Moreover, these results suggest that postnatal licking-like tactile stimulation may be an effective intervention against the adverse neurodevelopmental consequences 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 (25,88) and has a high binding affinity to ESRRG (86,87), 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

490 bisphenol exposure on later-life phenotypes and gene expression in the prefrontal cortex
491 and amygdala at PND10 in male offspring (34). Therefore, it is possible that postnatal
492 maternal licking/grooming can mitigate the impacts of prenatal bisphenol exposure in male
493 offspring, but the underlying biological mechanisms are currently unclear and/or are not
494 strongly related to estrogen receptor signaling. As an example, previous work using another
495 estrogenic endocrine disrupting chemical (Aroclor 1221) found estradiol levels predicted
496 behavioral phenotypes in prenatal Aroclor 1221-exposed females while dopamine-related
497 gene expression predicted behavioral phenotypes in prenatal Aroclor 1221-exposed male
498 offspring (89). These sex-specific impacts are worth exploring in future studies.
499
500 Our WGCNA analysis indicated that including postnatal nest attendance in our statistical
501 models was required to unveil the significant interactions between the 150 μg/kg Mixed BP
502 exposure group and maternal licking/grooming but was not significantly associated with
503 these modules by itself. Postnatal nest attendance includes licking/grooming and other
504 forms of pup contact, including thermotactile contact. This suggests that postnatal
505 licking/grooming is the primary driver of these interactions and overall pup contact plays a
506 minor but possibly important role (47) in gene expression changes for prenatal bisphenol-
507 exposed female pups. Directly manipulating licking-like tactile stimulation while keeping
508 other forms of pup contact consistent would confirm these findings and is an ongoing area
509 of investigation in our lab.
510
511 Our DMR analysis indicates postnatal maternal licking/grooming has a substantial
512 influence on DNA methylation changes in prenatal bisphenol-exposed pups. The finding
513 that more DMRs were added than lost when including postnatal maternal licking/grooming
514 as a covariate suggests that postnatal maternal licking/grooming plays more of a
515 moderating role in shaping the DNA methylome in prenatal bisphenol-exposed pups,
516 consistent with our prior findings (34,39). However, it was apparent that the differential
517 methylation findings did not map consistently onto differential gene expression. While it is
518 assumed that changes in DNA methylation and gene expression would be strongly linked,
519 there are several reasons why there may be a discordance. First, we did not implement any
520 substantial environmental challenges to the pups immediately prior to brain collection.
521 While DNA methylation changes are relatively stable, gene expression changes are
522 dependent on both the availability of chromatin to allow transcription and transcriptional
523 activity itself. Therefore, the associations between DNA methylation and gene expression
524 changes may be stimulus-dependent. The second reason is that there are inherent
525 differences in the approach in which these changes are assessed. While we assessed the
526 whole transcriptome, we only probed a small portion of CpG sites in the genome. In
527 addition, many of these CpG sites are intergenic and we have an incomplete understanding
528 of the role of DNA methylation in distal CpG sites on gene expression. Finally, in any
529 particular cell, DNA methylation is mainly binary (methylated or unmethylated with rare
530 instances of hemi-methylation) while gene expression changes can have a large range. This
531 could complicate analyses when using bulk tissue and would need to be resolved at the
532 single-cell level. Nevertheless, when we integrated the DNA methylation and gene
533 expression data using MOFA, we generally replicate the gene expression findings. This

534 confirms that the significant interactions between the 150 µg/kg Mixed BP exposure group
535 and maternal licking/grooming in female pups apply to both gene expression and DNA
536 methylation.
537
538 We explored potential transcription factor binding sites associated with DNA methylation
539 changes with prenatal bisphenol exposure and postnatal maternal licking/grooming. To do
540 so, we adapted the SArKS method to RRBS data so we can correlate DNA methylation
541 changes in prenatal bisphenol-exposed pups with and without postnatal maternal
542 licking/grooming as a covariate to motifs and possible transcription factor binding sites. As
543 a comparison, we also used MEME Suite to identify motifs based on the changes in DMRs
544 with and without postnatal maternal licking/grooming as a covariate. The main difference
545 with using a correlative-based method is that it accounts for the continuous range of DNA
546 methylation changes (0-100%) instead of a binary representation (i.e., it is differentially
547 methylated or not differentially methylated). A correlative-based method can be
548 particularly advantageous with more complex study designs where multiple independent
549 variables are assessed. When a binary representation is applied to DMR data with and
550 without covariates, there is an assumption that all DMRs that change will also have the
551 same effect size changes and would be equally important for motif discovery. In contrast,
552 accounting for the continuous range of differential methylation and effect size changes
553 with covariates can provide a more accurate representation of the potential transcription
554 factors involved with differential methylation at specific loci. With the SArKS method, we
555 found numerous motifs and transcription factor binding site predictions that change when
556 including postnatal maternal licking/grooming as a covariate, as we would have expected
557 based on the DMR analysis. This was also the case with the MEME Suite method and the
558 changes were sometimes even more pronounced, possibly due to the binary
559 representation of the input sequences as described above.
560
561 A consistent theme throughout our analyses was that the genes and transcription factor
562 binding site predictions associated with prenatal bisphenol exposure and postnatal
563 maternal licking/grooming as well as their interactions were either related to estrogen
564 receptor signaling or were estrogen-responsive genes. In prior transcriptome analyses in
565 the PND10 prefrontal cortex and amygdala, we found consistently strong and significant
566 enrichment of ESR1 in the Transcription Factor Protein-Protein Interactions module in
567 Enrichr (34). This was also the case in the PND10 MPOA, but we also found significant
568 (though weaker) enrichment of ESR1 in the ENCODE and ChEA Consensus TFs from ChIP-X
569 module. These findings are consistent with our hypothesis that the interactions between
570 prenatal bisphenol exposure and postnatal maternal licking/grooming converge on
571 estrogen receptor signaling, with some brain region-specific differences, and that we are
572 primarily finding secondary effects of estrogen receptor signaling changes by PND10.
573
574 It is worth noting that the mitigating effects of maternal licking/grooming on ESRRG
575 signaling are speculative since they are derived from RNA-seq data. Future work will need
576 to test these impacts more directly, such as with chromatin immunoprecipitation or
577 luciferase reporter assays for ESRRG. One other 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 (90,91), 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). While the dosages used in the current study were informative for discovering potential biological mechanisms, additional studies using lower doses are needed to ensure the translational potential of our current findings. Finally, we do not have any data on how maternal licking/grooming might mitigate the impacts of prenatal bisphenol exposure on MPOA-dependent behaviors later in life, such as social behavior and maternal care provisioning to the next generation. However, given that there is already evidence that both prenatal bisphenol exposure and postnatal maternal care can impact these later-life phenotypes (25,92–98), it is likely that interactions between these two early-life environmental exposures exist.

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, such as postnatal touch stimulation, to mitigate the effects of prenatal bisphenol exposure and improve health and well-being in human populations.

4. Materials and Methods

Supplementary Table S12 displays all the code files and data files used for the bioinformatic analyses, as well as the information from all samples used for analyses.

4.1 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. The rat pups used for the current study were part of a larger cohort that has been described in previous publications (34,39). Briefly, breeder female and male Long-Evans rats (Charles River) were housed in same-sex pairs

622 on a 12:12 hour inverse light-dark cycle with ad libitum access to standard chow diet
623 (#5LL2, Lab Diet) and water. To limit external exposure to bisphenols and other
624 xenoestrogens, all rats were provided glass water bottles, polysulfone cages, and aspen
625 wood shavings for bedding material.
626
627 **4.2 Gestational bisphenol administration**
628 Male and female rats were screened for breeding receptivity by pairing one male with one
629 or two females and observing mounting behavior for the male and lordosis for the
630 female(s). From gestational day 8 until parturition, cage-pairs of dams were randomly
631 assigned to receive control Corn Oil, 50 µg/kg BPA, 50 µg/kg Mixed Bisphenols (BP), or 150
632 µg/kg Mixed BP. This time period includes the start of brain sexual differentiation and when
633 the expression of estrogen receptors is apparent in the fetal brain (99). Stock solutions of
634 bisphenol A (BPA; #B0494, TCI, ≥ 99 %), bisphenol S (BPS; #A17342, Alpha Aesar, ≥ 99 %),
635 or bisphenol F (BPF; #A11471, Alfa Aesar, ≥ 98 %) were dissolved in corn oil (#405435000,
636 Acros Organics). Equal parts BPA, BPS, and BPF were used for both Mixed BP treatment
637 groups. Experimenters involved with the administration of treatments were blinded to the
638 treatment groups. After parturition (postnatal day 0; PND0), offspring were sex-determined
639 using relative anogenital distance and culled to six male and six female pups per litter.
640 Following the maternal care video recordings at PND10, the brains from one male pup and
641 one female pup per litter were collected and flash-frozen in hexanes on dry ice. All brains
642 were stored at -80°C until cryosectioning.
643
644 **4.3 Postnatal maternal care observations and quantification**
645 Home cage maternal behavior was recorded for one hour per day starting one hour after
646 lights-off from PND1-10 using Raspberry Pi 3B+ minicomputers. Maternal behavior was
647 scored by either manual coding or through the AMBER pipeline (100). All postnatal
648 maternal care measures were normalized to seconds of observed behavior per day and
649 then scaled and centered where the average is 0 and standard deviation is 1 for all
650 downstream bioinformatic analyses. Based on prior analyses (34,39) we used
651 licking/grooming and nest attendance from PND1-5 as independent variables or covariates
652 for the current study. Maternal behavior measures used in the current study did not vary
653 between prenatal bisphenol exposure groups (34).
654
655 **4.4 RNA extraction and 3' tag sequencing (Tag-seq)**
656 Power analyses were done using the R package PROPER for RNA-seq data with the Gilad
657 dataset (medium biological variation) and 25 data simulations to calculate effect size.
658 Analyzing 6 biological samples per group allowed 72% power to correctly identify
659 differentially expressed genes with an FDR value < 0.05 and log fold change of at least 0.5.
660 PND10 brains (n = 6 per sex per prenatal treatment group) were cryosectioned in 50 µm
661 slices using a ThermoFisher Scientific CryoStar NX50 cryostat or a Leica CM3050S
662 cryostat. The medial preoptic area (MPOA; 0.20 to -0.60 mm Bregma) was microdissected
663 using an atlas for the developing rat brain (101) and a supplementary atlas for the PND10
664 rat brain (102). RNA was extracted using the MagMAX FFPE DNA/RNA Ultra Kit (#A31881,
665 ThermoFisher Scientific). For unfixed frozen tissue, 210 µl of protease solution was

666 pipetted onto each MPOA sample and allowed to incubate at 55°C at 900 rpm for at least
667 one hour. The homogenate was immediately processed for RNA and DNA extraction using
668 the Kingfisher Flex System following the manufacturer's instructions. RNA quantity was
669 assessed with the Quant-iT RNA Assay kit (#Q33140, ThermoFisher Scientific) and RNA
670 quality was assessed using the RNA 6000 Pico Assay kit (#5067-1513, Agilent
671 Technologies). All RNA samples were diluted to 20 ng/µl before submission for library
672 preparation and tag-seq (103,104) at the Genome Sequence and Analysis Facility at the
673 University of Texas at Austin. Reads were sequenced on the NovaSeq S1 (100 bp single-end
674 reads).
675
676 **4.5 DNA extraction and oxidative reduced representation bisulfite sequencing**
677 **(oxRRBS)**
678 DNA was extracted using the MagMAX FFPE DNA/RNA Ultra Kit (#A31881, ThermoFisher
679 Scientific). A subset of samples (two 150 µg/kg Mixed BP females, one 150 µg/kg Mixed BP
680 male) did not contain sufficient DNA for library preparation for oxRRBS. Another subset of
681 samples (one 50 µg/kg BPA male, one 150 µg/kg Mixed BP female, one Corn Oil female)
682 likely had substantial anterior pituitary gland contamination based on the tag-seq results
683 (described in the tag-seq preprocessing section) and were not processed for oxRRBS. To
684 maintain a sample size of 6 per sex per prenatal treatment group, DNA was extracted from
685 additional MPOA samples using the MagMAX DNA Multi-Sample Ultra 2.0 Kit following the
686 manufacturer's instructions for tissue samples (#A45721, ThermoFisher Scientific). DNA
687 quantity was assessed with the Quantifluor dsDNA system (#E2670, Promega).
688
689 Library preparation for oxRRBS was done using the Ovation RRBS Methyl-Seq System with
690 the TrueMethyl® oxBS module (Tecan) using 55 ng of input DNA. Between 12-20 samples
691 were processed per batch of library preparation with a total of 5 batches, balanced by
692 prenatal treatment group and sex. The oxidation of DNA prior to bisulfite conversion
693 converts 5hmC to 5fC and allows for discrimination between 5mC and 5hmC at single
694 base-pair resolution. For this study, oxidative bisulfite conversion was used to remove
695 5hmC and detect only 5mC in the samples. Final libraries were quality-checked using the
696 Agilent Technologies High Sensitivity DNA Kit. Five libraries (one 50 µg/kg BPA female, one
697 50 µg/kg Mixed BP male, one 50 µg/kg Mixed BP female, and two 150 µg/kg Mixed BP
698 females) were removed due to insufficient final yield. The remaining libraries (n = 4-6 per
699 sex per prenatal treatment group) were pooled with 8-9 libraries per pool (balanced by
700 prenatal treatment group and sex) and submitted for an additional Ampure XP bead clean-
701 up and sequencing to the Genome Sequence and Analysis Facility at the University of Texas
702 at Austin. Reads were sequenced on the NovaSeq SP (150 bp paired-end reads).
703
704 **4.6 Pipeline and Analyses: Tag-seq**
705 **4.6.1 Preprocessing** The tag-seq pipeline overview can be found in **Fig 1** and all data and
706 code for the differential gene expression and weighted gene co-expression network
707 analysis can be found at https://github.com/SLauby/bisphenols_maternalcare_epigenome.
708 The analysis pipeline followed previously published tag-seq analyses (34). Adaptor
709 sequences, poly-A tails, and low-quality reads were trimmed (https://github.com/z0on/tag-

710 based_RNAseq) followed by cutadapt (version 1.18). Trimmed reads were mapped to the
711 rat genome (mRatBN7.2; RefSeq) using STAR (version 2.7.3a) with the default settings and
712 sorted using samtools (version 1.10). About 90% of reads were uniquely mapped to the rat
713 genome for all samples. Counts for each gene were calculated for each sample using the
714 bedtools (version 2.27.1) multicov function. Three samples (one 50 µg/kg BPA male, one
715 150 µg/kg Mixed BP female, one Corn Oil female) had unusually high counts (over 100x of
716 the other samples) for anterior pituitary gland-specific genes, including *Pomc, Gnrhr,* and
717 *Prl*. These samples were removed from the dataset prior to all downstream analyses.
718
719 **4.6.2 Differential gene expression analysis** For all downstream tag-seq analyses,
720 comparisons between prenatal treatment groups and interactions with prenatal treatment
721 group and postnatal maternal care were always done relative to the Corn Oil group. To
722 examine differential expression of individual genes, including all estrogen and estrogen-
723 related receptors, gene count data was normalized and analyzed for differential expression
724 using the DESeq2 R package (version 1.34.0), separated by sex. Genes with more than 5
725 counts for more than 6 samples were analyzed. Prenatal treatment and the postnatal
726 maternal care measures (licking/grooming, nest attendance) from PND1-5 were used as
727 independent variables. The estrogen and estrogen-related receptors (*Esr1, Esr2, Esrra,*
728 *Esrrb, Esrrg*) were specifically examined for significant interactions between each prenatal
729 treatment group and licking/grooming. Other potential differentially expressed genes
730 (DEGs) were identified with a false discovery rate (FDR) alpha ≤ 0.10 and ≤ 0.05 with
731 Benjamani-Hochburg correction. All effects were reported as statistically significant if $p \leq$
732 0.05 and marginally significant if $p \leq 0.10$.
733
734 **4.6.3 Weighted gene co-expression network analysis (WGCNA)** To examine differential
735 expression of co-expressing gene proflies, gene networks were created with the WGCNA R
736 package (version 1.72-1) using normalized and log-transformed gene count data, split by
737 sex. Genes with very low counts and variation were filtered out using the
738 goodSamplesGenes function in R (verbosity = 3). WGCNA calculates Pearson correlations
739 of count data between every gene for network construction and clusters highly correlated
740 genes into eigengene modules (at least 30 genes per module). A soft power threshold of 8
741 was used for network construction. We excluded modules with eigengene values that were
742 characterized by one extreme outlier (> 0.90) and low variation following outlier removal
743 (standard deviation < 0.10) from further analyses. The eigengene values from the remaining
744 modules were analyzed using the lm function in R. Prenatal treatment and the postnatal
745 maternal care measures (licking/grooming, nest attendance) from PND1-5 were used as
746 independent variables. For any eigengene module that showed statistically significant
747 interactions, the gene sets were extracted and analyzed using Enrichr (105,106) using the
748 full gene list used for network construction as the background
749 (https://maayanlab.cloud/Enrichr/). To examine if the gene sets were enriched in estrogen-
750 responsive genes, ESR1 was examined in the ENCODE and ChEA Consensus TFs from
751 ChIP-X module. To examine if the gene sets were enriched in genes that are known to
752 interact with estrogen receptors, ESR1 was examined in the Transcription Factor Protein-
753 Protein Interaction module. To examine the degree of connectivity with the co-expressed

754 gene modules with specific estrogen receptors, the kME was calculated for all estrogen
755 and estrogen-related receptors in the WGCNA package. The kME determines how closely a
756 gene of interest is related to a particular module and is represented as a positive or
757 negative percentage of all genes that have highly correlated expression to the gene of
758 interest. Negative values indicate inverse relationships. All effects were reported as
759 statistically significant if $p \leq 0.05$ and marginally significant if $p \leq 0.10$.
760
761 **4.7 Pipeline and Analyses: oxRRBS**
762 **4.7.1 Preprocessing** The oxRRBS pipeline overview can be found in **Fig 1** and all code for
763 the differentially methylated region analysis can be found at
764 https://github.com/SLauby/bisphenols_maternalcare_epigenome. The bismark-processed
765 DNA methylation dataset can be accessed in Zenodo
766 (https://doi.org/10.5281/zenodo.17273659). Adaptor sequences and low-quality reads ($q <$
767 30) were trimmed using trim galore (version 0.6.10) and cutadapt (version 4.6). Diversity
768 sequences and reads without the MspI site (CGG) were removed using a custom python
769 script provided by the manufacturer of the oxRRBS kit
770 (https://github.com/nugentechnologies/NuMetRRBS). Paired-end reads were mapped to
771 the rat genome (mRatBN7.2; RefSeq) using bismark (version 0.22.3) and bowtie2 (version
772 2.5.2) with the default settings. About 75% of reads were uniquely mapped to the rat
773 genome for all samples. PCR duplicates were removed based on the genomic coordinates
774 of the reads and a 6-bp unique molecular identifier found in the read index using a custom
775 python script provided by the manufacturer of the oxRRBS kit
776 (https://github.com/tecangenomics/nudup). An average of about 8.5 million uniquely
777 aligned, de-duplicated reads per sample were analyzed for DNA methylation levels using
778 the bismark methylation extractor. Bisulfite conversion rate was > 99% for all samples.
779
780 **4.7.2 Differentially methylated region (DMR) analysis** For all downstream oxRRBS
781 analyses, pairwise comparisons between prenatal treatment groups were always done
782 relative to the Corn Oil group. Differentially methylated regions (500 bp) between prenatal
783 treatment groups were examined using Methylkit (version 1.26.0), separated by sex.
784 Regions were included in the analysis if there were over 10x reads for at least 4 samples per
785 prenatal treatment group. Batch of library preparation was used as a covariate in all
786 analyses. DMRs were identified with an FDR q-value ≤ 0.05 using the SLIM method and had
787 > 5% overall methylation difference between groups. DMR analyses are currently limited to
788 pairwise comparisons, so an alternative approach was undertaken to examine potential
789 interactions between prenatal bisphenol exposure and postnatal maternal
790 licking/grooming. Each comparison between the prenatal treatments groups and Corn Oil
791 group was done including and excluding postnatal maternal licking/grooming as a covariate
792 and the degree of overlap in DMRs was identified. DMRs that were lost or added when
793 including postnatal maternal licking/grooming as a covariate in the analysis were
794 considered to be influenced by postnatal maternal licking/grooming. More specifically,
795 DMRs that were added were assumed to be due to interactions between prenatal
796 bisphenol exposure and postnatal maternal licking/grooming.
797

4.7.3 Differentially methylated motif and transcription factor binding site analysis: **Suffix Array Kernel Smoothing (SArKS)** To adapt the SArKS method to RRBS data, genomic regions with methylation data were created as input sequences for the SArKS algorithm to identify overrepresented sequence motifs. The code for creating those input sequences can be found at https://github.com/denniscwylie/prenatal_bp_x_lg_epigenome_analysis. For each individual sample, observed methylation sites were partitioned into disjointed 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 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 (https://github.com/denniscwylie/sarks; (62)) 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. Step-by-step instructions and pseudocode for SArKS can be found at https://bioconductor.posit.co/packages/3.23/bioc/vignettes/sarks/inst/doc/sarks-vignette.pdf.

4.7.4 Differentially methylated motif and transcription factor binding site analysis: **MEME Suite** DMRs that were found using Methylkit were used to identify overrepresented sequence motifs with MEME Suite (Version 5.5.7; (107)) using previously published

parameters (108) 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 (109). E-values ≤ 0.05 were considered statistically significant.

4.8 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. 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 and 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. 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 ≤ 0.05 and marginally significant if p ≤ 0.10.

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