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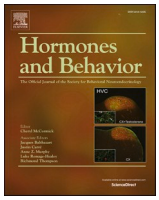

Interplay between prenatal bisphenol exposure, postnatal maternal care, and offspring sex in predicting DNA methylation relevant to anxiety-like behavior in rats

Samantha C. Lauby [a,b], Isha Agarwal [a], Hannah E. Lapp [a], Melissa Salazar [a], Sofiia Semyrenko [a], Danyal Chauhan [a], Amy E. Margolis [c], Frances A. Champagne [a,b,*]

[a] Department of Psychology, College of Liberal Arts, University of Texas at Austin, Austin, TX, United States of America
[b] Center for Molecular Carcinogenesis and Toxicology, University of Texas at Austin, Austin, TX, United States of America
[c] Department of Psychiatry, The Ohio State Univeristy, Columbus, OH, United States of America

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ABSTRACT

Prenatal exposure to endocrine disrupting chemicals, such as bisphenols, can alter neurodevelopmental trajectories and have a lasting neurobehavioral impact through epigenetic pathways. However, outcomes associated with prenatal bisphenol exposure may also be shaped by the postnatal environment and collectively these environmental effects may be sex-specific. Thus, an integrative research design that includes multiple early life exposures and considers sex differences may be essential for predicting outcomes. In the current study, we use a multivariate approach to examine the contributions of prenatal bisphenol exposure, postnatal maternal care, and offspring sex to variation in DNA methylation of well-studied candidate genes (NR3C1, BDNF, OXTR) in the ventral hippocampus and amygdala of adult Long-Evans rats. Main effects of postnatal maternal care and interactions with prenatal bisphenol exposure were consistently found for DNA methylation within the NR3C1 gene (ventral hippocampus) and within the BDNF and OXTR genes (amygdala). Sex-specific effects were also found across all analyses. Overall, our findings suggest that both early-life factors (prenatal and postnatal) and offspring sex contribute to variation in DNA methylation in genes and brain regions relevant for the expression of anxiety-like behavior. These results highlight the need to consider the brain region-specific effects of multiple exposures in males and females to understand the lasting effects of early environments.

1. Introduction

Environmental exposures occurring during the perinatal period can have a profound influence on neurodevelopment with consequences for later-life mental health and behavioral outcomes, including the risk for anxiety disorders. Both risk and protective factors contribute to the vulnerability of an individual to develop an anxiety disorder (Erzin and Guloksuz, 2021). Early-life risk factors include exposure to endocrine disrupting chemicals, low socio-economic status, childhood maltreatment and other adverse childhood experiences (Cardenas-Iniguez et al., 2022). Sex and gender may also contribute to differential risk, with women more likely to be diagnosed with an anxiety disorder than men (e.g., McLean et al., 2011; Vesga-López et al., 2008). Protective factors against anxiety disorders and anxiety-like behavior in early-life include social support, particularly from the primary caregivers (Caldji et al., 2000; Luby et al., 2022). Understanding the interplay between risk and protective factors and the underlying mechanisms involved in this interplay is critical to elucidating the links between early-life environmental exposures and anxiety disorders and identifying possible targets for intervention (Appleton et al., 2016; Tamiz et al., 2022).

Epigenetic modifications may serve as a mechanistic link between early life environmental exposures and anxiety-like behavior. While exposures are likely to have broad epigenome-wide effects, exploration of the link between exposures and behavioral outcomes has typically focused on candidate genes with established roles in neurodevelopment. In the case of anxiety, the genes encoding the glucocorticoid receptor (NR3C1), brain-derived neurotrophic factor (BDNF), and oxytocin receptor (OXTR) are of particular interest due to their plasticity in expression and their mechanistic role in stress responsivity, neural plasticity, and psychiatric risk. NR3C1 is widely expressed in the brain

* Corresponding author at: 108 E. Dean Keeton St., Austin, TX 78712, United States of America.
E-mail address: franceschampagne@utexas.edu (F.A. Champagne).

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and peripheral tissues and is involved in the response to glucocorticoids released following stress exposure. BDNF promotes neurogenesis and neuronal survival in the brain and is thought to buffer the negative effects of stress (Taliaz et al., 2011). Conversely, BDNF expression also decreases in response to stress (Murakami et al., 2005). OXTR signaling in the amygdala, specifically in the central nucleus, is associated with fear attenuation and stress reduction (Huber et al., 2005; Knobloch et al., 2012). Similar to BDNF, OXTR signaling in the hippocampus can regulate neurogenesis and synaptic plasticity which could also have indirect effects on stress and anxiety (Lin and Hsu, 2018).

Early-life social environments have been linked to changes in DNA methylation and transcript levels of NR3C1, BDNF and OXTR, suggesting a possible biological mechanism linking early-life environmental exposures and later-life anxiety-like behavior. Early-life stress and low levels of postnatal maternal care have been associated with increased DNA methylation levels in the promoter region of NR3C1, particularly within the NGFIA binding site in the Exon 1_7 promotor, in the hippocampus of rats (Weaver et al., 2004). These alterations in DNA methylation also affect NR3C1 transcript levels in the hippocampus and are associated with changes in anxiety-like behavior and corticosterone levels following restraint stress (Caldji et al., 2000; Liu et al., 1997; Weaver et al., 2004). Early-life maternal maltreatment or social enrichment has been associated with DNA methylation levels of several BDNF transcript variants and/or mRNA expression in multiple brain regions, including the hippocampus and amygdala (Branchi et al., 2013; Roceri et al., 2002; Roth et al., 2009, 2014). Finally, postnatal maternal care has been associated with DNA methylation levels in the MT2 region of OXTR in humans and prairie voles (Danoff et al., 2021; Perkeybile et al., 2019). In rats, postnatal maternal care has been associated with DNA methylation levels in the MT2 region of OXTR in blood but not the brain (Beery et al., 2016). Epigenetic plasticity within these genes may play an important role in regulating variation in stress-associated outcomes such as anxiety, though there is much that is unknown regarding the pathways through which this is achieved.

Studies of the impact of early life environmental exposures in animal models have not typically combined multiple exposures that vary in developmental timing. In environmental health, the concept of "mixtures" when considering exposures, is of increasing priority and reflects the real-world phenomenon of both cumulative and interacting chemical exposures. However, the concept of "mixtures" can also be extended to cumulative and interactive exposures consisting of both physical and social environments experienced at different developmental timepoints. These exposures may represent both risk and protective factors that interact to shape neurobehavioral outcomes such as anxiety. For example, our previous studies of prenatal exposure to endocrine disrupting chemicals in rodents suggest that behavioral and gene expression outcomes are the product of an interaction between prenatal bisphenol exposure and levels of postnatal maternal care (Kundakovic et al., 2013; Lauby et al., 2024). We have consistently found that prenatal bisphenol exposure, both bisphenol A and a bisphenol mixture (bisphenol A, bisphenol S, bisphenol F), is associated with impairments in later-life neurodevelopmental and behavior outcomes for rat offspring, but only after controlling for postnatal maternal care (Lauby et al., 2024; Fig. 1A). These interactions were often dependent on the daily dose of bisphenols administered and offspring sex. This was also evident within measures of anxiety-like behavior (Lauby et al., 2024), where we found significant interactions between prenatal bisphenol exposure (50 μg/kg/day bisphenol A and 50 μg/kg/day bisphenol mixture) and postnatal maternal care on performance in the open field test in male offspring only (Lauby et al., 2024). This finding likely

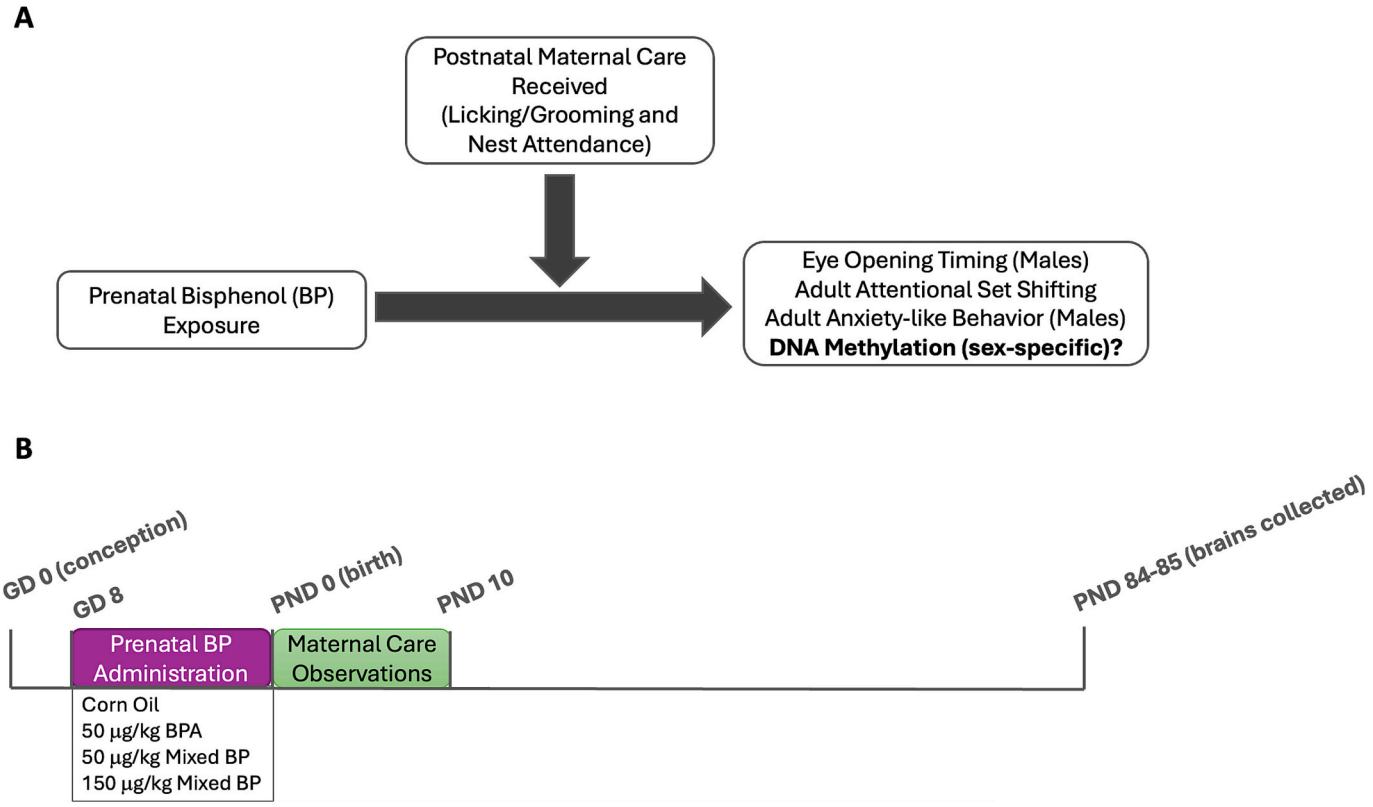

Fig. 1. (A) Previous work in our lab has found a moderating relationship between prenatal bisphenol exposure and postnatal maternal care (licking/grooming, nest attendance) on later-life neurodevelopmental and behavior outcomes in a sex-specific manner. The current study hypothesized a similar moderation between prenatal bisphenol exposure and postnatal maternal care on DNA methylation in candidate genes and brain regions relevant for anxiety-like behavior in a sex-specific manner. (B) Experimental design and timeline for prenatal bisphenol exposure, maternal care observations, and brain collection. Offspring brains were collected at postnatal days (PND) 84–85.

reflects the known impact of both prenatal bisphenol exposure and postnatal maternal care on this behavioral outcome (Kubo et al., 2003; Kundakovic et al., 2013; Ohtani et al., 2017; Perera et al., 2016; Wiersielis et al., 2020; Bonaldo et al., 2023; Boudalia et al., 2014; Catanese and Vandenberg, 2017; Kundakovic et al., 2013). The impact of these interactive effects on DNA methylation is currently unknown, though we have previously observed transcriptomic effects and the potential for opposing epigenetic effects of prenatal vs. postnatal environmental effects.

The aim of the current study is to examine the contributions of prenatal bisphenol exposure and postnatal maternal care and the interaction of these experiences to variation in DNA methylation within the ventral hippocampus and amygdala. Both the hippocampus and amygdala have been implicated in the expression of stress responsivity, anxiety and anxiety-like behavior and these brain regions also exhibit plasticity in response to early-life environmental exposures. Here we focus on DNA methylation levels of NR3C1, BDNF, and OXTR as potential links between early-life environmental exposures and later-life anxiety-like behavior within a research design that considers the potential sex-specificity of these effects. Sex differences in outcomes associated with early life environmental exposures are likely the norm rather than the exception and our previous studies have shown sex differences in response to prenatal bisphenol exposure, variation in postnatal maternal care, and the interaction between these variables. We hypothesized that DNA methylation levels in regulatory regions within the NR3C1, BDNF, and OXTR genes in the adult ventral hippocampus or amygdala would be associated with the effects of early-life prenatal bisphenol exposure and postnatal maternal care in a sex-specific manner.

2. Methods and materials

2.1. Animal housing

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 h 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.

2.2. Breeding and prenatal bisphenol (BP) exposure

The prenatal treatment groups and experimental timeline can be found in Fig. 1B. Briefly, 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 ($n = 22$). 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), 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.

2.3. Home-cage maternal behavior assessment

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 postnatal days (PND) 1–10. Maternal behavior was scored by either manual coding (when the dam was on the nest for 2 min or less during the video) or through the AMBER pipeline (Lapp et al., 2023). All postnatal maternal care measures were normalized to seconds of observed behavior per day to account for missing observations. Based on prior analyses (Lauby et al., 2024) we used licking/grooming and nest attendance from PND 1–5 as independent variables for the current study. There were no major impacts of prenatal bisphenol exposure found for the postnatal maternal care measures used in this study (Lauby et al., 2024).

2.4. Brain tissue collection and DNA extraction

At PND 84–85, all adult offspring were sacrificed by rapid decapitation; brains were flash-frozen in hexanes and stored at −80 °C until cryosectioning. One male and one female from a total of 23 litters were assessed for DNA methylation levels. Adult brains ($n = 5$–6 per sex per prenatal treatment group) that had undergone anxiety-like behavior assessment were cryosectioned in 100 μm slices using a Leica CM3050S cryostat. The amygdala (−1.8 to −3.4 mm Bregma) and ventral hippocampus (−4.2 to −5.8 mm Bregma) were identified and microdissected using a rat brain atlas (Paxinos and Watson, 2014). DNA was extracted using the MagMAX™ DNA Multi-Sample Ultra 2.0 Kit with the Kingfisher Flex™ system according to manufacturer's instructions for tissue samples (#A36570, ThermoFisher Scientific) with the optional RNAse A step (PureLink™ RNase A (20 mg/mL); #12091021, ThermoFisher Scientific). DNA quantity was assessed with the QuantiFluor® dsDNA System (#E2670, Promega). All DNA samples were submitted to EpigenDx (Hopkinton, Massachusetts, USA) for bisulfite conversion and DNA methylation (DNAm) assessment using next-generation sequencing at targeted regions within the glucocorticoid receptor (NR3C1), brain-derived neurotrophic factor (BDNF), and oxytocin receptor (OXTR) genes (Supplemental Table 1). A total of 33 CpG sites within NR3C1, 27 CpG sites within BDNF, and 23 CpG sites within OXTR were assessed for DNA methylation levels. These regions include the promoter region (including the promoter of Exon 1_7 and the NGFIA binding site) of NR3C1, the promoter region of Exon I as well as upstream and within Exon IV of BDNF, and the homologous MT2 region as well as Exon 1 (including the estrogen responsive element) of the OXTR gene.

2.5. Bisulfite conversion and PCR

500 ng of extracted DNA samples were bisulfite modified using the EZ-96 DNA Methylation Kit (#D5004, ZymoResearch) per the manufacturer's protocol with minor modification. The bisulfite modified DNA samples were eluted using M-elution buffer in 46 μL. All bisulfite modified DNA samples were amplified using separate multiplex or simplex PCRs. PCRs included 0.5 units of HotStarTaq (#203205, Qiagen), 0.2 μM primers, and 3 μL of bisulfite-treated DNA in a 20 μL reaction. All PCR products were verified using the Qiagen QIAxcel Advanced System (v1.0.6). Prior to library preparation, PCR products from the same sample were pooled and then purified using the QIAquick PCR Purification Kit columns or plates (#28106 or 28,183). Samples were run alongside established reference DNA samples with a range of methylation. They were created by mixing high- and low-methylated DNA to obtain samples with 0, 5, 10, 25, 50, 75, and 100 % methylation. The high-methylated DNA is in vitro enzymatically methylated genomic DNA with >85 % methylation. The low-methylated DNA is chemically and enzymatically treated with <5 % methylation. These

samples were first tested on numerous gene-specific and global methylation assays using pyrosequencing.

2.6. Library preparation, sequencing, and DNA methylation calculations

Libraries were prepared using a custom Library Preparation method created by EpigenDx. Library molecules were purified using Agencourt AMPure XP beads (#A63882, Beckman Coulter). Barcoded samples were then pooled in an equimolar fashion before template preparation and enrichment were performed on the Ion Chef™ system using Ion 520 & Ion 530 ExT Chef reagents (#A30670, Thermo Fisher). Following this, enriched, template-positive library molecules were sequenced on the Ion S5™ sequencer using an Ion 530 sequencing chip (#A27764). FASTQ files from the Ion Torrent S5 server were aligned to the *mRatBN7.2* rat reference genome using the open-source Bismark Bisulfite Read Mapper program (version 0.12.2) with the Bowtie2 alignment algorithm (version 2.2.3). Methylation levels were calculated in Bismark by dividing the number of methylated reads by the total number of reads. An R-squared value (RSQ) was calculated from the controls set at known methylation levels to test for PCR bias. Average number of reads per CpG site and RSQ information can be found in Supplemental Table 1.

2.7. Statistical analysis

All statistical analyses were performed using R version 4.1.3. Extreme outliers (> 3 standard deviations from the mean) within each CpG site were identified and removed from subsequent analyses. Inter-site correlations between proximal CpG sites within each gene were assessed using the psych R package and visualized using corrplot. Significant correlations between proximal sites were considered regions; the DNA methylation levels of all the CpG sites within these regions were averaged prior to further filtering and analyses. Three regions with significant inter-site correlations were identified, one from each gene (Supplemental Fig. 1). CpG sites and regions with 4 % or greater DNA methylation range were included for further analyses. CpG sites within known transcription factor binding sites were also included for further analyses. For NR3C1, two CpG sites (NR3C1.1980 and the NR3C1 3' NGFIA binding site) met the criterion for further analysis. The NR3C1 5' NGFIA binding site was also included in the analysis based on its role in transcriptional regulation even though it did not meet the criterion for DNA methylation range. For BDNF, one region that spanned 8 CpG sites (BDNF.3802–3881) and two separate CpG sites (BDNF.3946 and BDNF.3951) met the criterion for further analysis. For OXTR, one region that spanned 16 CpG sites (OXTR.5624–5775) met the criterion for further analysis. The OXTR Estrogen Responsive Element (ERE) site, located in the OXTR.5624–5775 region, was also analyzed separately. Details on the CpG sites and regions analyzed can be found in Table 1.

To examine the effects and interactions of prenatal bisphenol exposure and offspring sex on DNA methylation levels, 4 × 2-factor linear mixed models were performed with the lme4 R package and F-tests were calculated with the ANOVA (Type III) function in the cars R package. To examine the effects of postnatal maternal care and interactions with prenatal bisphenol exposure on DNA methylation levels, moderated regression analyses were performed using linear mixed models with the lme4 R package and F-tests were calculated with the ANOVA (Type III) function in the cars R package. Significant or marginal main effects of prenatal treatment, interactions with offspring sex, and interactions with postnatal maternal care on DNA methylation levels were followed with *t*-tests between the corn oil group and each of the BP treatment groups using the lmerTest R package. Batch of breeding was used as a random factor. All effects were reported as statistically significant if $p \leq 0.05$ and marginally significant if $p \leq 0.10$.

3. Results

3.1. Sex by prenatal treatment effects on DNA methylation

The t- and *p*-values from the significant pair-wise comparisons between the prenatal bisphenol treatment groups and interactions between the prenatal bisphenol treatment groups and offspring sex relative to the corn oil group can be found in Supplemental Table 2. Significant interactions were found between offspring sex and prenatal treatment when predicting DNA methylation in the NR3C1 gene within the amygdala (NR3C1.1980, $F_{(3,36)} = 3.27$, $p = 0.03$, Fig. 2A; marginal effect in NR3C1 3' NGFIA binding site, $F_{(3,33.498)} = 2.84$, $p = 0.05$, Fig. 2B) and within the BDNF gene within the ventral hippocampus (BDNF.3951, $F_{(3,33.156)} = 2.91$, p = 0.05, Fig. 2C). Within the amygdala, NR3C1.1980 CpG methylation was elevated in corn oil-treated male offspring compared to females, while this pattern was reversed within the mixed BP treatment groups (Fig. 2A). In contrast, within the NR3C1 3' NGFIA binding site in the amygdala, males had elevated DNA methylation compared to females within the 150 μg/kg Mixed BP exposure group with no sex differences observed in the other treatment groups (Fig. 2B). Within the ventral hippocampus, DNA methylation within the BDNF gene was elevated in corn oil-treated females compared to males, while this pattern was reversed within the BPA and 150 μg/kg Mixed BP exposure groups (Fig. 2C).

Significant main effects of treatment (but no sex by treatment interactions) were found within the OXTR gene in the amygdala (OXTR.5624–5775, $F_{(3,35.715)} = 5.76$, $p = 0.002$, corn oil < BPA and 50 μg/kg Mixed BP, Fig. 2D). Main effects of sex (but no sex by treatment interactions) were found in the amygdala within the OXTR gene (OXTR.5624–5775 region, $F_{(1,33.438)} = 10.55$, $p = 0.003$; females > males, Fig. 2D; OXTR ERE, $F_{(1,33.832)} = 4.20$, p = 0.05; females > males,

Table 1
List of CpG sites measured for DNA methylation (DNAm) levels and included in the final analysis, including location, basic statistics, and any known transcription factor binding sites.

Gene	CpG/Region ID	Gene Coordinates (rn7)	Location	Minimum DNAm levels	Maximum DNAm levels	DNAm Range	Known Transcription Factor Binding Sites
NR3C1	NR3C1.1980	Chr18:31362756	Exon 1_7 promoter	0.0	7.0	7.0	
	NR3C1.2020	Chr18:31362716	Exon 1_7 promoter	0.0	1.3	1.3	5' NGFIA Binding Site
	NR3C1.2026	Chr18:31362710	Exon 1_7 promoter	0.0	4.2	4.2	3' NGFIA Binding Site
BDNF	BDNF.3802–3881	Chr3:96163843–96,163,922	Exon I promoter	2.7	8.0	5.3	
	BDNF.3946	Chr3:96163987	Exon I promoter	0.7	8.2	7.4	
	BDNF.3951	Chr3:96163992	Exon I promoter	3.0	8.0	5.0	
OXTR	OXTR.5624–5775	Chr4:145614051–145,613,900	Exon 1	4.3	9.5	5.2	Estrogen Responsive Element
	OXTR.5750	Chr4:145613925	Exon 1	0.0	9.5	9.5	Estrogen Responsive Element

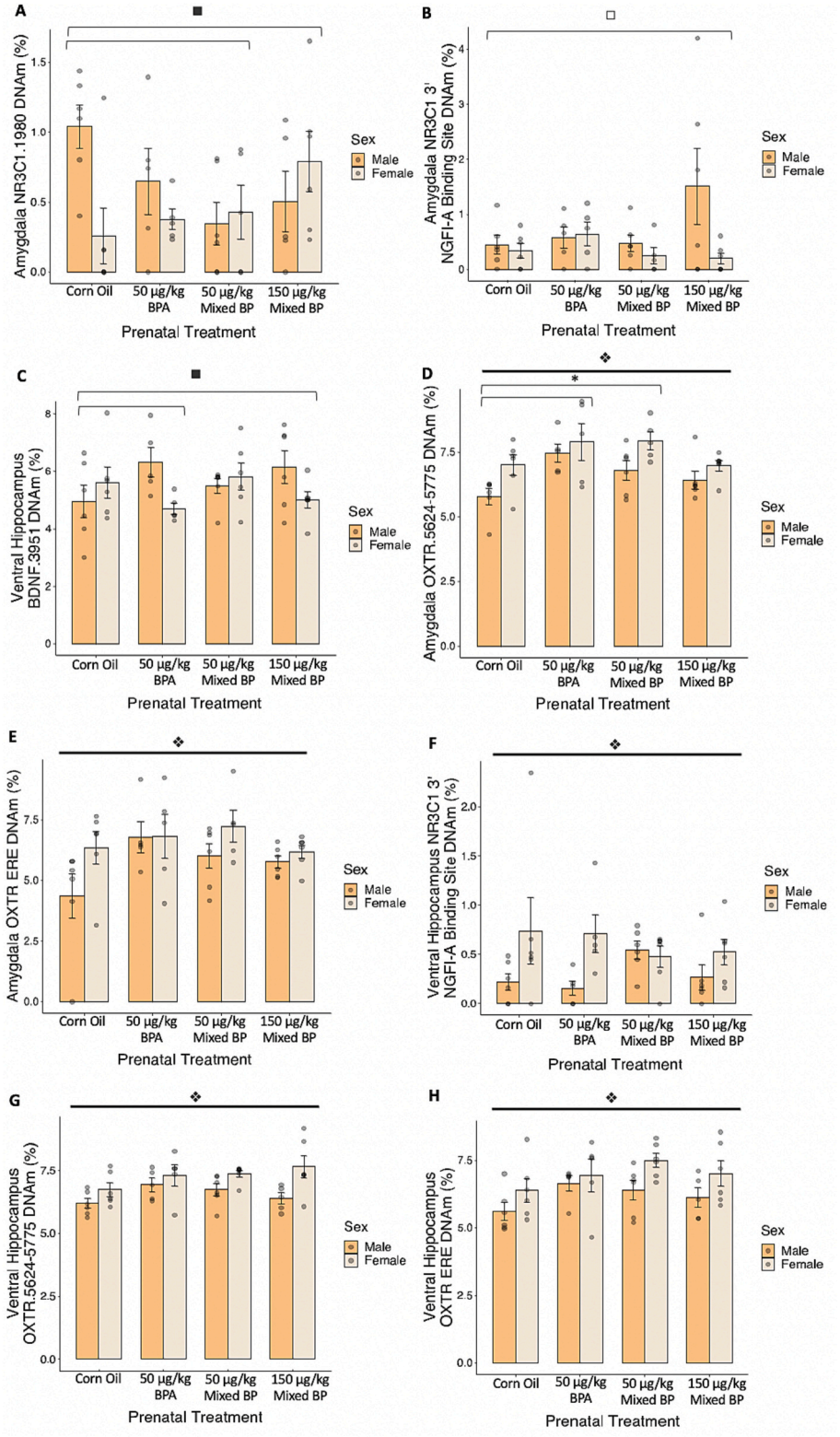

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Fig. 2. Main effects of prenatal bisphenol exposure, offspring sex, and interactions between prenatal bisphenol exposure and offspring sex on DNA methylation (DNAm) levels. Interactions were found between offspring sex and prenatal treatment for DNAm levels in the (A) NR3C1.1980 CpG site and (B) NR3C1 3' NGFIA binding site within the amygdala and in the (C) BDNF.3951 CpG site within the ventral hippocampus. Significant main effects of prenatal treatment were found in the (D) OXTR.5624–5775 region within the amygdala. Main effects of sex were found in the amygdala for (D) OXTR.5624–5775 region and (E) OXTR ERE and in the ventral hippocampus for the (F) NR3C1 3' NGFIA binding site, (G) OXTR.5624–5775 region, and (H) OXTR ERE. Bar plots are displayed with mean +/− SEM with individual datapoints. Brackets between two groups indicate significant pair-wise differences between the prenatal bisphenol treatment groups relative to the corn oil group. A thick line across all groups indicates significant main effects of offspring sex across all prenatal treatment groups.

Fig. 2E) and in the ventral hippocampus within the NR3C1 gene (NR3C1 3' NGFIA binding site, $F_{(1,38)} = 7.14$, $p = 0.01$; females > males, Fig. 2F) and OXTR gene (OXTR.5624–5775, ($F_{(1,37)} = 11.62$, p = 0.002; females > males, Fig. 2G; OXTR ERE, $F_{(1,33.728)} = 7.43$, p = 0.01; females > males, Fig. 2H).

3.2. Postnatal maternal care by prenatal treatment effects on DNA methylation

Given the presence of sex by treatment effects in DNA methylation levels, male and female siblings were analyzed separately. The t- and *p*-values from the significant pair-wise interactions between the prenatal bisphenol treatment groups and maternal care relative to the corn oil group can be found in Supplemental Table 3.

For male offspring, significant interactions were found between postnatal nest attendance and prenatal treatment within the NR3C1 gene in the ventral hippocampus ($F_{(1,13.674)} = 6.45$, $p = 0.006$, Fig. 3A) and the BDNF gene in the amygdala (BDNF.3946, $F_{(3,12.044)} = 4.09$, $p = 0.03$, Fig. 3B) and ventral hippocampus (BDNF.3951, $F_{(3,15)} = 6.13$, p = 0.006, Fig. 3C). In the ventral hippocampus, higher time spent on nest was associated with higher methylation levels in the NR3C1.1980 CpG site in the 50 μg/kg Mixed BP exposure group only (Fig. 3A). In contrast, higher time spent on nest was associated with lower methylation levels in the BDNF.3951 CpG site for the corn oil-treated offspring while it was associated with higher methylation levels in the BPA and 150 μg/kg Mixed BP exposure groups (Fig. 3B). In the amygdala, higher time spent on nest was associated with lower methylation levels in the BDNF.3946 CpG site in the 50 μg/kg Mixed BP exposure group only (Fig. 3C). No significant main effects of postnatal maternal care were found in male offspring on DNA methylation measures.

For female offspring, significant interactions were found between postnatal nest attendance and prenatal treatment within the NR3C1

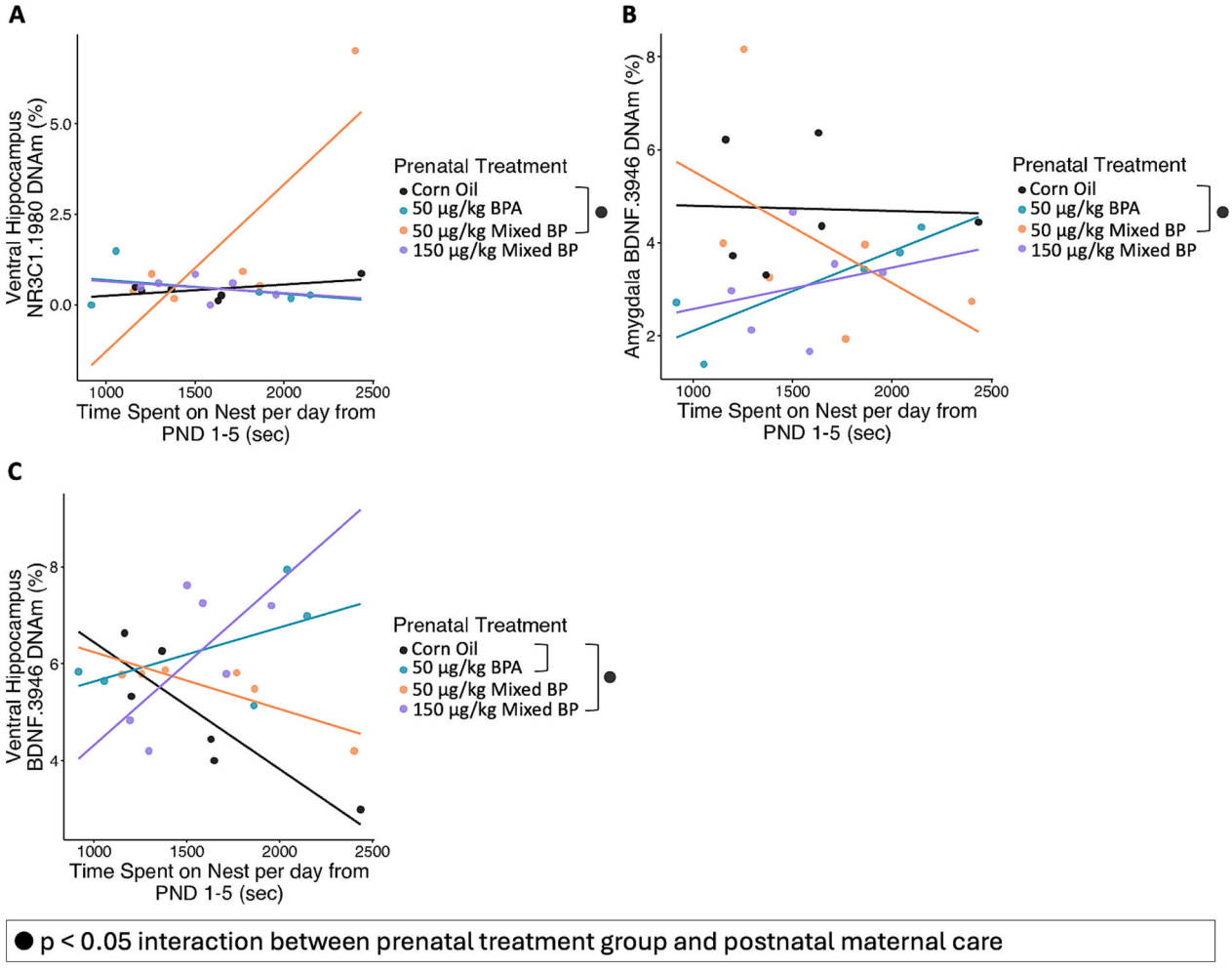

Fig. 3. Interactions between prenatal bisphenol exposure and postnatal nest attendance on DNA methylation (DNAm) levels in male offspring. Significant interactions were found between postnatal nest attendance and prenatal treatment in the (A) NR3C1.1980 CpG site and within the ventral hippocampus, the (B) BDNF.3946 CpG site gene within the amygdala and the (C) BDNF.3951 CpG site within the ventral hippocampus. Scatterplots are displayed with linear regression lines for each prenatal treatment group. Datapoints represent individual rats. Brackets between two groups indicate significant pair-wise interactions between the prenatal bisphenol treatment groups and maternal care relative to the corn oil group.

gene in the ventral hippocampus (NR3C1 3' NGFIA binding site, $F_{(3,15)} = 6.42$, $p = 0.005$, Fig. 4A) and the OXTR gene in the amygdala (OXTR.5624–5775, $F_{(3,12.938)} = 4.42$, $p = 0.02$; Fig. 4B). Within the ventral hippocampus, higher time spent on nest was associated with higher methylation levels in the NR3C1 3' NGFIA binding site in the corn oil-treated offspring only (Fig. 4A). Within the amygdala, higher time spent on nest, especially for offspring within the BPA exposure group, was associated with higher methylation in the OXTR.5624–5775 region (Fig. 4B).

In female offspring, significant main effects of postnatal licking/grooming were found within the NR3C1 gene in the ventral hippocampus (NR3C1 3' NGFIA binding site, $F_{(1,15)} = 4.87$, $p = 0.04$; positive association, Fig. 4C), and within the BDNF gene in the amygdala (BDNF.3802–3881, $F_{(1,14)} = 6.01$, $p = 0.03$; positive association, Fig. 4D; marginal effect in BDNF.3946, $F_{(1,14)} = 4.45$, $p = 0.05$; negative association, Fig. 4E; marginal effect in BDNF.3951, $F_{(1,14)} = 3.34$, $p = 0.09$; positive association, Fig. 4F). Significant main effects of postnatal nest attendance were found within the BDNF gene in the amygdala (BDNF.3802–3881, $F_{(1,14)} = 6.16$, $p = 0.03$; positive association, Fig. 4G; marginal effect in BDNF.3951, $F_{(1,14)} = 3.16$, $p = 0.096$; positive association, Fig. 4H) and the OXTR gene in the amygdala (OXTR.5624–5775, $F_{(1,11.146)} = 6.72$, $p = 0.02$; positive association, Fig. 4B).

4. Discussion

In this study, we examined the effects of prenatal bisphenol exposure, postnatal maternal care, and offspring sex on DNA methylation of well-studied candidate genes (NR3C1, BDNF, OXTR) in the amygdala and ventral hippocampus. We found main effects of postnatal maternal care and interactions with prenatal bisphenol exposure for NR3C1 in the ventral hippocampus and for BDNF and OXTR in the amygdala. The more prominent effects of prenatal bisphenol exposure and postnatal maternal care on the amygdala have also been found in our previous work examining genome-wide transcriptomic changes in postnatal rat pups and weanling rats (Lapp et al., 2022; Lauby et al., 2024). Sex-specific effects were found across all analyses, also consistent with previous literature examining the effects of prenatal bisphenol exposure. Overall, our findings suggest that both prenatal bisphenol exposure and postnatal maternal care as well as offspring sex contribute to the prediction of DNA methylation levels in a brain region specific manner. These results highlight the need to consider the effects of multiple exposures in males and females in multiple brain regions to understand the mechanisms conferring risk and resilience to psychiatric outcomes.

While main effects of prenatal BPA on a broad range of neural and behavioral outcomes have been observed (Kundakovic and Champagne, 2011), our recent exploration of the impact of BPA and mixed bisphenols suggest that transcriptional and behavioral outcomes are predicted by complex interactions between sex, postnatal care, and prenatal exposures (Kundakovic et al., 2013; Lauby et al., 2024). In the current study, we observed this pattern of results at the level of DNA methylation. Sex-specific effects of prenatal bisphenol exposure are consistent with the existing literature and may be in part due to the estrogen disrupting effects of bisphenols during sexual differentiation of the brain (Kubo et al., 2003; MacLusky and Naftolin, 1981). In addition, DNA methylation itself is involved in the sexual differentiation of the brain (Forger, 2016; Nugent et al., 2015), with sex differences and interactions between sex and early-life exposures predicting DNA methylation likely to be common and widespread. Interplay between different early life experiences in shaping developmental trajectories is also likely a widespread phenomenon and may account for significant variations in predicted outcomes. Our findings suggest that the expression of epigenetic and behavioral outcomes associated with prenatal exposures can vary dependent on the characteristics of the postnatal environment, reflecting the dynamic influence of the environment at different developmental stages. Ultimately, adult outcomes are a consequence of these "mixtures" of environmental experience.

Epigenetic effects of postnatal maternal care in rodents have been established using candidate-gene approaches that have contributed to our understanding of plasticity and stability in these molecular mechanisms (Champagne, 2016; Weaver et al., 2004). Our analyses indicate significant effects of postnatal maternal licking/grooming and nest attendance on DNA methylation in female offspring. Interestingly, most of these effects were positive relationships, such that higher maternal care was associated with higher DNA methylation. Previous studies have found that the experience of high compared to low levels of licking/grooming during postnatal development in rats is associated with hypomethylation of the NGFIA 3′ binding site in NR3C1 gene in the hippocampus of male offspring and hypomethylation of the estrogen receptor alpha (ESR1) promoter region in the hypothalamus (Champagne et al., 2006; Weaver et al., 2004). Disrupted postnatal maternal care has typically been associated with higher BDNF DNA methylation, though this relationship is likely dependent on the transcript variant studied (Exon I vs. Exon IV or Exon IX), brain region, and offspring sex (Roth et al., 2014). However, consistent with our findings, higher DNA methylation in the NGFIA 3′ binding site in NR3C1 has been observed in the hippocampus of female offspring that received higher levels of licking/grooming when considering within and between litter levels of licking/grooming (Pan et al., 2014). Our analyses of OXTR DNA methylation also suggest site specific positive and negative correlations with postnatal maternal care. Low care experienced by rodents is typically associated with increased OXTR DNA methylation (a negative relationship), though this effect may not be observed across all brain regions (Beery et al., 2016; Perkeybile et al., 2019). Methodological differences in the characterization of postnatal maternal care may account for some of these discrepancies and it is unlikely that epigenetic effects of maternal care are expressed similarly across the genome and across the brain.

Sex differences in gene transcription and DNA methylation have been observed within the brain and have been attributed to sex differences in circulating hormones at varying life stages. Our analyses indicate a consistent pattern of sex differences in adulthood, where females compared to males have elevated DNA methylation within OXTR (amygdala and ventral hippocampus) and NR3C1 (ventral hippocampus). These sex differences persist regardless of prenatal or postnatal environmental conditions. A similar pattern of sex differences has previously been observed in adolescent rats, where increased hippocampal DNA methylation of NR3C1 was observed in females compared to males, regardless of litter composition (Kosten et al., 2014). This increased DNA methylation may account for decreases in the expression of glucocorticoid receptors that have been observed within brain of females compared to males and mediate the increased hypothalamic-pituitary-adrenal axis activity observed in females compared to males (Bangasser, 2013). The sex differences in DNA methylation proximal to the OXTR ERE that we identified is consistent with previous findings of sex differences in OXTR binding in the amygdala and hippocampus, where females have lower OXTR than males (Dumais et al., 2013). These findings highlight the importance of including males and females with a research design to determine potential risk vs. resilience associated with sex.

To our knowledge, this is the first study to identify effects of early-life experiences and offspring sex on DNA methylation levels proximal to the OXTR ERE in the rat. Previous work has suggested a functional role of DNA methylation at this site for OXTR transcription in the mouse brain (Harony-Nicolas et al., 2014), though no sex differences were found in the brain regions studied (olfactory bulb and cerebellum). We found high inter-site correlations between proximal CpG sites in this region, which often occurs at putative regulatory regions (Jajoo et al., 2023; Min et al., 2020). In addition, both prenatal bisphenol exposure and higher maternal care were associated with higher DNA methylation in this region and the epigenetic effects of maternal care were stronger in female offspring. Both bisphenol exposure and postnatal maternal care are associated with changes in estrogen receptor signaling (Champagne

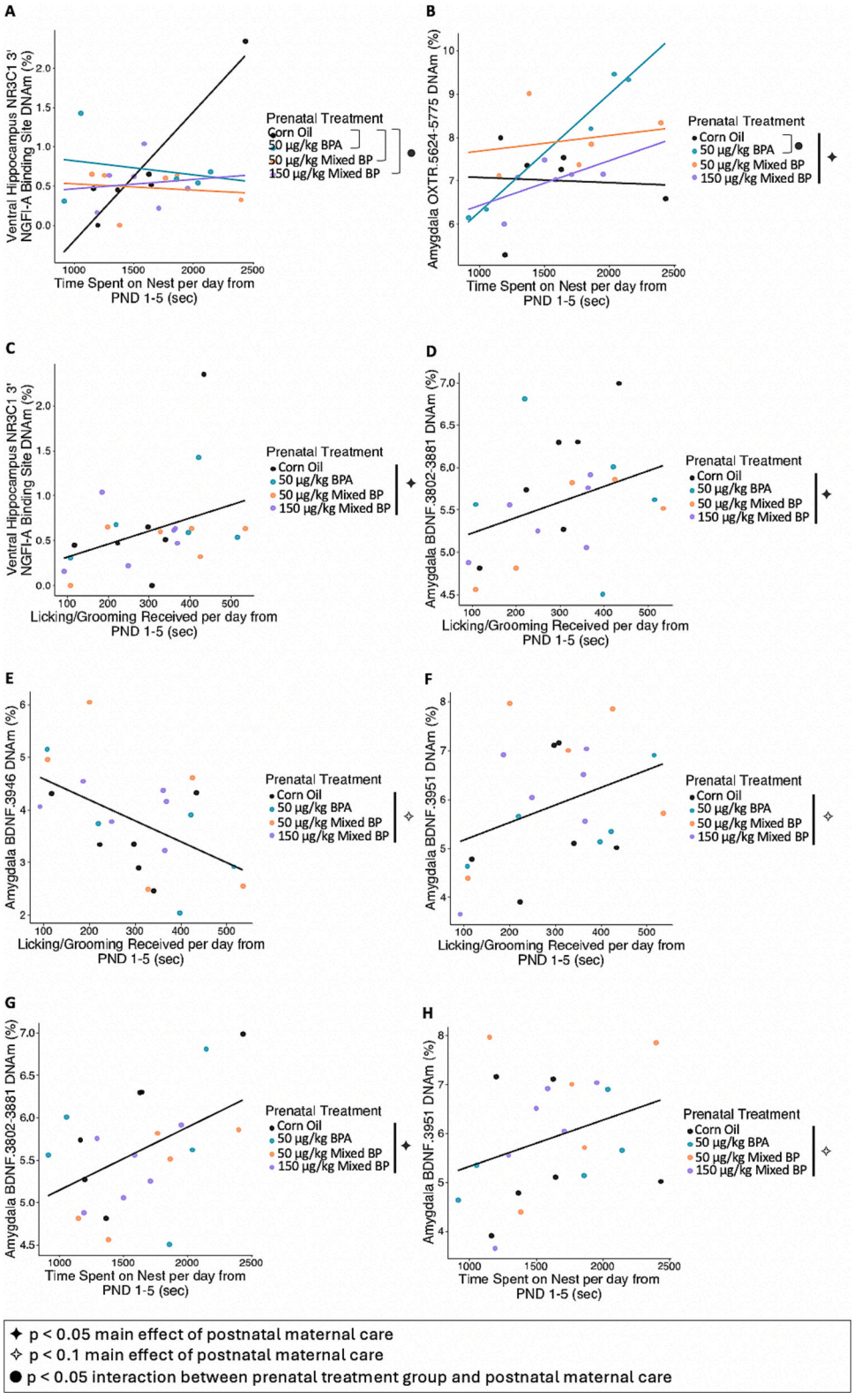

Fig. 4. Main effects of postnatal maternal care and interactions between prenatal bisphenol exposure and postnatal maternal care on DNA methylation (DNAm) levels in female offspring. Significant interactions were found between postnatal nest attendance and prenatal treatment in the (A) NR3C1 3' NGFIA binding site within the ventral hippocampus and the (B) OXTR.5624–5775 region within the amygdala. Significant main effects of postnatal licking/grooming were found in the (C) NR3C1 3' NGFIA binding site within the ventral hippocampus and in the (D) BDNF.3802–3881 region, (E) BDNF.3946 CpG site, and (F) BDNF.3951CpG site within the amygdala. Significant main effects of postnatal nest attendance were found in the (G) BDNF.3802–3881 region, (H) BDNF.3951 CpG site, and (B) OXTR.5624–5775 region in the amygdala. Scatterplots are displayed with linear regression lines for each prenatal treatment group. Datapoints represent individual rats. Brackets between two groups indicate significant pair-wise interactions between the prenatal bisphenol treatment groups and maternal care relative to the corn oil group. A thick line across all groups indicates significant main effects of maternal care across all prenatal treatment groups.

et al., 2003; Champagne and Curley, 2008; Jorgensen et al., 2016), so it is possible that the ERE would be a target for the effects of these early-life exposures.

There are several limitations to note with this study. First, our sample sizes within each prenatal treatment group and sex were small, which also potentially limited the range of observed postnatal maternal care and DNA methylation levels analyzed. Future work should include larger sample sizes that focus on the most promising candidate CpG sites and regions found in this study. Second, we did not measure gene expression levels associated with the CpG sites and regions analyzed in this study. While we primarily focused on CpG sites and regions that have been previously shown to regulate expression of those genes in the brain, the regulatory role of several of the CpG sites (in NR3C1 and BDNF) examined in this study have yet to be examined. Finally, while our study design did not allow us to link DNA methylation modifications to anxiety-like behavior directly, this will be an important avenue to examine in future work. Though previous studies have shown that DNA methylation modifications occurring early in life will persist into adulthood, there is little known about the downstream effects of DNA methylation modifications on neural function and circuit formation throughout neurodevelopment. There are well-known bidirectional projections between the amygdala and ventral hippocampus that contribute to the expression of fear responses (Ishikawa and Nakamura, 2006; Nguyen et al., 2023), so it is possible that brain-region specific variation in DNA methylation emerging in early life could impact the molecular profile and function of other brain regions that regulate behavioral phenotypes. This dynamic developmental process is challenging to establish and would require the integration of in vivo and in vitro approaches.

5. Conclusions

Using a multivariate approach, we found that multiple perinatal experiences contribute to DNA methylation levels within known or potential regulatory regions in NR3C1, BDNF, and OXTR in brain regions relevant for anxiety-like behavior in a rat model. These findings are consistent with human studies that are increasingly reporting the contributions of multiple early-life factors and gender on the risk of psychiatric disorders at adulthood. The results underscore the need to study multiple brain regions and their circuitry to fully understand the mechanisms linking early-life experiences, DNA methylation modifications in the brain, and changes in adult behavior. Examining these mechanisms will be important to understand how DNA methylation modifications in peripheral tissues could serve as a useful biomarker for studying potential mediators between early-life experiences and psychiatric outcomes in human studies.

CRediT authorship contribution statement

Samantha C. Lauby: Writing – original draft, Visualization, Methodology, Investigation, Formal analysis, Conceptualization. **Isha Agarwal:** Writing – review & editing, Methodology, Investigation, Conceptualization. **Hannah E. Lapp:** Writing – review & editing, Investigation, Formal analysis. **Melissa Salazar:** Investigation. **Sofiia Semyrenko:** Investigation. **Danyal Chauhan:** Investigation. **Amy E. Margolis:** Writing – review & editing, Funding acquisition, Conceptualization. **Frances A. Champagne:** Writing – review & editing, Supervision, Funding acquisition, Conceptualization.

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.yhbeh.2025.105745.

Data availability

Data will be made available on request.

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