Impact of prenatal polycyclic aromatic hydrocarbon exposure on behavior, cortical gene expression, and DNA methylation of the *Bdnf* gene ☆

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ARTICLE INFO

ABSTRACT

Prenatal exposure to polycyclic aromatic hydrocarbons (PAH) has been associated with sustained effects on the brain and behavior in offspring. However, the mechanisms have yet to be determined. We hypothesized that prenatal exposure to ambient PAH in mice would be associated with impaired neurocognition, increased anxiety, altered cortical expression of *Bdnf* and *Grin2b*, and greater DNA methylation of *Bdnf*. Our results indicated that during open-field testing, prenatal PAH–exposed offspring spent more time immobile and less time exploring. Females produced more fecal boli. Offspring prenatally exposed to PAH displayed modest reductions in overall exploration of objects. Further, prenatal PAH exposure was associated with lower cortical expression of *Grin2b* and *Bdnf* in males and greater *Bdnf IV* promoter methylation. Epigenetic differences within the *Bdnf IV* promoter correlated with *Bdnf* gene expression but not with the observed behavioral outcomes, suggesting that additional targets may account for these PAH-associated effects.

1. Introduction

Prenatal exposure to toxins can have a lasting impact on the brain and behavior with implications for development and health in subsequent generations (Skinner et al., 2008; Wolstenholme et al., 2012). Polycyclic aromatic hydrocarbons (PAH) are a class of pollutants produced during the incomplete combustion of organic materials. In US cities, traffic emissions are one of the most abundant sources of outdoor PAH. High levels of PAH exposure can originate from indoor sources (eg, space heating, cooking, smoking, burning incense or candles) as well as from outdoor traffic sources that penetrate indoors (Jung et al., 2010). In human cohort studies, our group at the Columbia Center for Children's Environmental Health (CCCEH) and others have shown that prenatal exposure to PAH was linked to reduced head circumference

and birth weight (Dejmek et al., 2000), lower IQ (Perera et al., 2009), anxious and depressive symptoms, attention problems (Perera et al., 2012), reductions in white matter surface, and slower cognitive processing speed in later childhood (Peterson et al., 2015).

The molecular mechanisms of PAH-induced disruption to neurodevelopment have yet to be determined. In laboratory rodents, prenatal exposure to low doses of the PAH benzo[a]pyrene impaired learning and memory, in part by reducing neural plasticity by disrupting glutamate signaling (Brown et al., 2007) and inducing toxic effects to glial cells (Dutta et al., 2010; Wormley et al., 2004). Environmentally induced alterations in DNA methylation within the brain that may suppress gene expression have been demonstrated following prenatal exposure to stress (Mueller and Bale, 2008), endocrine-disrupting chemicals (Kundakovic et al., 2013), and variation in the nutritional environment (Cho et al., 2013) and may serve as a molecular mechanism that mediates the sustained effects of prenatal and postnatal PAH environmental exposures. For example, in vitro studies demonstrated transcriptional changes of interspersed element-1 (LINE-1), a mammalian retrotransposon, in response to benzo[a]pyrene exposure; these were attributed to reductions in the expression of DNA methyltransferase-1 (*Dnmt1*) and *Dnmt1*

recruitment to the LINE-1 promoter (Teneng et al., 2011). In zebrafish, exposure to benzo[a]pyrene was shown to induce both genomewide transcriptional changes in embryos (Fang et al., 2015) and gene-specific and genomewide reductions in DNA methylation (Fang et al., 2013). In human cohorts, global reductions in DNA methylation cord blood were associated with prenatal exposure to elevated PAH levels (Herbstman et al., 2012). Overall, these studies provide converging evidence implicating epigenetic changes in mediating the effects of PAH.

In the current study, we conducted analyses of the impact of prenatal exposure to PAH on behavior, brain gene expression, and DNA methylation in mice exposed to an aerosolized mixture that simulates the prenatal PAH exposure of a CCCEH cohort of children from Northern New York City (Chu et al., 2013; Yan et al., 2014). Within this study, we assessed behavioral outcomes related to cognition, activity levels, and anxiety-like phenotypes and focused on the expression of 2 candidate genes implicated in neural plasticity: brain-derived neurotrophic factor (*Bdnf*) and the ionotropic glutamate receptor *N*-methyl D-aspartate 2B (*Grin2b* or *Nmdar2b*). Previous studies have demonstrated altered expression and DNA methylation of *Bdnf* exon IV in response to in utero exposure to endocrine-disrupting chemicals in mice and humans (Kundakovic et al., 2015) and postnatal exposure to stressful rearing conditions in rats (Roth et al., 2009). In humans, prenatal exposure to related combustion products, namely, fine particulate matter, was associated with lower *Bdnf* expression in the placenta (Saenen et al., 2015). Closure of a highly polluting, coal-fired power plant was associated with higher mature BDNF protein levels in cord blood and improved neurocognitive development measured at 2 years of age (Tang et al., 2014). Similarly, studies in rodents indicated altered *Grin2b* expression and promoter DNA methylation in response to prenatal adversity (Kundakovic et al., 2015; Kleiber et al., 2013), and *Grin2b* genetic variations predicted risky decision making and attention problems in human cohorts (Riva et al., 2015; Ness et al., 2011).

We hypothesized that prenatal exposure to PAH would be associated with impaired neurocognitive function and increased anxiety-like behaviors. Second, we hypothesized that prenatal exposure to PAH would be associated with decreased expression of *Bdnf* and *Grin2b* in the cerebral cortex and increased DNA methylation of *Bdnf* IV. Finally, based on previous studies indicating sex differences in neurodevelopmental and epigenetic outcomes associated with prenatal exposure to stress and endocrine disrupting chemicals (Mueller and Bale, 2008; Kundakovic et al., 2013), we predicted that both behavioral and gene expression/epigenetic outcomes associated with prenatal exposure to PAH would vary by offspring sex.

2. Materials and methods

2.1. Animals

Nine-week-old BALB/cByj female mice weighing 20-22 g were obtained from Charles River and housed 4-5/cage in temperature-regulated (20°C), ventilated cabinets with a 12-hour light/12-hour dark cycle (09:00 to 21:00). Animals were provided ad libitum access to a standard diet and water and were housing acclimated in this controlled environment for at least 1 week before any experiments. After 1 week, mice were mated, and the protocol summarized in Fig. 1 was initiated. Animal experiments were carried out in strict accordance with the principles and procedures of the *Guide for the Care and Use of Laboratory Animals*. The protocol was approved by the Institutional Animal Care and Use Committee, Columbia University Medical Center.

2.2. PAH exposure

The PAH mixture was produced by the Lovelace Respiratory Research Institute to replicate the proportions of individual PAH that was measured among a cohort of more than 700 pregnant women using personal air sampling devices (Chu et al., 2013; Yan et al., 2014; Miller et al., 2004). Briefly, the negative control aerosol solution consisted of 99.97% purified water, 0.02% Tween 80, and 0.01% antifoam (Sigma-Aldrich, St Louis, MO). The mixed PAH solution was added to yield the final concentration of 7.29 ng/m^3. Solutions were delivered via nebulizers (Unomedical Inc, McAllen, TX) connected to filtered compressed air in a chamber set to achieve a flow of 12.5 to 13.0 L/min (Chu et al., 2013; Yan et al., 2014). Four dams were exposed in 1 cage at a time with full access to food and water for 5 hours a day, 5 days a week, beginning on gestational day 0.5-2.5 until birth of litter (gestational day 19-21). Upon completion of the daily exposure session, mice were returned to their usual housing conditions. Previously, we determined chamber pyrene levels of 23.24 ± 3.05 ng/m^3 (range, 7.38-40 ng/m^3) from 3 weekly filters extracted together (Chu et al., 2013), suggesting that levels ambient in the chamber may be higher than levels ambient in the NYC urban environment. Prenatally exposed offspring were weaned at postnatal day 21 and housed in same-sex, same-condition groups of 4 mice per cage until testing.

2.3. Behavioral phenotyping

At postnatal day 60, male and female offspring (sample sizes for behavioral analyses consisted of n = 18 male/n = 18 female control offspring and n = 14 male/n = 16 female PAH-exposed offspring) were assessed on a battery of behavioral tests that included indices of activity, exploration, anxiety-like responses, and recognition memory (Fig. 1). Tests were conducted sequentially with 3-4 days between tests in the following order: open field, elevated plus, light-dark box, and novel object recognition.

2.3.1. Open-field testing

Behavior during open-field assessment is a standard measure of activity and anxiety-like responses in rodents (Belzung and Griebel, 2001). The open-field apparatus used was a 24 × 24 × 16-in Plexiglas box. On the day of testing, the mouse was placed directly into one corner of the open-field. After a 10-minute session, the mouse was returned to its home cage. All testing was conducted under red lighting conditions. Behaviors were video recorded. Behaviors scored using AnyMaze software (version 4.82) included (1) total distance traveled (meters), (2) time spent immobile (seconds), and (3) time spent in the center area (the inner 12 × 12-in region of the field) (seconds). Counts of fecal boli produced during testing also were assessed.

2.3.2. Elevated plus maze

The elevated plus maze is a standard measure of anxiety-like behavior and exploration in rodents (Walf and Frye, 2007). The
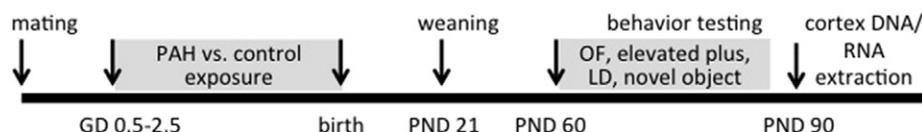

Fig. 1. Study design indicating timing of treatment and assessments of offspring. Abbreviations: OF = open field; LD = light-dark box; GD = gestational day; PND = postnatal day.

Plexiglas testing apparatus consisted of 2 open arms and 2 closed arms, each extending 18 in in length and 4 in in width. The apparatus was elevated 24 in above the floor. All testing was conducted under red lighting conditions. At the start of testing, the animal was placed in the center of the maze, and a 10-minute exploration session was video recorded and scored with AnyMaze. Behaviors scored included (1) total distance traveled (meters), (2) latency to enter the open arms (seconds), (3) time spent in the open arms (seconds), and (4) time spent in the closed arms (seconds).

2.3.3. Light-dark box

Behavioral assessment in the light-dark box is a standard laboratory measure of anxiety-like behavior and exploration in rodents (Bourin and Hascoet, 2003). The light-dark testing apparatus was a $11.5 \times 19 \times 11$-in (outer dimensions) Plexiglas box consisting of a light compartment (11×11 in) and a smaller dark compartment (11×7 in) connected by a door (3×3 in) in the center of the wall separating the 2 compartments. A 60-W bulb located 16 in above the center of the light compartment provided bright illumination. Mice were placed in the center of the dark compartment facing the door and were allowed to explore the box for 10 minutes. Measures scored (AnyMaze) included (1) time spent in the light compartment (seconds) and (2) time spent in the dark compartment (seconds).

2.3.4. Novel object test

The novel object recognition test has been previously used to assess memory deficits (Broadbent et al., 2010). Testing consisted of 3 phases: habituation, acquisition, and test phase. On the first day, mice were habituated to the novel object testing arena (a $24 \times 24 \times 16$-in Plexiglas box) for 10 minutes in the absence of any objects. The following day, mice were tested for object recognition using acquisition and test trials separated by a 30-minute delay. During the acquisition trial, mice were exposed to 2 identical objects for 15 minutes. During the 15-minute test trial, mice were exposed to the original object and a new ("novel") object. Following data collection using video recording, all observations were coded using Observer XT software (Noldus Information Technology, version 9.0). The variables of interest were total amount of time spent exploring (sniffing and direct contact) the objects and preference for the novel object. Preference score was calculated as the amount of time spent investigating the novel object divided with the total time spent investigating both the novel and familiar objects. We also categorized mice as having a preference for the novel object if their preference score exceeded 0.5.

2.4. RNA and DNA extraction

Brains were extracted 1 week following the completion of behavioral testing and stored at $-80°C$. The cerebral cortex was dissected in a cryostat at $-20°C$. Total RNA was extracted using Trizol (Invitrogen) following the manufacturer's protocol. RNA was treated with DNase I (Ambion, Applied Biosystems). RNA quality was determined using Nano Drop ND-1000 (Thermo Fisher Scientific Inc), and the OD A260/A280 ratio was calculated to assess quality. For DNA extraction, 30 mg of cerebral cortex was quickly washed and incubated in 900 μL of lysis buffer (50 mmol/L Tris-Cl pH 8.0, 10 mmol/L EDTA, 100 mmol/L NaCl, 1% SDS) and 0.1 mg proteinase K (Promega) with shaking for 4 hour at 55°C. The genomic DNA was separated by 2 rounds of phenol-chloroform extraction, and traces of phenol were removed with chloroform. Genomic DNA was precipitated with sodium acetate/isopropanol followed by washing with 75% ethanol, and it was then dissolved in Tris-EDTA buffer and stored at $-20°C$.

2.5. Gene expression

Relative gene expression was measured with real-time semiquantitative polymerase chain reaction (PCR) on a 96-well CFX Connect Real-Time PCR Detection System (BioRad) as previously described (Yan et al., 2014). All primers were designed to span exons and had >85% efficiency in a standard curve with single melt peaks. Calculations of relative gene expression were conducted with the $2^{\text{Delta–Delta CT}}$ method normalized by Gapdh and to control offspring (Schmittgen and Livak, 2008). Analyses included transcript specific expression of Bdnf (exon III and IV; Fig. 2A) and Grin2b (spanning exon III, IV; Fig. 2B). Forward and reverse primer sequences are indicated in Table 1.

2.6. DNA methylation

To determine levels of DNA methylation within Bdnf exon IV, 250 ng of genomic DNA underwent bisulfite modification using the EZ DNA Methylation-Direct Kit (Zymo Research). The bisulfite-converted DNA was resuspended in 40 μL of Tris-EDTA buffer and stored at $-80°C$. Primers for pyrosequencing were designed with PyroMark Assay Design Software 2.0 (Qiagen) (Table 1). PCR was performed according to standard techniques with the following components: 3.0 mmol/L MgCl₂, 200 mmol/L dNTPs, 0.2 mmol/L primers, 1.25 U HotStar Taq DNA polymerase (Qiagen), and approximately 10 ng of bisulfite-converted DNA per 50-mL reaction. Following purification, biotinylated PCR (20 μL) products were analyzed using a PyroMark Q24 system (Qiagen). The DNA methylation status of each CpG site was analyzed individually as a T/C SNP by the PyroMark Assay Design Software 2.0 (Qiagen) as shown in Fig. 2A.

2.7. Statistical analyses

Behavioral, gene expression, and DNA methylation data were analyzed using a 2-way analysis of variance (repeated measures for DNA methylation) with prenatal treatment (control vs PAH) and sex (male vs female) as independent variables and litter as a covariate. Tukey post hoc test was conducted in cases of sex by treatment interactions. Novel object preference was analyzed using a χ^2 test. Spearman rank correlation coefficients were used to examine associations between gene expression, DNA methylation, and behavior. Values of $P < .05$ were regarded as statistically significant.

3. Results

Prenatal PAH exposure had no significant impact on reproductive success (n = 11 litters were generated for each exposure condition) or litter size (PAH mean 4.36 ± 0.45, control mean 5.09 ± 0.68, nonsignficant).

3.1. Behavioral impact of prenatal PAH exposure

3.1.1. Open-field test

Total distance traveled during testing was not altered by PAH exposure ($P = .27$; Fig. 3A). Analyses of behavioral data collected during open-field testing indicated that prenatal PAH exposure resulted in more time spent immobile ($F_{1,66} = 4.46$, $P < .05$; Fig. 3B) and less time spent in the inner area of the field ($F_{1,66} = 5.67$, $P < .05$; Fig. 3C). A sex by treatment interaction ($F_{1,66} = 4.21$, $P < .05$) was found on counts of fecal boli produced during testing, with PAH-exposed female offspring generating more fecal boli (Tukey; $P < .01$) than control offspring (with no effect observed in male offspring; Fig. 3D).

3.1.2. Elevated plus maze and light-dark box

No significant differences were observed in behavioral measures assessed during elevated plus and light-dark box testing (Table 2).

A. *Bdnf* gene:
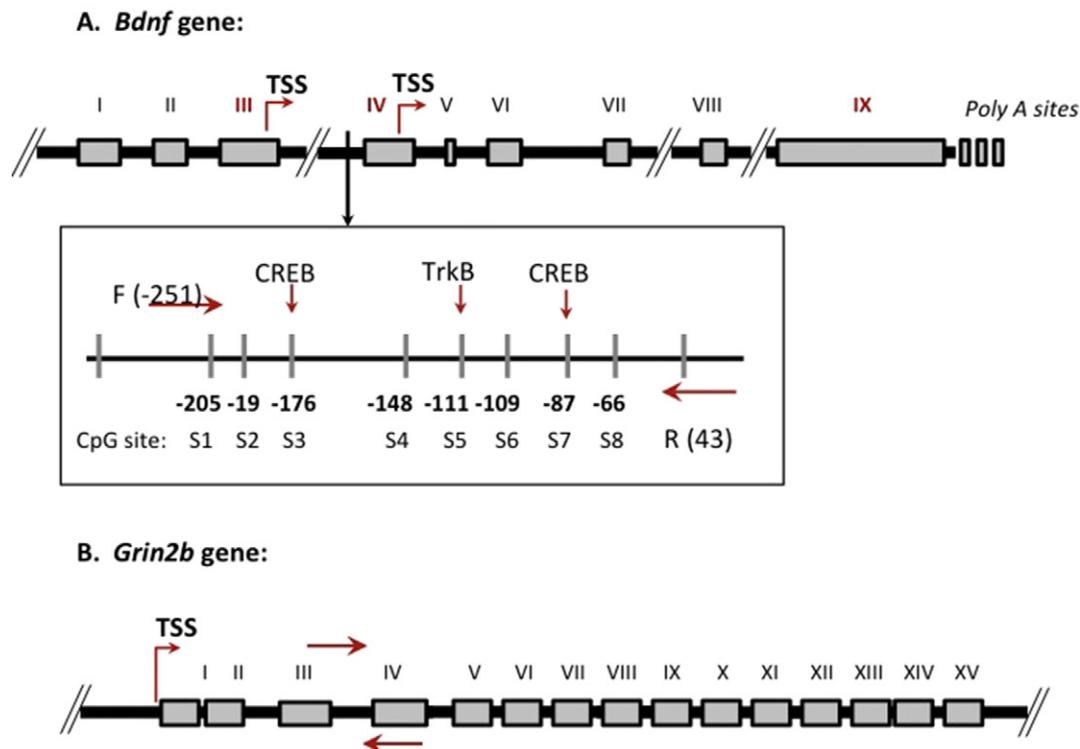
B. *Grin2b* gene:

Fig. 2. Schematic of *Bdnf* and *Grin2b* gene structure. A, Proximal regions of the *Bdnf* promoter and exon III. The CpG islands (S1-S8) included in DNA methylation analyses and proximal transcription factor binding sites are shown. The pyrosequencing primers were listed as F and R (forward primer started from −251 base pairs (bp) and reverse +43 bp from the transcription start site of exon IV). (B) *Grin2b* gene. The real-time PCR primers were designed to cover an exon-exon junction to eliminate gDNA amplification. Arrows demonstrate region amplified. Abbreviations: TSS = transcription start site; TrkB = tyrosine receptor kinase B.

3.1.3. Novel object test

A trend suggesting a possible effect of prenatal PAH exposure on total time spent investigating the objects ($F_{1,66} = 3.76$, $P = .06$; Fig. 4A), with PAH exposure associated with reductions in object exploration time, was observed. There was also a marginal association of PAH exposure on novel object preference score ($F_{1,66} = 2.4, P = .12$; Fig. 4B) suggestive of a possible reduction in novel object preference in PAH-exposed males. This effect was more evident in analyses of the percentage of offspring demonstrating a novel object preference ($\chi = 5.64$, $P < .05$) and indicated a significant reduction in number of male PAH-exposed mice (compared with controls) exhibiting a novel object preference ($\chi = 8.57$, $P < .01$), with no significant effect on this measure observed in females ($\chi = 0.20$, $P = .65$; Fig. 4C). Among

control males, 61% (11/18) exhibited a novel object preference, whereas 0% (0/14) of PAH-exposed males exhibited a preference for the novel object. Among female offspring, 67% (12/18) of control and 56% (9/16) of PAH-exposed mice displayed a preference for the novel object.

3.2. Cerebral cortex gene expression and DNA methylation

Analyses of mRNA levels of *Grin2b*, *Bdnf* III, and *Bdnf* IV revealed a significant sex by exposure interaction for all gene targets (*Grin2b*: $F_{1,40} = 40.42$, $P < .001$; *Bdnf* III: $F_{1,40} = 44.46$, $P < .001$; *Bdnf* IV: $F_{1,40} = 69.71$, $P < .001$; Fig. 5A-C). PAH-exposed males had reduced expression levels compared with controls ($P < .001$). This effect of

Table 1
Primers used for RT-PCR and pyrosequencing

Gene	Primers	Sequence (5′-3′)	Product size (bp)	Method
Bdnf exon III	Forward	tcatacttcggttgcatgaagg	137	RT-PCR
	Reverse	agacctctcgaacctgccc		
Bdnf exon IV	Forward	catatcggccaccaaagact	177	RT-PCR
	Reverse	gggggtgaggaaagagaaag		
Grin2b	Forward	gccatgaacgagactgaccc	107	RT-PCR
	Reverse	gcttcctggtccgtgtcatc		
Gapdh	Forward	aggtcggtgtgaacggatttg	95	RT-PCR
	Reverse	ggggtcgttgatggcaaca		
Bdnf exon IV (1)	Forward 1	ccttcaaccaaaaactccatttaatct	315	Pyrosequencing (1)
	Reverse 1	biotin-taggattggaagtgaaaatatttataaagt		
	Sequencing 1	agaggaggtattatatgatag		
Bdnf exon IV (2)	Forward 2	biotin-ccttcaaccaaaaactccatttaatct	315	Pyrosequencing (1)
	Reverse 2	taggattggaagtgaaaatatttataaagt		
	Sequencing 2	aaaaactaaaaaatttcatactaac		

Primers were designed based on NCBI accession number NM_001048139 for *Bdnf*, NM_008171for *Grin2b*, and NM_008084 for *Gadph*.
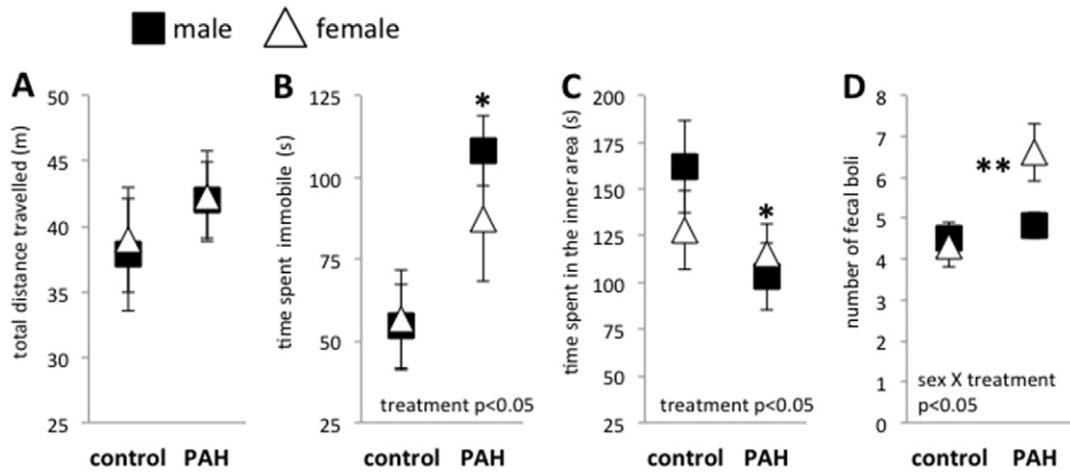
Fig. 3. Prenatal PAH exposure effects on open-field behavior. A, Total distance traveled (meters) during testing was not significantly impacted by prenatal PAH exposure. B, Prenatal PAH exposure was associated with greater time (seconds) spent immobile and (C) reduced time (seconds) spent exploring the inner area of the field. D, A sex by treatment effect was found on the number of fecal boli produced during testing, with PAH-exposed females emitting more boli compared with controls (Tukey, $P < .01$). Sample sizes for behavioral analyses consisted of n = 18 male/n = 18 female control offspring and n = 14 male/n = 16 female PAH-exposed offspring. $*P < .05$, $**P < .01$.

PAH was not observed in PAH-exposed females. Repeated-measures analyses of DNA methylation levels in the 8 CpG sites analyzed within the *Bdnf* IV gene (Fig. 2A) indicated a significant effect of treatment ($F_{1,40} = 15.31$, $P < .001$), with PAH exposure associated with greater *Bdnf* IV DNA methylation within the cortex in both males (Fig. 6A) and females (Fig. 6B). This effect was similarly observed using *t* tests that compare average methylation of all CpG sites, with DNA methylation at BDNF exon IV significantly higher following PAH exposure (compared with controls) in males (PAH 30.5 ± 0.37% vs control 27.5 ± 0.54%, $P < .001$) and females (30.14 ± 0.27% vs 28.45 ± 0.6%, $P = .02$). Site-specific analyses indicated a significant effect of PAH exposure on DNA methylation at S4 ($F_{1,40} = 9.23$, $P < .01$), S5 ($F_{1,40} = 4.85$, $P < .05$), S6 ($F_{1,40} = 5.91$, $P < .05$), and S7 ($F_{1,40} = 14.90$, $P < .001$). Consistent with the hypothesized relationship between DNA methylation and gene silencing, elevated DNA methylation of *Bdnf* IV was associated with reduced *Bdnf* IV mRNA ($r = -0.61$, $P < .01$). However, DNA methylation and mRNA were not correlated with any behavioral measure assessed.

4. Discussion

Consistent with our hypotheses, prenatal PAH exposure was associated with altered behavioral indices of anxiety and cognition, reductions in gene expression, and increased *Bdnf* DNA methylation within the cortex in adulthood. Moreover, in some analyses, the impact of PAH varied by offspring sex. During open-field testing, PAH-exposed offspring spent more time immobile and less time exploring the inner area of the field—both indices of increased anxiety-like responses (Belzung and Griebel, 2001). Increased physiological stress is associated with increased fecal boli production in rodents (Monnikes et al., 1993), and here we observed that females prenatally exposed to PAH produced more boli during open-field testing, with no effect observed in males. Even though analyses of

other open-field measures did not reveal a sex by treatment effect, as can be observed in Fig. 3C-D, behavioral effects of prenatal PAH were more pronounced in male offspring. However, no effects of prenatal PAH were observed on any measure assessed during elevated plus maze and light-dark box testing. During the novel object test, adult offspring prenatally exposed to PAH displayed modest reductions in overall exploration of the objects (consisting of sniffing and direct contact). PAH-exposed males failed to exhibit a preference for the novel object. Within the brain, prenatal PAH exposure was associated with reduced expression of *Grin2b* and *Bdnf* (exons III, IV) in males compared with controls, with no effect observed in females. Finally, repeated-measures analyses indicated a significant increase in DNA methylation within the *Bdnf IV* promoter in both PAH-exposed males and females compared with controls. These epigenetic changes within the *Bdnf IV* promoter were correlated with *Bdnf* gene expression but not with the observed behavioral outcomes, suggesting that other molecular or neurobiological targets may account for these PAH-associated effects.

4.1. Behavioral and cognitive effects of PAH

In the CCCEH birth cohort, elevated prenatal exposure to PAH has been associated with higher anxious/depressed symptom scores in childhood (Perera et al., 2012). Our data suggest that this phenotype also emerged in adult mice prenatally exposed to a PAH mixture that mimics the human cohort exposure levels. We observed 2 behavioral indices of increased anxiety-like/depressed behavior (greater immobility and reduced exploration; Fig. 3C-D) in PAH-exposed mice. Balb/c mice displayed novelty-induced reductions in locomotor activity compared with other inbred mouse strains (Griebel et al., 2000), and this response appeared to be heightened following PAH exposure. However, it was notable that no treatment effects were observed on the measure of total distance traveled during testing, suggesting a pattern of

Table 2

Control and prenatal PAH-exposed offspring behavior during elevated plus maze and light-dark testing (mean ± SE)

| Treatment	Sex	Elevated plus maze				Light-dark box (s)	
		Distance traveled (m)	Open arm entry latency (s)	Time in open arm (s)	Time in closed arm (s)	Time in light box (s)	Time in dark box (s)
Control	M	10.63 ± 0.89	24.41 ± 14.90	35.85 ± 11.20	441.78 ± 28.90	74.09 ± 23.32	525.49 ± 23.25
	F	10.63 ± 1.01	8.23 ± 1.79	38.29 ± 11.48	444.54 ± 28.56	86.85 ± 18.20	511.89 ± 18.30
PAH	M	10.54 ± 0.98	20.02 ± 9.74	23.40 ± 7.60	497.32 ± 24.80	91.66 ± 36.13	508.34 ± 36.13
	F	10.46 ± 0.88	12.83 ± 5.03	28.00 ± 11.95	471.13 ± 27.74	85.99 ± 28.08	514.01 ± 28.08
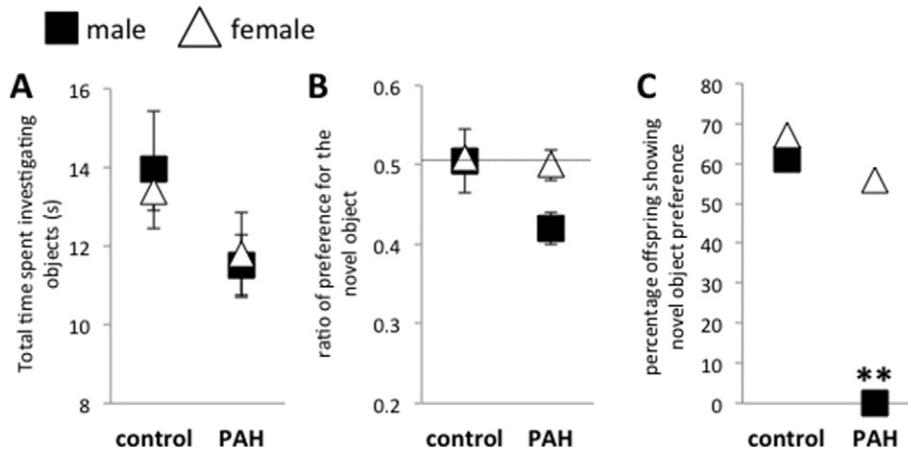
Fig. 4. Prenatal PAH exposure and novel object investigation. A, Prenatal PAH exposure predicted modest (but not significant; $P < .06$) reductions in time spent exploring both novel and familiar objects. B, Ratio preference for the novel object was modestly (though nonsignificantly; $P < .12$) lower in PAH-exposed males. Dashed line indicates cutoff ratio for demonstrating preference (>0.5 indicates greater that 50% time exploring the novel object as a function of total time exploring novel and familiar objects), with values above the time indicating novel object preference and values below the fine indicating familiar object preference. C, Percentage of PAH-exposed and control male and female offspring exhibiting a novel object preference, indicating reductions in novel object preference in PAH-exposed males. Sample sizes consisted of n = 18 male/n = 18 female control offspring and n = 14 male/n = 16 female PAH-exposed offspring. **$P < .01$.

activity characterized by freezing/immobility and rapid movement within the field. This movement was restricted to the periphery of the field as indicated by the decreased time spent in the inner area of the field by PAH-exposed mice. The greater production of fecal boli during testing in females prenatally exposed to PAH indicated that, although anxiety-like responses were elevated in both sexes, the physiological manifestation of anxiety may have differed between males and females. The absence of any indices of anxiety-like behavior during elevated-plus or light-dark testing may have indicated that the novelty of testing was necessary to reveal PAH-associated differences in anxiety-like responses. Thus, the effects induced by prenatal exposure to PAH may be subtle and contextually dependent.

Disruptions to the prenatal environment also have been associated with a lasting impact on cognition. In humans, elevated in utero PAH exposure has been linked to reductions in verbal IQ (Jedrychowski et al., 2015) and decreased processing speed during cognitive testing (Peterson et al., 2015). Our analyses indicated a reduced preference for the novel object in PAH-exposed males, attributed to the greater preference for the familiar vs the novel object (see Fig. 4B). This finding was consistent with the behavioral effects previously reported

in mice prenatally exposed to benzo[a]pyrene from embryonic day 14 to 17 (Sheng et al., 2010). The novel object test is typically used to assess recognition memory and assumes that presentation of a novel object (as compared with a familiar object) will result in increased exploration/investigation time when object recall is intact (ie, when novel vs familiar can be differentiated) (Ennaceur and Delacour, 1988). Inactivation of the hippocampus during novel object training phases resulted in impaired encoding and consolidation of memory for the familiar object, resulting in decreased novel vs familiar object preference (Cohen et al., 2013). However, the patterns of novel object investigation observed in the current and previous studies of PAH exposure were more consistent with active avoidance of the novel object rather than an inability to recognize novelty. Chronic increases in levels of corticosterone (a model of anxiety/depression) similarly led to reduced exploration of a novel object in adult male mice (Darcet et al., 2014). This reduced novelty preference also was observed in our previous studies of prenatal bisphenol A (BPA) exposure, and similar to the current study, the effects of BPA on novel object preference were only observed in male offspring (Kundakovic et al., 2015).
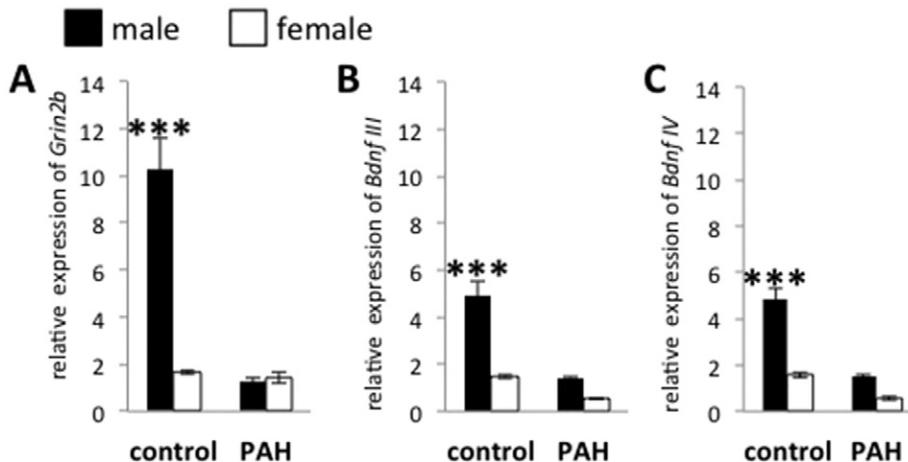
Fig. 5. Cortical gene expression in prenatal PAH vs control mice. Prenatal PAH-exposed males (but not females) had reduced cortical mRNA levels of (A) *Grin2b*, (B) *Bdnf* III, and (C) *Bdnf* IV. n = 10/sex/treatment. ***$P < .001$. Ten males and 10 females per experimental exposure were studied.
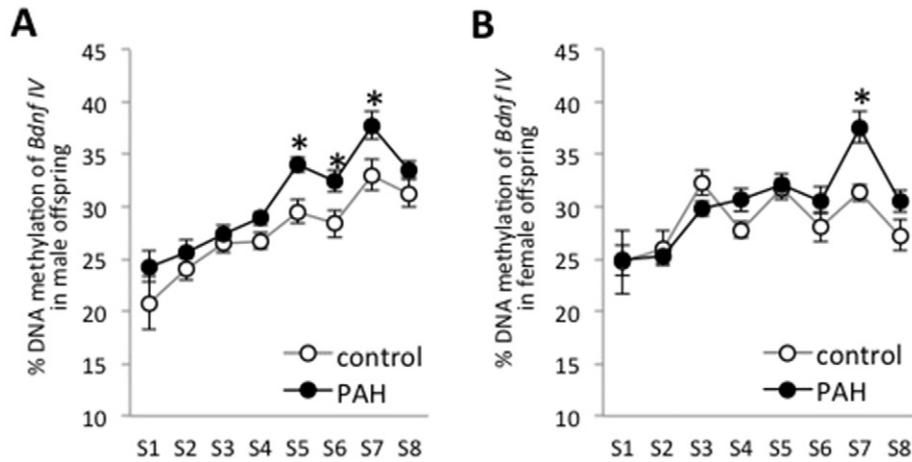
Fig. 6. Prenatal PAH exposure effects on *Bdnf* IV promoter DNA methylation in the cortex. Percentage DNA methylation was significantly higher in prenatal PAH-exposed compared with control (A) males (at S5-S7) and (B) females (at S7). n = 10/sex/treatment *P < .05.

4.2. Impact of PAH on Bdnf and Grin2b

Elevated prenatal PAH exposure in humans was associated with reduced cortical white matter (Peterson et al., 2015), and in the current study, we examined the possible cortical gene targets that may account for the impaired cognition linked to this neurodevelopmental effect. In rodents, in utero benzo[a]pyrene exposure was associated with diminished hippocampal long-term potentiation (Wormley et al., 2004), suggesting reduced neural plasticity. Cortical development also was disrupted by in utero benzo[a]pyrene exposure (Sheng et al., 2010), and exposure during late gestation (embryonic days 14-17) was associated with reduced *Nmda2b* (*Grin2b*) subunit expression in the cortex (Brown et al., 2007; McCallister et al., 2008). *Bdnf* also plays a critical role in neuroplasticity, and protein levels of this neurotrophin were downregulated in adult rats exposed chronically to oral benzo[a]pyrene compared with controls (Chengzhi et al., 2011). Studies in humans also indicated altered *Bdnf* levels in response to PAH, with elevated prenatal PAH predicting reduced cord blood protein levels (Perera et al., 2015). Reductions in environmental PAH were associated with higher cord blood *Bdnf* levels and improved neurodevelopmental outcomes (Tang et al., 2014). Here we demonstrated that inhaled PAH during gestation in mice resulted in reduced expression of both *Bdnf* and *Grin2b* in the adult cerebral cortex but that this effect was only observed in males. Although the basis for this sex difference is unclear, it is consistent with recent findings that exposure to the persistent organic pollutant polychlorinated biphenyls decreased *Bdnf* (but not *Grin2b*) protein levels and *Bdnf*-mediated signaling in the adult male rat cerebral cortex (Bavithra et al., 2015). We previously observed a male-specific effect of prenatal BPA exposure on reductions in hippocampal *Bdnf* and *Grin2b* gene expression and cognitive performance, and these sex-specific effects were likely the typical response to prenatal adversity (Kundakovic et al., 2015) and may have been mediated by chromosomal, hormonal, and epigenetic differences between males and females during fetal development (Silkaitis and Lemos, 2014; Slob et al., 1980; Spiers et al., 2015).

4.3. Epigenetic effects of prenatal PAH

The *Bdnf* IV promoter displays significant epigenetic plasticity in response to both prenatal and postnatal environmental experiences (Kundakovic et al., 2015; Roth et al., 2009). Our data suggested that prenatal PAH exposure induced a sustained impact on DNA methylation levels within this genomic region in the cortex. Unlike expression of *Bdnf*, this epigenetic effect was observed in both males

and females. In addition, as can be seen in Fig. 6A and B, PAH-associated elevations in DNA methylation were only observed at CpG S7 in females, whereas this effect spans CpGs S5-7 in males. Our findings are consistent with previous experimental work demonstrating an epigenetic impact of PAH on DNA methylation in noncortical tissues, and with human studies indicating a global reduction in DNA methylation in cord blood in humans prenatally exposed to elevated PAH (Herbstman et al., 2012). We also found a significant negative correlation between *Bdnf* IV DNA methylation and *Bdnf* IV expression, suggesting that the epigenetic effects of PAH exposure may account for the observed reductions in *Bdnf*. However, the mechanism through which PAH exposure leads to altered *Bdnf* IV DNA methylation remains unclear. In vitro studies indicated PAH-associated reductions in DNMT1 binding to gene regulatory regions (Teneng et al., 2011), and our previous studies illustrating an epigenetic effect of in utero BPA exposure in the cortex also revealed cortical reductions in *Dnmt1* expression associated with elevated prenatal BPA exposure (Kundakovic et al., 2015). Thus, disruption to the prenatal environment may alter DNA methylation through targeting of *Dnmt1*. Another possible mechanistic pathway may involve cAMP response element-binding protein (CREB) interactions with the *Bdnf* promoter. CREB binding to the *Bdnf* promoter was associated with transcriptional activation (Lonze and Ginty, 2002), and recruitment of CREB to the promoter region may have prevented epigenetic silencing of *Bdnf*, a process that could be disrupted following PAH exposure. The CpG S7 within our analyses resides proximal to a CREB binding site (Martinowich et al., 2003), suggesting a functional significance of DNA methylation at this loci and the possible role of CREB in PAH-mediated effects on gene expression. These hypothetical mechanisms would predict broad epigenetic changes within the brain that extend beyond *Bdnf* IV DNA methylation.

Although higher *Bdnf* IV DNA methylation in the cortex was predicted by PAH exposure and was predictive of *Bdnf* IV cortical gene expression, this epigenetic effect was not associated with the observed behavioral/physiological effects of PAH assessed in this study. There are several possible explanations for this finding. First, our analyses were limited to the cortex and did not extend to other brain regions that could influence anxiety-like behavior or cognition (eg, hippocampus, hypothalamus) and also did not examine subregions within the cortex. Second, our epigenetic analyses were limited to a small number of CpG sites within the *Bdnf* IV promoter and did not extend to other genes involved in neuroplasticity and development. Complex behavioral phenotypes are dependent on cascades of gene expression in multiple brain regions and circuits. Thus, our

focused analyses may not have had sufficient depth to draw conclusive mechanistic insights into the origins of the PAH-associated phenotypes that emerged. Third, our analysis of gene expression and DNA methylation was conducted on mice that had undergone a battery of behavioral tests. It has been demonstrated that epigenetic changes in *Bdnf* are dynamically altered in response to neuronal activity (Martinowich et al., 2003). The behavioral/cognitive tests may have generated epigenetic changes that obscured the detection of relationships to behavioral outcomes. Finally, we only captured one time point in the offspring and not multiple developmental stages when examining PAH-associated effects.

5. Conclusion

In conclusion, prenatal PAH exposure induced multiple alterations in behavioral indices of anxiety and cognition, cortical *Bdnf* and *Grin2b* gene expression, and *Bdnf* DNA methylation in adult mice. Select sex effects were evident. The epigenetic changes measured within the *Bdnf IV* promoter negatively correlated with *Bdnf* gene expression, as predicted. However, the epigenetic changes did not correlate with the measured behavioral outcomes, suggesting that other molecular or neurobiological targets may account for these PAH-associated effects. Future studies will be needed to determine the timing and mechanisms that are critical for these observed PAH-induced effects. Enhancing the translational potential of this research would also require the analyses of the correlation between PAH-associated changes in cortical DNA methylation and those tissues that can be more readily assessed in humans, such as blood and buccal cells.

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