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

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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.

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INTRODUCTION

Prenatal exposure to toxins can have a lasting impact on the brain and behavior with implications for development and health in subsequent generations [1, 2]. Polycyclic aromatic hydrocarbons (PAH) are a class of pollutants produced during the incomplete combustion of organic materials. In U.S. cities, traffic emissions are one of the most abundant sources of outdoor PAH. High levels of PAH exposure can originate from indoor sources (e.g., space heating, cooking, smoking, burning incense or candles) as well as from outdoor traffic sources that penetrate indoors [3]. 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 [4], lower IQ [5], anxious and depressive symptoms, attention problems [6], reductions in white matter surface, and slower cognitive processing speed in later childhood [7].

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 [8] and inducing toxic effects to glial cells [9, 10]. Environmentally-induced alterations in DNA methylation within the brain that may suppress gene expression have been demonstrated following prenatal exposure to stress [11], endocrine disrupting chemicals [12], and variation in the nutritional environment [13] 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 [14]. In zebrafish, exposure to benzo[a]pyrene was shown both to induce genome-wide transcriptional changes in embryos [15] and gene-specific and genome-wide reductions in DNA methylation [16]. In human cohorts, global reductions in DNA methylation cord blood were associated with prenatal exposure to elevated PAH levels [17]. 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 [18, 19]. Within this study, we assessed behavioral outcomes related to cognition, activity levels, and anxiety-like phenotypes and focused on the expression of two candidate genes implicated in neural plasticity: brain derived neurotrophic factor (Bdnf) and the ionotropic glutamate receptor, N-methyl D-aspartate 2B (Grin2b, or...
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 [20], and postnatal exposure to stressful rearing conditions in rats [21]. In humans, prenatal exposure to related combustion products, namely fine particulate matter, was associated with lower Bdnf expression in the placenta [22]. 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 [23]. Similarly, studies in rodents indicated altered Grin2b expression and promoter DNA methylation in response to prenatal adversity [20, 24], and Grin2b genetic variations predicted risky decision making and attention problems in human cohorts [25, 26].

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 BdnfIV. Finally, based on previous studies indicating sex differences in neurodevelopmental and epigenetic outcomes associated with prenatal exposure to stress and endocrine disrupting chemicals [11, 12], we predicted that both behavioral and gene expression/epigenetic outcomes associated with prenatal exposure to PAH would vary by offspring sex.

**MATERIALS AND METHODS**

**Animals**

Nine-week-old BALB/cByj female mice weighing 20–22g were obtained from Charles River and housed 4–5/cage in temperature-regulated (20°C), ventilated cabinets with a 12 h light/12 h 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 prior to any experiments. After 1 week, mice were mated and the protocol summarized in Figure 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.

**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 over 700 pregnant women using personal air sampling devices [18, 19, 27]. 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/m³. Solutions were delivered via nebulizers (Unomedical Inc., McAllen, Texas) connected to filtered compressed air in a chamber set to achieve a flow of 12.5 to 13.0 liters per minute [18, 19]. Four dams were exposed in one cage at a time with full access to food and water for 5 hours a day, 5 days a week, beginning on gestational day (GD) 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/m³, range 7.38–40 ng/m³ from 3 weekly filters extracted together [18], suggesting levels ambient in the chamber may be higher than levels ambient in the NYC urban environment. Prenatally exposed offspring were weaned at postnatal day (PND) 21 and housed in same-sex, same-condition groups of 4 mice per cage until testing.

**Behavioral phenotyping**

At PND 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 (Figure 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.

**Open-field testing**—Behavior during open-field assessment is a standard measure of activity and anxiety-like responses in rodents [28]. The open-field apparatus used was a 24 × 24 × 16-inch Plexiglas box. On the day of testing, the mouse was placed directly into one corner of the open-field. After a 10-min 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 travelled (m), (2) time spent immobile (s), and (3) time (s) spent in the center area (the inner 12 × 12-inch region of the field). Counts of fecal boli produced during testing also were assessed.

**Elevated plus maze**—The elevated plus maze is a standard measure of anxiety-like behavior and exploration in rodents [29]. The Plexiglas testing apparatus consisted of two open-arms and two closed arms, each extending 18 inches in length and 4 inches in width. The apparatus was elevated 24 inches 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-min exploration session was video recorded and scored with AnyMaze. Behaviors scored included: (1) total distance travelled (m), (2) latency to enter the open-arms (s), (3) time spent in the open-arms (s), and (4) time spent in the closed arms (s).

**Light-dark box**—Behavioral assessment in the light-dark box is a standard laboratory measure of anxiety-like behavior and exploration in rodents [30]. The light-dark testing apparatus was a 11.5 × 19 × 11-inch (outer dimensions) Plexiglas box, consisting of a light compartment (11 × 11 inches) and a smaller dark compartment (11 × 7 inches) connected by a door (3 × 3 inches) in the center of the wall separating the two compartments. A 60-Watt bulb located 16 inches 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 min. Measures scored (AnyMaze) included: (1) time (s) spent in the light compartment and (2) time (s) spent in the dark compartment.

**Novel Object Test**—The novel object recognition test has been previously used to assess memory deficits [31]. Testing consisted of three phases: habituation, acquisition, and test phase. On the first day, mice were habituated to the novel object testing arena (a 24 × 24 ×
16-inch Plexiglas box) for 10 min in the absence of any objects. The following day, mice were tested for object recognition using acquisition and test trials separated by a 30-min delay. During the acquisition trial, mice were exposed to two identical objects for 15 min. During the 15 min 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.

**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 manufacture’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 calculated to assess quality. For DNA extraction, 30 mg of cerebral cortex were quickly washed and incubated in 900 µL lysis buffer (50 mM Tris-Cl pH 8.0, 10 mM EDTA, 100 mM NaCl, 1% SDS), and 0.1 mg proteinase K (Promega) with shaking for 4 h at 55°C. The genomic DNA was separated by two 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 (TE) buffer and stored at −20°C.

**Gene expression**

Relative gene expression was measured with real-time semi-quantitative polymerase chain reaction (PCR) on a 96-well CFX Connect™ Real-Time PCR Detection System (BioRad) as previously described [19]. 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^ΔΔCT method normalized by Gapdh and to control offspring [32]. Analyses included transcript specific expression of Bdnf (exon III and IV; Figure 2A) and Grin2b (spanning exon III, IV; Figure 2B). Forward and reverse primer sequences are indicated in Table 1.

**DNA methylation**

To determine levels of DNA methylation within Bdnf exon IV, 250 ng of genomic DNA underwent bisulfite modification utilizing the EZ DNA Methylation-Direct Kit (Zymo Research). The bisulfite-converted DNA was resuspended in 40 µl TE 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 mM MgCl2, 200 mM dNTPs, 0.2 mM primers, 1.25 U HotStar Taq DNA polymerase (Qiagen), and approximately 10 ng of bisulfite-converted DNA per 50 µl reaction. Following purification, biotinylated PCR (20 µl) products were analyzed using a

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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 Figure 2A.

**Statistical analyses**

Behavioral, gene expression and DNA methylation data were analyzed using a two-way ANOVA (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’s post-hoc test was conducted in cases of sex by treatment interactions. Novel object preference was analyzed using a Chi-square test. Spearman rank correlation coefficients were used to examine associations between gene expression, DNA methylation and behavior. Values of p < 0.05 were regarded as statistically significant.

**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, nonsignificant (n.s.)).

**Behavioral impact of prenatal PAH exposure**

**Open-field test**—Total distance travelled during testing was not altered by PAH exposure (p=0.27; Figure 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<0.05; Figure 3B] and less time spent in the inner area of the field [F(1,66)=5.67, p<0.05; Figure 3C]. A sex by treatment interaction [F(1,66)=4.21, p<0.05] was found on counts of fecal boli produced during testing, with PAH exposed female offspring generating more fecal boli (Tukey’s; p<0.01) than control offspring (with no effect observed in male offspring; Figure 3D).

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

**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=0.06; Figure 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=0.12; Figure 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 (χ²=5.64, p<0.05) and indicated a significant reduction in number of male PAH-exposed mice (compared to controls) exhibiting a novel object preference (χ²=8.57, p<0.01), with no significant effect on this measure observed in females (χ²=0.20, p=0.65; see Figure 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.
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<0.001; *Bdnf* III: F(1,40)=44.46, p<0.001; *Bdnf* IV: F(1,40)=69.71, p<0.001; Figure 5A–C). PAH exposed males had reduced expression levels compared to controls (p<0.001). This effect of 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 (Figure 2A) indicated a significant effect of treatment [F(1,40)=15.31, p<0.001], with PAH exposure associated with greater *Bdnf* IV DNA methylation within the cortex in both males (Figure 6A) and females (Figure 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 to controls) in males (PAH 30.5±0.37% vs. control 27.5±0.54%, p<0.001), and females (30.14±0.27% vs. 28.45±0.6%, p=0.02). Site-specific analyses indicated a significant effect of PAH exposure on DNA methylation at S4 [F(1,40)=9.23, p<0.01], S5 [F(1,40)=4.85, p<0.05], S6 [F(1,40)=5.91, p<0.05] and S7 [F(1,40)=14.90, p<0.001]. 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<0.01). However, DNA methylation and mRNA were not correlated with any behavioral measure assessed.

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 [28]. Increased physiological stress is associated with increased fecal boli production in rodents [33], 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 Figures 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 to 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 to controls. Theses 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.
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 [6]. 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 two behavioral indices of increased anxiety-like/depressed behavior (greater immobility and reduced exploration; see Figure 3C–D) in PAH exposed mice. Balb/c mice displayed novelty-induced reductions in locomotor activity compared to other inbred mouse strains [34] 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 travelled during testing, suggesting a pattern of 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, while 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 [35] and decreased processing speed during cognitive testing [7]. 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 Figure 4B). This finding was consistent with the behavioral effects previously reported in mice prenatally exposed to benzo[a]pyrene from embryonic day 14–17 [36]. The novel object test is typically used to assess recognition memory and assumes that presentation of a novel object (as compared to a familiar object) will result in increased exploration/investigation time when object recall is intact (*i.e.* when novel vs. familiar can be differentiated) [37]. 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 [38]. 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 [39]. 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 [20].

Impact of PAH on Bdnf and Grin2b

Elevated prenatal PAH exposure in humans was associated with reduced cortical white matter [7] 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
**Epigenetic effects of prenatal PAH**

The *Bdnf* IV promoter displays significant epigenetic plasticity in response to both prenatal and postnatal environmental experiences [20, 21]. 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 Figure 6A,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 non-cortical tissues, and with human studies indicating a global reduction in DNA methylation in cord blood in humans prenatally exposed to elevated PAH [17]. 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 [14], 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 [20]. 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 [47], 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 [48], 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 (e.g. hippocampus, hypothalamus) and also did not examine sub-regions 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 [48]. 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.

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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REFERENCES


HIGHLIGHTS

- Prenatal PAH exposed offspring spent more time immobile and less time exploring an open-field.
- Prenatal PAH exposed females produced more fecal boli during open-field testing.
- Prenatal PAH exposed males failed to exhibit a preference for a novel object.
- Prenatal PAH exposed males exhibited reduced cortical expression of *Grin2b* and *Bdnf*.
- Prenatal PAH exposed offspring exhibited elevated DNA methylation in *Bdnf IV*. 

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Figure 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
Figure 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, and reverse +43 base pairs 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; CREB=cAMP response element-binding protein; TrkB= tyrosine receptor kinase B.
Figure 3.
Prenatal PAH exposure effects on open-field behavior. (A) Total distance travelled (m) during testing was not significantly impacted by prenatal PAH exposure. (B) Prenatal PAH exposure was associated with greater time (s) spent immobile and (C) reduced time (s) 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 to controls (Tukey’s, 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<0.05, ** p<0.01
Figure 4.
Prenatal PAH exposure and novel object investigation. (A) Prenatal PAH exposure predicted modest (but not significant; \( p<0.06 \)) reductions in time spent exploring both novel and familiar objects. (B) Ratio preference for the novel object was modestly (though non-significantly; \( p<0.12 \)) lower in PAH exposed males. Dashed line indicates cut-off ratio for demonstrating preference (>0.5 indicates greater than 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 line 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. ** \( p<0.01 \)
Figure 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) BdnfIII, and (C) Bdnf IV. n=10/sex/treatment. ***p<0.001. 10 males and 10 females per experimental exposure were studied.
Figure 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 to control (A) males (at S5–S7), and (B) females (at S7). n=10/sex/treatment *p*<0.05.
Table 1

Primers used for RT-PCR and pyrosequencing

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Primers were designed based on NCBI accession number NM_001048139 for *Bdnf*, NM_008171 for *Grin2b*, and NM_008084 for *Gapdh*.
Table 2
Control and prenatal PAH exposed offspring behavior during elevated plus maze and light-dark testing (mean ±SE).

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Sex</th>
<th>Elevated Plus Maze</th>
<th>Light-Dark Box (s)</th>
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<tr>
<td></td>
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<td>Distance travelled (m)</td>
<td>Open arm entry latency (s)</td>
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<tr>
<td>Control</td>
<td>M</td>
<td>10.63 ±0.89</td>
<td>24.41 ±14.90</td>
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<td>F</td>
<td>10.63 ±1.01</td>
<td>8.23 ±1.79</td>
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<tr>
<td>PAH</td>
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<td>F</td>
<td>10.46 ±0.88</td>
<td>12.83 ±5.03</td>
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</table>

Neuroepigenetics. Author manuscript; available in PMC 2017 March 01.