Prenatal Cocaine Exposure Alters BDNF-TrkB Signaling in the Embryonic and Adult Brain

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Keywords

Cocaine · Brain-derived neurotrophic factor · Prenatal exposure · Tyrosine kinase B · Epigenetics

Abstract

Prenatal cocaine exposure remains a major public health concern because of its adverse effects on cognitive function. Although the molecular mechanisms underlying the cognitive impairment are not fully understood, brain-derived neurotrophic factor (BDNF) signaling via its receptor tyrosine kinase B (TrkB) is emerging as a potential candidate. We used a mouse model to examine the impact of ongoing cocaine exposure on BDNF expression in the dorsal forebrain on embryonic day 15 (E15) as well as the long-term effects of prenatal cocaine exposure on BDNF-TrkB signaling in the frontal cortex in early postnatal (postnatal day 16; P16) and adult (P60) male and female mice. We found that ongoing cocaine exposure decreased BDNF expression in the E15 dorsal forebrain, prenatal cocaine exposure did not alter BDNF or TrkB (total or phosphorylated) expression in the frontal cortex at P16, and that the prenatal cocaine exposure produced significant increases in BDNF, the activated (phosphorylated) form of TrkB, as well as *Bdnf* mRNA in

the frontal cortex at P60. The increase in BDNF protein and mRNA expression at P60 was concurrent with hyperacetylation of histone H3 at the *Bdnf* promoter in the frontal cortex. The increase in frontal cortical BDNF and activated TrkB at P60 occurred in male but not female mice. Thus, our data demonstrate that ongoing cocaine exposure produces a decrease in BDNF expression in the embryonic brain, and that prenatal cocaine exposure produces a sex-specific increase in frontal cortical BDNF-TrkB signaling at P60 only in male mice. Lastly, hyperacetylation of histone H3 at the *Bdnf* promoter is one epigenetic mechanism mediating the effects of prenatal cocaine exposure on *Bdnf* expression at P60 in male mice.

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Introduction

Cocaine abuse continues to be a major public health concern in the USA and across the world. A 2014 survey showed that 1.5 million individuals aged 12 years or old-

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er (approximately 0.6% of the population) reported current cocaine use in the USA (National Survey on Drug Use and Health, 2014). While cocaine abuse is a major public health issue, cocaine use by pregnant women entails potential harm to the unborn child as well as the mother. In the USA, among pregnant women aged 15–44 years, 5.4% were current illicit drug users [1] and, according to a study published in 2009, over 7.5 million children were prenatally exposed to cocaine [2]. However, the risk for fetal drug exposure as a result of mothers' drug use may be much higher than that revealed by these statistics because nearly 28% of women become aware of pregnancy on average 2–3 months after conception [3].

The consequences of prenatal cocaine exposure for the brain and behavior remain a subject of debate, with opinions ranging from fears about a lost generation of “crack babies” to the notion that prenatal cocaine exposure may produce only subtle effects on cognitive ability [4–6]. The inherent variability in data from human studies and the lack of ecologically valid experimental models continue to fuel the debate. However, there is general agreement from human and animal studies that prenatal cocaine exposure contributes to deficits in a variety of cognitive functions, including attention, executive function, and language [6–12].

Molecular changes in the brain are useful points of convergence of behavioral and cellular biological phenomena, and thereby have the potential to serve as a common mechanistic denominator for behavioral outcomes from studies of prenatal cocaine exposure in human subjects and experimental animal models. Alterations in brain-derived neurotrophic factor (BDNF) signaling via its receptor tyrosine kinase B (TrkB) is an example of a molecular mechanism mediating the effects of cocaine on the brain and behavior in humans as well as in animal models. Recent studies report significant impairments in BDNF-TrkB signaling in multiple experimental animal models of cocaine exposure [13–19; reviewed in 20]. Although a definitive link between BDNF signaling and cocaine exposure has not been established in humans, there is a correlation between BDNF signaling and cognitive functions, such as attention in children and adults [21–25]. With this in mind, we examined the expression of BDNF and TrkB (total and phosphorylated) proteins, as well as the epigenetic regulation of *Bdnf* mRNA expression in a mouse model of cocaine exposure. We focused on the frontal cortex because it is a key regulator of higher cognitive functions, many of which are mediated via BDNF-TrkB signaling, and are reported to be impaired by cocaine [26–

29]. We included both male and female offspring in our studies so as to examine the role of sex in potential cocaine-BDNF interactions.

Our data show that prenatal cocaine exposure produces significant changes in the expression of BDNF and the activated form of TrkB (phospho-TrkB), as well as *Bdnf* mRNA. In addition, acetylation of histones at specific *Bdnf* promoters was also increased, suggesting an epigenetic mechanism that may be involved in this process. The effects of cocaine exposure were evident during the embryonic period, while cocaine administration was ongoing, and these effects persisted into adulthood, long after the exposure had ceased. The long-term effects were sex specific and evident in the frontal cortex of only male mice.

Materials and Methods

Animals

Timed-pregnant Swiss Webster mice were used. The day of vaginal plug detection was considered embryonic day 0 (E0) and the day of birth postnatal day 0 (P0). Pregnant dams of comparable body weight were assigned to prenatal saline or cocaine exposure groups on the 6th day of pregnancy. Singly housed dams received twice daily (7 a.m. and 7 p.m.) subcutaneous injections of cocaine (20 mg/kg/injection; total daily dose 40 mg/kg/day) or saline (same volume and time of administration as the cocaine injection) from the 8th day of pregnancy until the day of birth for analysis of postnatal offspring. The dose of cocaine used here (20 mg/kg, subcutaneous) results in a fetal plasma cocaine concentration of 2.3 μ M (30 min postinjection) and 0.6 μ M (120 min postinjection), which is comparable to the 1.1 μ M concentration reported in humans following 0.6 mg/kg intravenous cocaine administration [30]. All offspring were cross-fostered to drug-naïve dams within 2 days of birth to eliminate potential effects of the experimental interventions on mother-infant interactions. The offspring were weaned on P21. Embryos were collected by hysterotomy on deeply anesthetized dams (ketamine, 50 mg/kg; xylazine, 10 mg/kg body weight) on the 15th day of pregnancy (i.e. embryonic day 15; E15), with the last cocaine injection occurring at 7 p.m. on E14. The age of each embryo was ascertained by examination of the external morphological features [31, 32]. Embryos or entire litters that did not meet the relevant criteria were discarded from analysis. Postnatal mice were euthanized on P16 and P60 by anesthetic overdose and the brains were removed. We did not evaluate estrous cycle phases prior to euthanasia. All of the experimental procedures were in full compliance with institutional guidelines at Florida State University and the NIH guide for the ethical care and use of laboratory animals.

Western Blotting

The dorsal forebrain (E15) or the frontal cortex (P16 and P60) was micro-dissected from each hemisphere based on anatomical landmarks [33–35] and the samples from the 2 hemispheres were pooled. Whole tissue extracts were used for Western blot [36, 37].

We did not determine the sex of the embryo prior to tissue collection. Integrated density values (% IDV) were calculated for each sample by dividing the optical density values of the bands for the protein of interest by the density values for the loading control (GAPDH or histone H3). Samples from 4–6 mice from 4–5 litters were used for each experiment.

The following antibodies were used. Anti-BDNF antibody diluted 1:700 (Aviva Systems Biology, San Diego, CA, USA; ARP41970_P050), anti-TrkB 1:600 (Novus Biologicals, Lilleton, CO, USA; NB100-92063), anti-pTrkBY705 1:1,000 (Abcam, Cambridge, MA, USA; Ab52191), anti-pTrkBY515 1:1,000 (Abcam; Ab109684), anti-diacetyl lysine 9, lysine14 histone H3 (H3K9K14ac2; AcH3) 1:1,000 (EMD Millipore, Billerica, MA, USA; 06-599), anti-trimethyl lysine 9 histone H3 (H3K9me3, MeH3) 1:700 (EMD Millipore; 07-523), anti-histone H3 1:500 (EMD Millipore; 07-690), and anti-GAPDH 1:1,000 (Abcam; MAB374).

RNA Extraction and Reverse Transcription

RNA was extracted using the RNeasy kit (Qiagen, Valencia, CA, USA) [36, 38]. Reverse transcription reactions were performed using the SuperScript VILO cDNA synthesis kit (Life Technologies, Grand Island, NY, USA). Primer sequences for *Gapdh* and *Bdnf* were chosen based on previously published data [36, 38, 39]. Quantitative real-time PCR was performed in an iCycler (Bio-Rad, Hercules, CA, USA) using SYBR-green PCR Master Mix (Life Technologies) through 50 PCR cycles (95°C for 30 s, 57°C for 60 s, 72°C for 90 s). The threshold cycle for each sample was chosen from the linear range and converted to a starting quantity by interpolation from a standard curve run on the same plate for each set of primers. Levels of mRNA were normalized to *Gapdh*. Samples from 4–6 mice were used for each experiment.

Chromatin Immunoprecipitation

Association of AcH3 with *Bdnf* promoters was assessed by chromatin immunoprecipitation (ChIP) [36, 37]. Briefly, samples were fixed with formaldehyde (10 μ L of 1% formaldehyde to 1 mg of tissue) and suspended in SDS lysis buffer containing protease inhibitors. Lysates were sonicated into 200- to 1,000-bp fragments (Branson Cell Disruptor 350), centrifuged, and diluted with ChIP dilution buffer. The diluted sample (1% or 20 μ L) was set aside and designated as “input” DNA. Each sample (2 mL) was incubated overnight with 25 μ L Dynabeads Protein A (Life Technologies, Grand Island, NY, USA) and 5 μ g of AcH3 antibody (06-599; Millipore). Negative controls included no antibody mock and IgG. After crosslink reversal, all samples (ChIP and input) were digested with 20 μ g of proteinase K and DNA recovered with phenol/chloroform extraction followed by ethanol precipitation.

Real-time thermal cycling was performed using ChIP-derived DNA (1 μ L), 2 $\times$ SYBR Green Master Mix, 0.5 μ M of each primer and an iCycler thermal cycler (Bio-Rad), with continuous SYBR Green monitoring using iCycler software. All PCR reactions were performed (iCycler and SYBR Green) in triplicate and included negative (no DNA) and positive controls (serial dilutions of genomic DNA). Target DNA sequence quantities were estimated from threshold amplification cycle numbers (T_c). For every gene sequence studied, a ΔT_c value was calculated for each sample by subtracting the T_c value for the immunoprecipitated sample from the T_c value for the corresponding input DNA to normalize for differences in ChIP sample aliquots. DNA quantities were ex-

pressed as percentages of the corresponding input using the following equation: antibody ChIP as a percentage of input = $2^{(\Delta T_c)} \times 100$. Finally, DNA quantities (normalized to input) were compared for immunoprecipitated versus mock-immunoprecipitated samples; only when immunoprecipitated samples contained >1.5 times as much DNA as in the mock sample were they considered to have sufficient DNA for analysis.

Exon-specific BDNF primers were designed based on previously published sequences [40, 41] and used for real-time PCR analysis. BDNF exon I: forward 5'-GCAGTTGGACAGTCATTG-GTAACC-3' and reverse 5'-ACGCAAACGCCCTCATTCTG-3'. BDNF exon II: forward 5'-GCAGAGTCCATTCAGCACCTTG-3' and reverse 5'-TGGCTTGACAGCGAGGAAAAG-3'. BDNF exon IV (CREB and MeCP2 binding sites): forward 5'-AA-CAAGAGGCTGTGACAC TATGCTC-3' and reverse 5'-CAG-TAAGTAAAGGCTAGGGCAGGC-3'. BDNF exon VI: forward 5'-TTTGGGGCAGACGAGAAAGC-3' and reverse 5'-GGCAGTGGAGTCACATTGTTGTC-3'. A total of 4–6 mice were used.

Statistical Analyses

The main effects of prenatal treatment, sex, and interaction between prenatal treatment and sex were analyzed using a 2-way analysis of variance (ANOVA). Post hoc pairwise comparisons were performed using a Bonferroni correction. A 2-tailed Student *t* test was used when differences between only 2 groups were compared.

Results

BDNF Protein Expression

Cocaine exposure significantly decreased BDNF expression in the forebrain of the E15 embryo (*t* test, BDNF [mean $\pm$ SEM]: saline 22.5 ± 1.3 , cocaine 8.7 ± 4.0 , $t = 3.243$, $df = 6$, $p < 0.05$; Fig. 1a). Tissue from male and female embryos was pooled, and therefore separate analysis for each sex could not be performed. P16 and P60 frontal cortex samples were analyzed separately for male and female mice (Fig. 1b, c). At P16, there was no significant main effect of prenatal treatment on BDNF expression in the frontal cortex (2-way ANOVA, $F(1, 14) = 2.527$, $p > 0.05$; Fig. 1b). However, there was a significant effect of sex ($F(1, 14) = 210.6$, $p < 0.0001$) and no significant prenatal treatment $\times$ sex interaction ($F(1, 14) = 1.571$, $p > 0.05$). A Bonferroni multiple comparison post hoc test demonstrated significantly higher BDNF expression in female mice compared to males in both the prenatally saline- and cocaine-exposed groups (saline male vs. saline female: $t = 9.375$, $df = 14$, $p < 0.0001$; cocaine male vs. cocaine female: $t = 11.15$, $df = 14$, $p < 0.0001$).

At P60, prenatal treatment produced a significant effect on BDNF expression in the frontal cortex (2-way ANOVA, $F(1, 12) = 10.57$, $p < 0.01$), whereas there was

Fig. 1. Prenatal cocaine exposure and BDNF protein expression. BDNF expression normalized to the intensity of GAPDH (IDV) from E15 embryos (male and female pooled sample; **a**), P16 (**b**), and P60 (**c**) mice. Values are the mean $\pm$ SEM. Representative Western blots for BDNF and for GAPDH are shown corresponding to each bar graph. * $p < 0.01$, ** $p < 0.0001$.

no significant main effect of sex ($F(1, 12) = 0.069$, $p > 0.05$). However, there was a significant prenatal treatment $\times$ sex interaction ($F(1, 12) = 4.849$, $p < 0.05$). A Bonferroni multiple comparison post hoc test demonstrated significant increases in BDNF expression in the prenatally cocaine-exposed male, but not female, mice compared to their prenatally saline-exposed counterparts ($t = 3.856$, $df = 12$, $p < 0.01$; Fig. 1c).

Total TrkB and pTrkB Expression

We examined the expression of total TrkB as well as each of its 2 phosphorylated forms, namely pTrkB-Y705 (autophosphorylation site) and pTrkB-Y515 (MAPK/ERK intracellular signaling site), in the frontal cortex at P16 and P60 (Fig. 2, 3). At P16 a 2-way ANOVA showed a significant main effect of sex on total TrkB expression ($F(1, 11) = 5.551$, $p < 0.05$; Fig. 2a) and pTrkB-Y515 expression ($F(1, 11) = 94.70$, $p < 0.0001$; Fig. 2c) but no significant main effect of prenatal treatment (total TrkB: $F(1, 11) = 1.191$; $p > 0.05$; pTrkB-Y515: $F(1, 11) = 0.4374$, $p > 0.05$), or treatment $\times$ sex interaction (total TrkB: $F(1, 11) = 0.936$, $p > 0.05$; pTrkB-Y515: $F(1, 11) = 3.452$, $p > 0.05$) for either protein. Bonferroni multiple comparison post hoc analysis demonstrated a significant increase in pTrkB-Y515 expression in female mice compared to male mice in both the prenatal treatment groups at P16 (saline male vs. saline female: $t = 5.447$, $df = 11$, $p < 0.001$; cocaine male vs. cocaine female: $t = 8.375$,

$df = 11$, $p < 0.0001$). However, Bonferroni multiple comparison post hoc analysis did not reveal a significant change in total TrkB expression in female mice compared to male mice in either prenatal treatment group (saline male vs. saline female: $t = 2.299$, $df = 11$, $p > 0.05$; cocaine male vs. cocaine female: $t = 1.004$, $df = 11$, $p > 0.05$). Lastly, neither prenatal treatment nor sex produced significant effects on pTrkB-Y705 expression (2-way ANOVA, $p > 0.05$; Fig. 2b).

At P60, neither prenatal treatment nor sex produced significant effects on total TrkB or pTrkB-Y705 expression (2-way ANOVA, $p > 0.05$; Fig. 3a, b). However, with respect to pTrkB-Y515 expression, there were significant main effects of prenatal treatment (2-way ANOVA, $F(1, 9) = 6.18$, $p < 0.05$) and sex ($F(1, 9) = 51.7$, $p < 0.0001$), but no significant treatment $\times$ sex interaction ($F(1, 9) = 3.37$, $p > 0.05$; Fig. 3c). Bonferroni multiple comparison post hoc analysis demonstrated a significant increase in pTrkB-Y515 expression in the prenatally cocaine-exposed male mice compared to their prenatally saline-exposed counterparts ($t = 3.529$, $df = 9$, $p < 0.05$). There was no significant difference between female mice within a given prenatal treatment group. Additionally, post hoc analysis showed a significant decrease in pTrkB-Y515 expression in females compared to males in each prenatal treatment group (saline male vs. saline female: $t = 3.571$, $df = 9$, $p < 0.05$; cocaine male vs. cocaine female: $t = 6.826$, $df = 9$, $p < 0.001$).

Fig. 2. TrkB expression in P16 mice. Total TrkB (a), pTrkB-Y705 (b), and pTrkB-Y515 (c) band intensities normalized to the intensity of GAPDH (IDV) from male and female mice. Values are the mean $\pm$ SEM. Representative Western blots for total TrkB, pTrkB-Y705, and pTrkB-Y515 are shown corresponding to each bar graph. * $p < 0.001$, ** $p < 0.0001$.

Fig. 3. TrkB expression in P60 mice. Total TrkB (a), pTrkB-Y705 (b), and pTrkB-Y515 (c) band intensities normalized to the intensity of GAPDH (IDV) from male and female mice. Values are the mean $\pm$ SEM. Representative Western blots for TrkB, pTrkB-Y705, and pTrkB-Y515 are shown corresponding to each bar graph. * $p < 0.05$, ** $p < 0.001$.

Prenatal Cocaine Exposure Selectively Increases *Bdnf* Exon I mRNA Expression at P60

Given that a significant increase in BDNF and pTrkB-Y515 expression occurred at P60 and not P16, and only the male mice (and not female mice) in the prenatally cocaine-exposed group demonstrated the increase at P60, we performed the following additional analyses at P60 and in the male mice only. We measured the expression

of *Bdnf* exons I-, II-, IV-, and VI-containing transcripts (Fig. 4a) and found significant main effects of prenatal treatment (2-way ANOVA, treatment, $F(3, 22) = 3.35$, $p < 0.05$, and exon isoform, $F(1, 22) = 12.93$, $p < 0.05$); however, the prenatal treatment $\times$ exon interaction was not significant ($F(3, 22) = 1.017$, $p > 0.05$). The Bonferroni multiple comparison post hoc test indicated that prenatal cocaine exposure produced a significant increase

Fig. 4. *Bdnf* exon transcripts and the association of AcH3 in P60 mice. **a** Quantitative real-time PCR analysis of *Bdnf* exon (Ex) I-, II-, IV-, and VI-containing transcripts. Values are the mean $\pm$ SEM. **b** Representative Western blots of global acetylated histone 3 (H3K9K14ac2, AcH3) and methylated histone H3 (H3K9me3, MeH3) together with histone H3 as the loading control. AcH3 (**c**) and MeH3 (**d**) expression normalized to the intensity of histone H3 (IDV). Values are the mean $\pm$ SEM. **e** ChIP analysis of AcH3 association with *Bdnf* exon I. * $p < 0.05$, ** $p < 0.01$.

in the expression of the exon I-containing transcript ($t = 3.024$, $df = 22$, $p < 0.05$; Fig. 4a), and no significant change in the expression of the other *Bdnf* transcripts (exon-II: $t = 1.985$, $df = 22$, $p > 0.05$; exon-VI: $t = 0.6645$, $df = 22$, $p > 0.05$).

Epigenetic Regulation of *Bdnf* Exon mRNA Expression

To understand the epigenetic mechanisms that might contribute to changes in *Bdnf* transcript mRNA expression, we examined representative permissive (acetylated histone 3; H3K9K14ac2, AcH3) and repressive (methylated histone 3; H3K9me3, MeH3) markers of global transcription. While the AcH3 protein levels were significantly increased in the prenatally cocaine-exposed mice (t test [mean $\pm$ SEM], AcH3: saline 47.7 ± 8.8 , cocaine 141.8 ± 27.0 , $t = 3.922$, $df = 8$, $p < 0.01$; Fig. 4b, c), there was no significant change in MeH3 protein levels (MeH3: saline 40.9 ± 8.1 , cocaine 64.75 ± 13.9 , $t = 1.597$, $df = 10$, $p > 0.05$; Fig. 4b, d). ChIP combined with qPCR (ChIP-qPCR) demonstrated a significant increase in AcH3 association with the *Bdnf* exon I promoter in the prenatally cocaine-exposed mice (Student t test [mean $\pm$ SEM]: saline 5.4 ± 0.5 , cocaine 12.8 ± 2.3 , $t = 2.838$, $df = 7$, $p < 0.05$; Fig. 4e).

We did not examine epigenetic changes with the other promoters because their expression was not significantly affected by the prenatal cocaine exposure (Fig. 4a).

Discussion

Our findings demonstrate that while cocaine exposure decreases BDNF expression in the embryonic brain, prenatal cocaine exposure significantly increases BDNF and pTrkB expression in the frontal cortex of the adult (i.e. P60) brain. The increases in BDNF and pTrkB expression at P60 are sex specific and only occur in male mice. These effects are also region specific. In a companion paper [42], we report that BDNF or TrkB (total or pTrkB) levels were not significantly altered in the P60 striatum in our prenatal cocaine exposure mouse model. Moreover, the increased BDNF protein expression is associated with increased *Bdnf* mRNA expression, and acetylation of histone H3 is associated with the exon I promoter of the *Bdnf* gene in the frontal cortex at P60. TrkB phosphorylation is also selective in response to prenatal exposure to cocaine and only occurs at Y515, the shc adaptor protein

docking site, which leads to MAPK/ERK activation [43, 44]. Phosphorylation at Y705, the autophosphorylation site, is not significantly altered. Expression of BDNF, total TrkB or pTrkB was not significantly altered in the frontal cortex of male or female mice at P16.

Our data indicate that the effects of prenatal cocaine exposure on frontal cortical BDNF-TrkB signaling were influenced by the developmental stage. Thus, prenatal cocaine exposure did not produce significant changes in BDNF or TrkB (total or pTrkB) levels at P16, but produced significant increases in both at P60. We chose P16 and P60 for analysis because these 2 ages represent disparate points along the trajectory of postnatal development of the frontal cortex. For example, neuronal maturational events, such as dendritic arborization and synaptic remodeling, are robust at P16 and are essentially completed by P60. Since BDNF plays a major role in the regulation of these developmental processes, we reasoned that the effects of prenatal cocaine exposure might be different at P16 compared to P60, and in fact found it to be the case. Our data do not offer an explanation for the lack of effects of prenatal cocaine exposure at P16. However, we speculate that the developmental demand for BDNF imposed by the robust neuronal maturational events may have masked the effects of prenatal cocaine exposure on BDNF expression at P16, which were evident earlier (E15) and later (P60). The developmental increases in the neurotransmitter ligand-receptor expression with which BDNF is known to interact, especially the dopamine system [45, 46], may also contribute to the developmental changes in BDNF expression. In fact, cocaine produces significant changes in dopamine signaling in the developing brain [6, 20, 47], adding support to this possibility.

Our finding of reduced BDNF expression in the E15 dorsal forebrain is consistent with our earlier report, in which we found a cocaine-induced decrease in BDNF in the E15 dorsal and basal forebrain [18]. Thus, during embryonic development while cocaine exposure was ongoing (i.e. cocaine was present in the system), there was a decrease in BDNF expression, whereas at P60, when cocaine exposure had ceased, BDNF levels were increased. Therefore, it is likely that the 2 types of effects produced by cocaine (short-term effects of ongoing exposure vs. long-term effects of prenatal exposure observed after exposure had ceased) represent a combination of the type of exposure, the developmental stage, and the brain region examined.

We found that BDNF and TrkB (total and phosphorylated) expression in the frontal cortex was influenced by sex at P16. Female mice had higher frontal cortical BDNF,

total TrkB, and pTrkB than males at P16. The only significant sex difference in the effects of prenatal cocaine exposure at P60 was that pTrkB-Y515 expression was lower in females than in males. The influence of sex on BDNF-TrkB signaling has been previously reported. For example, pTrkB levels were significantly increased in the frontal cortex of adult male but not female BDNF heterozygous (BDNF+/-) mice [48]. In addition, altered BDNF-TrkB signaling was proposed as a basis for sex differences in the incidence of depressive disorders [49]. Importantly, interactions between estrogen and BDNF were also proposed as a potential mechanism for sex differences in BDNF signaling in the brain [50–53].

Although the mechanisms mediating developmental and sex-specific changes in BDNF expression and the responses of these intrinsic factors to the prenatal cocaine exposure remain elusive, the *Bdnf* gene with its multiple exons and promoters [39] may be particularly suited to differential regulation by intrinsic and extrinsic factors throughout the life of the organism. The mouse *Bdnf* gene is comprised of eight 5' untranslated exons and a 3' protein coding exon [38, 39]. Expression of exon-specific BDNF transcripts is differentially regulated, as each exon contains its own promoter region. Such diversity at the levels of exons and promoters is conducive to distinct expression profiles in different brain regions, at different periods of development, and in males versus females [39], as a result of chromatin remodeling at individual promoters [36, 37, 39]. It is therefore possible that the epigenetic changes associated with the *Bdnf* gene in the fetal brain produced by cocaine are further differentially modified or impacted in the male and female organisms [54]. As an example, estradiol can regulate frontal cortical BDNF expression as the *Bdnf* gene contains an estrogen response element [51, 52] and estrogen receptors colocalize with neurons that express BDNF [53], suggesting a relationship between the two and providing a mechanism whereby BDNF is differentially expressed in male and female mice. Future studies assessing alterations in BDNF/TrkB signaling throughout the estrous cycle in prenatally cocaine exposed mice would assist with understanding how sex steroid hormones may regulate BDNF/TrkB signaling and shed light on the sex-specific differences reported here.

Our approach in the present study was to illustrate the molecular mechanisms associated with prenatal cocaine exposure centered on BDNF and TrkB signaling in the frontal cortex. Our focus on the frontal cortex is based on reports that prenatal cocaine exposure is associated with significant alterations in higher cognitive functions regu-

lated by the frontal cortex [6]. In addition, the frontal cortex is also the focus of studies of prenatal cocaine exposure and BDNF signaling mechanisms in animal models [6; also see the companion paper, 42]. Therefore, BDNF-TrkB signaling in the frontal cortex became a highly justifiable choice for the present study. Overall, our finding of increased BDNF-TrkB signaling in the frontal cortex is consistent with earlier reports [13, 16]. However, our findings offer novel evidence to advance the field by illustrating the role of developmental stage and sex in mediating the effects of cocaine exposure, and also by demonstrating an epigenetic mechanism underlying the increases in frontal cortical BDNF levels.

Interpretation of findings from studies focused on the effects of prenatal exposure to cocaine (or to any other drug) is often associated with technical concerns in pre-clinical models, especially when behavioral parameters are analyzed. For example, whether the cocaine-exposed dams interact with their prenatally drug-exposed offspring “differently” during the nursing period, and whether such differences in mother-infant interaction contribute to the behavioral changes observed in the offspring in later life, is always a major concern. To address this concern, we cross-fostered the offspring from each experimental group to drug-naïve nursing mothers. We have previously shown in another study [55] that nutritional factors do not contribute to the developmental consequences of prenatal cocaine exposure in our mouse model, which is consistent with other reports on this subject [30, 56].

Our BDNF expression and BDNF-TrkB signaling data are from samples pooled from the medial and dorsal prefrontal cortices preventing resolution of potential regional differences within the frontal cortex. However, earlier reports showed that neither BDNF nor TrkB expression was altered in the medial prefrontal cortex of prenatally cocaine-exposed adult mice [16]. Therefore, the increased BDNF signaling observed in the present study is likely selective to the dorsal prefrontal cortex.

Finally, as described earlier, the *Bdnf* gene with its multiple exons and promoters appears to be especially vulnerable to the effects of multiple environmental factors, not just cocaine. Nicotine, a cholinergic drug, produces significant alterations in BDNF levels in humans and experimental animals. As an example, maternal cigarette smoking during pregnancy influences BDNF expression in the prenatally exposed offspring [57, 58]. Additionally, a number of studies using animal models also report significant effects of prenatal nicotine exposure on BDNF expression in multiple brain regions in both males

and females [59–62]. Lastly, prenatal stress results in decreased BDNF mRNA expression in the frontal cortex and hippocampus, with alterations in DNA methylation at the *Bdnf* gene proposed as the underlying mechanism [63]. These effects were specific to only male offspring as the effects of prenatal stress were not examined in female offspring in this study.

In conclusion, our findings illustrate multiple effects of cocaine exposure on BDNF signaling that depend on the type of exposure (ongoing vs. prenatal), developmental stage, brain region, and sex. Our study further advances the field by demonstrating an epigenetic mechanism underlying prenatal cocaine exposure-induced long-term changes in frontal cortical BDNF-TrkB signaling mechanisms.

Acknowledgements

We gratefully acknowledge technical assistance from Sarah E. Lowe, Penny Coshatt, and Gavin R. Sangrey.

This work was supported by the Jim and Betty Anne Rodgers Chair Funds (P.G.B.), the Florida State University Research Foundation GAP Grant Program (P.G.B.), and National Institutes of Health (NIDA R01 DA033641, NIDA R21 DA033641; G.S.-V.).

Disclosure Statement

All authors reported no biomedical financial interests or potential conflicts of interest in relation to this work.

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