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Intravenous prenatal nicotine exposure alters METH-induced hyperactivity, conditioned hyperactivity, and BDNF in adult rat offspring

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Abstract

In the United States, approximately 15% of women smoked tobacco cigarettes during pregnancy. *In utero* tobacco smoke exposure produces somatic growth deficits like intrauterine growth restriction and low birth weight in offspring, but it can also negatively influence neurodevelopmental outcomes in later stages of life, such as an increased incidence of obesity and drug abuse. Animal models demonstrate that prenatal nicotine (PN) alters development of the mesocorticolimbic system, which is important for organizing goal-directed behavior. In the present study we determined if intravenous (IV) PN altered the initiation and/or expression of methamphetamine (METH)-induced locomotor sensitization as a measure of mesocorticolimbic function in adult rat offspring. We also determined if PN and/or METH exposure altered protein levels of brain-derived neurotrophic factor (BDNF) in the nucleus accumbens, the dorsal striatum, and the prefrontal cortex of adult offspring. BDNF was of interest because of its role in the development and maintenance of the mesocorticolimbic pathway, and its ability to modulate neural processes that contribute to drug abuse, such as sensitization of the dopamine system.

Dams were injected with IV nicotine (0.05 mg/kg/injection) or saline, 3×/day on gestational days 8–21. Testing was conducted when offspring reached adulthood (~postnatal day 90). Following three, once daily habituation sessions animals received a saline injection and baseline locomotor activity was measured. PN and prenatal saline (PS)-exposed offspring then received 10, once daily injections of METH (0.3 mg/kg) to induce locomotor sensitization. Animals received a METH injection (0.3 mg/kg) to assess the expression of sensitization following a 14-day period of no injections. A day later, all animals were injected with saline and conditioned hyperactivity was assessed. Brain tissue was harvested 24-h later.

PN animals habituated more slowly to the activity chambers compared to PS controls. PN rats treated with METH showed significant enhancement of locomotor behavior compared to PS rats following acute and repeated injection; however, PN did not produce differential initiation or

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expression of behavioral sensitization. METH produced conditioned hyperactivity, and PN rats exhibited a greater conditioned response of hyperactivity relative to controls. PN and METH exposure produced changes in BDNF protein levels in all three regions and complex interactions were observed between these two factors. Logistic regression revealed that BDNF protein levels, throughout the mesocorticolimbic system, significantly predicted the difference in animals' conditioned hyperactive response: both correlations were significant, but the predicted relationship between BDNF and context-elicited activity was the stronger in PN (r=0.67) compared to the PS rats (r=0.42).

These findings indicate that low-dose PN exposure produces long-term changes in activity and enhanced sensitivity to the locomotor effects of METH. The enhanced METH-induced contextual conditioning shown by the PN animals suggests that offspring of *in utero* tobacco smoke exposure have greater susceptibility to learn about drug-related conditional stimuli, such as the context. The PN-induced alterations in mesocorticolimbic BDNF protein lend further support for the hypothesis that maternal smoking during pregnancy produces alterations in neuronal plasticity that contributes to drug abuse vulnerability. The current findings demonstrate that these changes are persistent into adulthood.

Keywords

prenatal nicotine; intravenous; locomotor sensitization; methamphetamine; conditioned hyperactivity; BDNF; rat

1. Introduction

In the USA, 15% of pregnant women, aged 15–44, smoked cigarettes within the past month; the incidence of maternal smoking has remained constant over the last decade e.g., ~15.4% in 2012–2013; [1]. Aside from the well documented somatic effects of *in utero* tobacco smoke exposure [2,3, e.g., low birth weight; 4], converging evidence indicates that maternal smoking also produces neurodevelopmental deficits during adolescence and adulthood [5–8]. Of particular interest here are the well documented findings showing that *in utero* tobacco smoke exposure is associated with an increased likelihood of drug abuse in offspring [9–11].

Kandel et al. [10] first suggested that PN exposure disrupts the development of the mesocorticolimbic system; a key neural pathway responsible for organizing goal-directed behavior [12]. It was hypothesized that nicotine's agonist effects on the cholinergic system increase vulnerability for drug abuse in this population of individuals, presumably through sensitization of the developing mesocorticolimbic system. Animal models of PN exposure have suggested that enduring changes in neurotransmitter content in developing offspring are associated with PN exposure, e.g., acetylcholine, catecholamines, orexin, and neurotrophic factors [13–19], therefore providing general support for this hypothesis.

Our laboratory uses a model of intravenous (IV) PN exposure in rats to mimic the pharmacokinetic profile of nicotine absorption with tobacco smoke inhalation [20,21]. Several studies from our laboratory have shown that 3×/day IV nicotine administration

produces significant and long-lasting neurobehavioral alterations in exposed offspring [17,21,22]. In the present study, we used adult male and female offspring exposed to IV PN or prenatal saline (PS) and assessed methamphetamine (METH)-induced hyperactivity and METH-induced sensitization. Behavioral sensitization is a progressive increase in activity following repeated exposure to a psychostimulant drug [23,24], and it putatively reflects functional changes in multiple neurotransmitter systems such as dopamine, glutamate, and GABA, throughout the mesocorticolimbic system [25,26]. Investigating sensitization of locomotor activity is a common measure to assess functional changes in the mesocorticolimbic system [23,27], although it is not a measure of drug reinforcement [28].

Our laboratory has also investigated neurochemical changes in the mesocorticolimbic system of offspring produced by PN exposure [17,18]. Brain-derived neurotrophic factor (BDNF) is an activity-dependent protein that is primarily responsible for the proliferation and differentiation of developing midbrain dopamine neurons, but is also integral for DA neuron survival in mature organisms [29,30]. Psychostimulant exposure increases BDNF protein and mRNA levels throughout the brain [31,32]. BDNF has been shown to modulate the reinforcing properties of abused drugs [33,34] and it is involved in the process of drug-induced sensitization [35,36]. A previous study from our laboratory investigated METH-induced locomotor activity and BDNF protein levels in the mesocorticolimbic system of adolescent, female and male offspring exposed to IV PN or PS [17]. PN exposure did not alter the initiation of METH-induced sensitization relative to controls; however, PN animals exhibited overall increases in protein levels of BDNF in the nucleus accumbens (NAc) and the dorsal striatum (dSTR), and there were no sex differences for this finding. Wei et al., [16] reported that continuous exposure to prenatal nicotine resulted in female offspring that exhibited decreased levels of BDNF in the prefrontal cortex and increased BDNF in the striatum [19]. Whether such PN-induced changes endure into adulthood has not been investigated. Therefore, in the present study adult male and female PN and PS offspring were injected with METH to produce behavioral sensitization and the expression of sensitization was assessed following a 14-day non-drug period [37]. METH-induced conditioned hyperactivity was assessed on the final day [38,39], and 24-h later, brain tissue was harvested for BDNF protein analysis. It was predicted that PN exposure would enhance the initiation and expression of METH-induced sensitization and modulate BDNF protein levels in the NAc, dSTR, and FC.

2. Methods

2.1 Animals

Sixty female and 30 male adult, nulliparous Sprague-Dawley rats were purchased from Harlan Industries, Inc. (Indianapolis, IN). Rats were maintained in the animal care facilities in the department of Psychology at the University of South Carolina. Rat chow (ProLab Rat/Mouse/Hamster Chow 3000) and water were provided *ad libitum* during the experiments, except when otherwise specified. Animals were provided with Nestlets (NestletsT; Ancare, Bellmore, NY) and Nylabones (durable chew; Nylabone, Inc.; Neptune, NJ) in their home cages throughout the duration of the study. A Nylabone was replaced when it was thoroughly chewed, and one Nestlet nesting product was placed in the animals' cage when

the cage was changed, which occurred 2× per week. The targeted environmental regulation was 21 ± 2° C, 50% ± 10% relative humidity and a 12L:12D cycle with lights on at 0700 h EST. The protocol for this research methodology was approved by the Institutional Animal Care and Use Committee at the University of South Carolina (animal assurance number: A3049-01).

2.2 Internalized jugular catheter surgeries

The females were catheterized with an indwelling jugular catheter at Harlan Industries prior to arrival at the University of South Carolina. Materials and the surgical procedure followed the methods of Mactutus et al. [40]. Rats were anesthetized with a mixture of xylazine (3.3 mg/kg/ml) and ketamine hydrochloride (100 mg/kg/ml). A sterile Intracath IV catheter (Becton, Dickinson and Co., Franklin Lakes, NJ), fitted with a Luer-Lok injection cap (Medex, Inc., Carlsbad, CA) was implanted into a subcutaneous space located on the dorsal side of the rat. The distal end of the catheter was then inserted into the left jugular vein and bound with a sterile suture. Animals were kept under periodic post-operative observation and returned to the colony at Harlan Industries following recovery. The catheters were flushed with 0.2 ml of heparinized saline daily to help maintain catheter patency. This began 24-h after surgery.

2.3 Breeding

All animals were allowed to habituate to the colony room for 7 days following arrival. The next week, female rats were housed three per cage. One male was placed in the cage with the females, overnight, from approximately 1700 to 0900 h for breeding. Vaginal lavage samples were analyzed under light microscopy (10×). The estrous cycle status and the presence of sperm were assessed to determine pregnancy. Upon identification of a positive sample the female was single-caged and that day was considered GD 0. Non-positive animals were returned to the group cage and bred the following night(s) until determined pregnant. The weight of the pregnant dams was recorded daily throughout gestation.

2.4 Drugs

Nicotine hydrogen tartrate (base weight) and METH hydrochloride (salt weight) were purchased from Sigma-Aldrich Pharmaceuticals (St. Louis, MO). Both compounds were dissolved in physiological saline (0.9%; Hospira, Inc. Lake Forest, IL). The pH of the nicotine solution was neutralized to 7.0 ± 0.2. Heparin was acquired from APP Pharmaceuticals (Schaumburg, IL), and a heparinized saline solution (2.5%) was used to flush the IV catheters.

2.5 Prenatal nicotine treatment

The dams were randomly assigned to the nicotine or the saline groups. Nicotine (0.05 mg/kg/injection) or saline was administered intravenously, 3×/day via the internalized IV catheters from GD 8–21. The injections were delivered through the Luer-Lok injection cap of the subcutaneously implanted injection port (20 seconds in duration). Following the first two daily injections, catheters were flushed with 0.2 ml of 0.9% physiological saline to clear out the entirety of the catheter volume. Heparinized saline was used to flush the catheter

after the third and final daily IV injection to maintain catheter patency. All post-flush injections were 20 seconds in duration. The IV injections were performed daily during the light portion of the photoperiod, at approximately 1000, 1300, and 1600 h.

2.6 Surrogate fostering, litter composition, and postnatal testing

The day of birth was considered postnatal day (PND) 0. On PND 1, litters were culled to 10 pups with 5 males and 5 females, whenever possible. All pups were surrogate-fostered to timed-pregnant, drug naïve dams on PND 1 to prevent poor maternal care attributable to prior nicotine treatment [41]. The developmental milestones of the righting reflex, negative geotaxis, and eye opening were assessed [42]. For the righting reflex, rats were placed on their backs and the latency (25 seconds maximum) to right themselves was recorded upon release. This was assessed in 3 trial blocks across 3 consecutive days, on PND 3–5. During negative geotaxis testing, animals were placed on a wire mesh grid positioned on a 25° downward angle. The latency (30 seconds maximum) for animals to turn to face upward on the slope (180°) was recorded. This behavior was measured in 3 trial blocks across 3 consecutive days, on PND 8–10. Both eyes of each animal were checked for degree of openness across 5 consecutive days, on PND 13–17. The degree of openness was rated on a scale of 0–3: 0 = completely closed; 1 = any opening exposing the cornea; 2 = cornea and pupil exposed but eye lids are not fully open; 3 = fully open. All animal weights were recorded on PND 1, 7, 14, and 21. The offspring were weaned and pair-housed with same sex cage mates on PND 21.

2.7 Experiment 1: METH-induced locomotor sensitization

2.7.1 Apparatus—Locomotor activity was assessed with 16 square (40 × 40 cm) chambers manufactured by Hamilton-Kinder (Kinder Inc., Poway, CA). The chambers were housed together in an isolated room and they were controlled by MotorMonitor software (Kinder Inc., Poway, CA) on a Pentium class computer equipped with Windows®. Each chamber had 32 emitter/detector pairs of infrared photocells and behavior was recorded as activity counts via infrared photocell interruptions made by the animal. Horizontal activity and distance travelled (cm) in the center portion of the locomotor apparatus (central 24%) was calculated using the MotorMonitor software. The chambers were converted to round compartments (~ 40 cm diameter) by adding Plexiglas inserts. Each emitter/detector pair was tuned by the manufacturer in order to accommodate the extra perspex width imposed by the Plexiglas. The use of round chamber-inserts reduces the likelihood that rats will remain stationary in one of the corners of the standard, square activity chamber [43–45].

2.7.2 Induction of locomotor sensitization—Adult male and female offspring, exposed to PS or PN, were randomly assigned to either saline (S) or METH (M) groups, and this yielded 8 total groups; the PS-S (females = 10; males =10), PS-M (females = 10; males = 10), PN-S (females = 10; males = 10), and PN-M (females = 10; males = 10) treatment conditions. In total, 24 litters were represented. Pups were randomly assigned to treatment groups. For all experiments, only one male and one female, randomly selected from each litter, were assigned to a group [46]. Five, 1 hour-long activity sessions were conducted per day in the 16 locomotor chambers. Two males and two females from each treatment group were represented in each of the 5 daily activity sessions.

Animals were habituated to the locomotor activity chambers for one hour/day over three consecutive days, and no injections occurred on these days. Twenty four hours after the third habituation test all rats received a subcutaneous (s.c.) saline injection, and five minutes later, were placed into the activity chambers for one hour to measure baseline activity. This day was referred to as the "saline baseline." The sensitization phase was conducted over the next 10 days. Animals were injected with either METH (0.3 mg/kg/injection; sc) or saline and were placed into the locomotor chamber five minutes later. Injections occurred once per day and activity was recorded for 60 minutes.

2.7.3 Expression of locomotor sensitization—Following the induction of sensitization, animals remained in the colony for 13 consecutive days. No injections were administered during this period. Animals were then administered the same injection they received during the induction of sensitization (saline or METH 0.3 mg/kg/injection) and placed in the locomotor activity chamber for one hour. This day represented the expression of locomotor sensitization.

2.7.4 Conditioned hyperactivity—Twenty-four hours after the measure of expression, all animals received a test of drug-induced conditioned activity [38,47]. This form of conditioning is the result of learning to associate the contextual cues of the apparatus with the unconditional stimulus (US) effects of the drug. Over repeated testing the subsequent presentation of the context alone elicits a conditional response (CR) of activity in the absence of drug injection (e.g., saline injection). In the present experiment, animals received a saline injection and locomotor activity was recorded for 1 hour, and this was the final locomotor test of the experiment (see Table 2). Assessing learning related to conditional stimuli is important because exaggerated attribution of salience to drug related cues are hypothesized to engender greater drug seeking behavior (Robinson and Berridge, 2003).

The dependent measures for all locomotor tests were horizontal activity and center distance travelled (cm). Center distance has been used as a measure of locomotor sensitization in previous studies [48–50].

2.8 Experiment 2: BDNF protein levels in the nucleus accumbens, dorsal striatum, and prefrontal cortex

2.8.1 Analysis of BDNF protein levels—Animals were rapidly decapitated twenty-four hours after the conditioned hyperactivity test. A 24-h interval was used so that rapid decapitation occurred at intervals that corresponded to the times they were injected and tested on previous days. Given that tissue was removed at the end of the experiment, BDNF protein levels prior to behavioral sensitization are not reported. The whole brain was extracted and flash frozen in isopentane and stored at −80° C. A BDNF Enzyme-linked immunosorbent assay kit (ELISA; Promega, Madison, WI) was used to analyze BDNF protein levels in the NAc, FC, and dSTR and the protocol provided with the kit was closely followed. In brief, tissue samples were thawed and microdissected to remove the NAc, PFC and dSTR and placed into 250 μl of RIPA cell lysis buffer (150 mM NaCl, 50mM Tris-Hcl, 1.0% NP-40, 0.5% sodium deoxycholate and 0.1% SDS) with added protease/phosphatase inhibitors (P5726, P8340, P0044). Regions from separate animals were not combined.

Tissue was then homogenized using a Fisher Scientific Model 705 sonic dismembrator for 3 s. Homogenates were then centrifuged at 14,000 g for 20 min at 4°C. To assess BDNF content, anti-BDNF monoclonal antibody (mAb) was added to a carbonate coating buffer (pH 9.7, per specifications of the Promega protocol) and 100 μl of the coating buffer was added to each well of a 96-well polystyrene ELISA plate (MaxiSorb, Nalge Nunc International, Rochester, NY) and incubated overnight at 4°C. All wells were washed using TBS-T wash buffer, incubated at room temperature for 1 hour, and nonspecific binding was blocked by adding block and sample 1× buffer to each well and incubated at room temperature for 1 hour. Following washing, the BDNF standard curve was prepared using the BDNF standard supplied from the manufacturer (1μg/ml). The standard was diluted in Block & Sample 1× buffer to achieve a concentration range of 0 to 500 pg/ml. Homogenized tissue samples (100 μl per well) were added, in duplicate, to the 96-well plate. Standards and samples were then incubated (with shaking) at room temperature for 2 h. The addition of anti-Human BDNF pAB to each well plate was followed by 2 h of incubation at room temperature. Finally, Anti-IgY horseradish peroxidase (HRP) conjugate was added to plate followed by a 1 h incubation period. Visualization was achieved by adding TMB solution to each well followed by an incubation period of 10 minutes at room temperature. The reaction was stopped by adding 1N hydrochloric acid to each well and the plate was read within 30 minutes of stopping the reaction.

2.9 Data Analysis

2.9.1 Litter parameters—The between-subjects factors for the litter parameter analyses were Sex (2) and Maternal Treatment (2; nicotine or saline), and the within-subjects factors were PND (4) and GD (4). A one-way ANOVA was conducted for the total number of pups born to PN and PS dams with Prenatal Treatment as the between-subjects factor. A Sex × Prenatal Treatment factorial ANOVA was used to analyze the ratio of males to females born to PN and PS dams. A Sex × Prenatal Treatment × PND mixed-factorial ANOVA was conducted for the pup weight gain, righting reflex, negative geotaxis data. A Kruskall-Wallis test was used to analyze each day of eye-opening with Prenatal Treatment and Sex as grouping factors.

2.9.2 Experiment 1: Initiation and expression of METH-induced locomotor sensitization and conditioned hyperactivity—The habituation sessions were analyzed with a Sex × Prenatal Treatment × Day mixed-design ANOVA. The data from the saline baseline day was analyzed using a Sex × Prenatal Treatment factorial ANOVA. A Sex × Prenatal Treatment × Adult Treatment × Day mixed-design ANOVA was used to analyze data from the initiation phase of the experiment. Data collected from the sixth day of sensitization was dropped from all analyses of the sensitization period because of a hardware malfunction. Thus, the analysis included 9 rather than 10 days of sensitization as a repeated measure. Analysis of partial data that was collected from the sixth day suggests no difference in the interpretation of results, i.e. *p*-values did not deviate from the analysis of 10 compared to 9 days of sensitization.

The data from the expression of sensitization phase was analyzed in two ways. First, a Sex × Prenatal Treatment × Adult Treatment factorial ANOVA was used to analyze data from the

single day expression was measured. Second, a Sex × Prenatal Treatment × Adult Treatment × Day mixed-design ANOVA, which added Day (the final day of induction and the challenge day) as a within-subjects measure, was used to determine if there was any change in activity from the final day of induction to the expression day. Finally, the data from the last day of the experiment (Saline Baseline 2) was analyzed with a Sex × Prenatal Treatment × Adult Treatment factorial ANOVA. The dependent measures for all locomotor testing days were analyzed over the 60-minute period.

2.9.3 Experiment 2: BDNF protein levels—The between subjects factors for the BDNF data analysis were Sex (2), Prenatal Treatment (2), and Adult Treatment (2). Separate Sex × Prenatal Treatment × Adult Treatment ANOVAs were conducted on the protein data from the NAc, dSTR, and PFC.

Multiple regression techniques were also used to characterize the potential relationship between locomotor activity (from Experiment 1) and BDNF protein levels (from Experiment 2). Pearson correlations were used to illustrate the linear relationship between the predicted and observed locomotor scores derived from the regression analysis. Statistical significance was determined using $\alpha \leq 0.05$ for all analyses. Any descriptive data presented in text represents the mean ± standard error ($M \pm$ SEM). Data analyses were performed using SPSS 19 (IBM Software) and GraphPad Prism 4 (GraphPad Software, Inc.) statistical programs.

It is important to note that the factor of Sex was included in all analyses. Sex is a significant factor in some of the analyses, particularly those examining METH-induced horizontal locomotor activity. The factor of Sex never interacted with Prenatal Treatment and this suggests that females, regardless of exposure to PS and PN, exhibited increased locomotor behavior in general and in response to METH. This sex difference in locomotor activity is well documented [51–53]. For all analyses in which Sex was significant it is stated; however; given that Sex did not interact with Prenatal Treatment all data are presented collapsed across sex [54].

3. Results

3.1 Litter parameters

Analysis of maternal weight gain, distribution of sex and total litter size, pup weight gain, negative geotaxis, the righting reflex, and eye opening revealed no significant differences between PN and PS-exposed dams and pups. The results are summarized in Table 1. There was a significant main effect of Sex [$F(1, 240) = 5.4$, $p \leq 0.05$]. Males gained slightly more weight (g; 18.8 ± 0.16) than females (18.3 ± 0.15); however, there was neither an effect of Prenatal Treatment, nor a Sex × Prenatal Treatment interaction, and so the weight gain data are collapsed across sex in table 1.

3.2 PN exposure produced enhanced spontaneous and METH-induced locomotor activity in offspring

3.2.1 Habituation—The 2 × 2 × 3 mixed-factorial ANOVA performed on the horizontal activity data indicates that the activity of all animals decreased across days [Day: $F(2, 152) = 11.2$, $p \leq 0.001$], and that females exhibited more activity than males [Sex: $F(1, 76) = 9.9$,

$p \leq 0.01$; data not shown]. The Prenatal Treatment × Day interaction was significant [$F(2, 152) = 3.8$, $p \leq 0.05$]; this interaction is driven by increased activity in PN compared to PS controls during habituation (Figure 1). No other main effects or interactions were significant.

Analysis of the center distance travelled data revealed a significant main effect of Day [$F(2, 152) = 4.6$. $p \leq 0.05$]. Animals showed an overall decrease in center distance travelled across the 3 habituation testing days (6155.88 ± 222.11, 6097.40 ± 214.58, 5679.64 ± 214.10 cm, respectively). No other main effects or interactions were significant.

3.2.2 Saline baseline—The 2 × 2 ANOVA for horizontal activity revealed that females exhibited more activity than males [Sex: $F(1, 76) = 24.5$, $p \leq 0.001$; data not shown], but no other significant main effects or interactions were observed. Thus, the PN and PS groups showed equivalent activity during the Saline Baseline test (see Figure 2A).

The 2 × 2 ANOVA for center distance travelled revealed no significant main effects or interactions. The PN and PS groups showed similar activity during the Saline Baseline test (see Figure 2B).

3.2.3. Induction of METH-induced locomotor sensitization: Days 1–10—Figure 2A shows the mean horizontal activity for the saline baseline day and the initiation of sensitization period for the PN-S, PN-M, PS-S, and PS-M groups. A three-way ANOVA revealed three significant main effects of Sex [$F(1, 72) = 38.7$, $p \leq 0.001$; data not shown], Adult Treatment [$F(1, 72) = 379.0$, $p \leq 0.001$], and Day [$F(8, 576) = 11.5$, $p \leq 0.001$]. Animals treated with METH exhibited greater horizontal activity relative to saline controls. Several two-way interactions were statistically significant, as well. METH treatment resulted in more activity in females relative to males across the entire sensitization period (Sex × Adult Treatment [$F(1, 72) = 11.4$, $p \leq 0.01$; data not shown]. Repeated METH induced locomotor sensitization [Day × Adult Treatment: $F(8, 576) = 12.9$, $p \leq 0.001$], and the increase in activity for the PS-M and PN-M groups was 15.9% and 15.1% greater on Day 10 compared to Day 1, respectively. A greater magnitude of METH-induced sensitization was suggested for the PN compared to the PS animals [Prenatal Treatment × Adult Treatment: $F(1, 72) = 3.2$, $p \leq 0.077$].

Figure 2B shows the mean center distance travelled for the saline baseline day and the sensitization period for the four treatment groups. A three-way ANOVA revealed significant main effects of Prenatal Treatment [$F(1, 72) = 10.0$, $p < 0.01$], Adult Treatment [$F(1, 72) = 406.8$, $p \leq 0.001$], and Day [$F(8, 576) = 8.6$, $p \leq 0.001$]. PN rats travelled significantly further than PS animals, repeated treatment with METH increased the distance traveled in the central zone relative to saline-injected animals and center distance travelled increased as a function of day. There were also two significant two-way interactions; Day × Adult Treatment [$F(8, 576) = 9.5$, $p \leq 0.001$] and Prenatal Treatment × Adult Treatment [$F(1, 72) = 15.3$, $p \leq 0.001$]. Animals repeatedly treated with METH travelled a significantly greater distance in the center of the arena than saline-treated animals, and behavioral sensitization increased as a function of day. PN rats treated with METH travelled a significantly greater distance relative to PS animals injected with METH. This effect together with the a non-significant Prenatal Treatment × Adult Treatment × Day interaction is important because

together they show that although IV PN treatment potentiated the unconditioned stimulus effects of METH, it did not differentially affect the induction of the sensitized response in either group administered METH. First, the PS-M and PN-M groups exhibited similar trajectories of sensitization, although the PN-M group showed greater center distance travelled than the PS-M group at every test (all t < .05, comparing PS-M and PN-M groups). Second, the relative magnitudes of behavioral sensitization on the final induction test were also similar: the PN-M and PS-M groups showed a 25.8% and 25.0% increase in center distance travelled, respectively, relative to the acute response (Day 1 of METH treatment). This relationship is shown in Figure 2B. Thus, PN exposure reliably potentiated the hyperactive response to METH throughout the induction phase relative to PS animals injected with METH. There were no main effects of Sex or interactions.

3.2.4 Expression of METH-induced locomotor sensitization—A three-way ANOVA revealed that METH treatment significantly increased horizontal activity compared to saline-treated animals [Adult Treatment: $F(1, 72) = 303.5$, $p \leq 0.001$]. METH-treated females were more active than METH-treated males, and they were also more active than males and females treated with saline [Sex × Adult Treatment: $F(1, 72) = 4.1$, $p \leq 0.05$; Sex: $F(1, 72) = 12.8$, $p \leq 0.001$; data not shown]. There was no main effect of Prenatal Treatment and there was no interaction between Prenatal Treatment and Sex.

Analysis of the center distance travelled data indicate that PN animals exhibited significantly greater distance travelled relative to PS animals (13,694.83 ± 587.27 and 11,391.56 ± 587.27 cm, respectively; Prenatal Treatment: $F(1, 72) = 7.7$, $p \leq 0.01$]. METH increased center distance travelled compared to saline-treated animals [Adult Treatment: $F(1, 72) = 301.0$, $p \leq 0.001$], and PN exposure potentiated distance travelled in the PN-M group relative to the PN-S, PS-M, and PS-S [Prenatal Treatment × Adult Treatment: $F(1, 72) = 5.5$, $p \leq 0.05$; data not shown].

3.2.5 Comparison of the initiation and expression of sensitization—A four-way ANOVA compared the final day of the initiation phase to the expression test day to assess behavioral sensitization following the 14-day drug-free period. The analysis of total horizontal activity revealed that the females were significantly more active than males [Sex: $F(1, 72) = 21.8$, $p \leq 0.001$]. The METH-injected groups exhibited significantly more horizontal activity compared to saline-treated groups [Adult Treatment: $F(1, 72) = 342.3$, $p \leq 0.001$], and METH-treated females exhibited more activity than the METH-treated males, and also showed greater activity than the male and female controls [Sex × Adult Treatment: $F(1, 72) = 6.9$, $p \leq 0.05$; data not shown]. There was neither a main effect of day nor any interaction with day as a factor, which indicates that the level of sensitized horizontal locomotor behavior did not change following the 14-day drug-free period.

The center distance travelled changed as a function of the drug-free period. The distance travelled increased significantly in all animals combined from day 10 [11,300.61 ± 2348.92 cm] to the expression test day [12,543.20 ± 415.26 cm; Day: $F(1, 72) = 27.39$, $p \leq 0.001$]. The PN animals travelled significantly more distance in the center than the PS groups during the two days of testing [13,021.96 ± 515.76 cm and 10,821.85 ± 515.76 cm, respectively; Prenatal Treatment: $F(1, 72) = 9.1$, $p \leq 0.01$], and the PN-M group exhibited increased center

distance travelled relative to the PS-M group and the PN-S and PS-S controls [Adult Treatment: $F(1, 72) = 367.3$, $p \leq 0.001$; Prenatal Treatment × Adult Treatment: $F(1, 72) = 9.3$, $p \leq 0.01$; data not shown]. Neither Prenatal Treatment nor Adult Treatment interacted with the factor of day for this measure, indicating that the enhanced sensitization effect observed in PN animals during initiation was also present during the test of expression; however, the magnitude of the sensitized effect did not change over the 14-day withdrawal period. There were no main effects or interactions with the factor of sex.

3.2.6 Post-sensitization Baseline—On the final day of locomotor testing, all groups were injected with saline and activity was measured for 60 minutes. Consistent with the first saline baseline measure, the 2 × 2 × 2 ANOVA conducted for horizontal activity revealed that females were more active than males [Sex: $F(1, 72) = 13.8$, $p \leq 0.001$; data not shown]. Rats previously injected with METH were significantly more active than rats that received saline throughout the initiation of sensitization phase [Adult Treatment: $F(1, 72) = 74.2$, $p \leq 0.001$; data not shown].

The center distance data are presented in Figure 3. The 2 × 2 × 2 ANOVA revealed that the PN animals travelled significantly more than the PS animals [Prenatal Treatment: $F(1, 72) = 4.9$, $p \leq 0.05$; Figure 3A] and that rats treated repeatedly with METH exhibited more center distance travelled compared to rats injected with saline [Adult Treatment: $F(1, 72) = 74.17$, $p \leq 0.001$]. Moreover, the interaction of Prenatal Treatment × Adult Treatment, which is shown in Figure 3B, indicates that the PN-M rats exhibited more activity than the PS-M rats and the PN-S and PS-S controls [$F(1, 72) = 4.4$, $p \leq 0.05$].

3.3 Prenatal nicotine exposure and METH treatment, separately or in combination, impacts BDNF protein levels in the mesocorticolimbic dopamine system

3.3.1 BDNF protein levels in the NAc, dST, and PFC—Analysis of NAc BNDF indicates increased levels of BDNF protein in PN animals relative to PS animals [Prenatal Treatment: $F(1, 65) = 8.1$, $p \leq 0.01$; Figure 4A], and rats administered METH, regardless of prenatal treatment, exhibited increased BDNF levels compared to those treated with saline vehicle [Adult Treatment: $F(1, 65) = 4.8$, $p \leq 0.05$; Figure 4B]. There were no main effects or interactions with the factor of Sex. The Prenatal Treatment × Adult Treatment interaction indicates that METH treatment during adulthood increased BDNF levels in rats prenatally treated with IV nicotine [$F(1, 65) = 5.2$, $p \leq 0.05$; Figure 4C].

Animals exposed to PN had significantly elevated levels of BDNF in the dSTR, relative to PS rats [Prenatal Treatment: $F(1, 64) = 5.2$, $p \leq 0.05$; Figure 4D]. Animals treated with METH showed a reduction of BDNF levels in the dSTR compared to saline-injected rats [Adult Treatment: $F(1, 64) = 10.3$, $p \leq 0.01$; Figure 4E]. There were no main effects or interactions with the factor of Sex. The Prenatal Treatment × Adult Treatment interaction suggests that the PN-induced effect of increasing striatal BDNF levels was resolved if animals received METH treatment in adulthood [$F(1, 64) = 5.2$, $p \leq 0.05$ Figure 4F].

Analysis of the PFC revealed that PN exposure [Prenatal Treatment: $F(1, 64) = 5.4$, $p < 0.05$; Figure 4G] and METH injections [Adult Treatment: $F(1, 64) = 8.1$, $p \leq 0.01$; Figure 4H] produced decreases of BDNF protein levels in this region. There were no main effects or

interactions with the factor of Sex. The Prenatal Treatment × Adult Treatment interaction was not significant. Figure 4I shows the parallel, downward shift in PFC BDNF levels as a function of PN and METH exposure.

3.3.2 Relationship between BDNF levels and conditioned locomotor activity—

The potential relationship between BDNF and locomotor activity was investigated using logistic regression. The center distance measure from the context-conditioned hyperactivity assessment on the final day of behavioral testing was used as the to-be-predicted behavior in the regression analysis. Data from the final day of testing was chosen because it represented the most proximal behavioral point in relation to collection of the brain tissue. Two separate regression analyses were conducted for PN and PS animals on the predicted center distance scores (derived from the regression equation) and the observed scores. A trimmed mean analysis was used for the correlation data, removing the potential bias of the lowest and highest scores from each group: the total number of data points used for each group was PN ($n = 28$) and PS ($n = 29$). Figure 5 shows the regression lines for each group. The correlation for PN [$r = 0.67$, $p \leq 0.05$] and PS [$r = 0.42$, $p \leq 0.05$] animals were both significant, but the strength of the association appeared relatively greater for the PN animals. Collectively, these analyses establish a predictive relationship between BDNF levels and locomotor activity, and also demonstrate that this relationship varied as a function of gestational exposure to nicotine.

4. Discussion

The present experiment assessed the influence of PN on the induction and expression of sensitization to METH, with both behavioral and neurochemical measures, on the functional integrity of the mesocorticolimbic system of adult offspring. The animals received no post-partum nicotine exposure and METH was not administered until the locomotor experiment began in adulthood, which was ~PND 100. The factors of Prenatal Treatment (PN vs PS) and Adult Treatment (METH vs Saline) altered locomotor behavior and BDNF protein levels relative to controls, and these factors interacted to further modulate locomotor activity and BDNF protein levels throughout the mesocorticolimbic system. The IV PN exposure method did not produce deficits in maternal weight or any of the postnatal milestones (e.g., litter composition, eye opening, negative geotaxis, righting reflex; [17,22,55,56] indicating that changes in adult offsprings' behavior and neurochemistry relative to controls is due to PN exposure. Surrogate fostering further restricted the developmental nicotine exposure to the prenatal period.

Habituation was delayed in PN treated rats relative to controls. Initially, the PN exposure group exhibited more spontaneous horizontal activity than the PS rats, and on the third day of testing, both groups showed similar levels of activity, suggesting comparable levels of habituation. The enhanced activity exhibited by IV PN offspring is consistent with previous experiments reporting that PN, administered through various routes of administration, produced hyperactive rodent offspring of various ages [5,14,19,57–60]. Notably, there are also reports of hypoactivity in PN-exposed offspring [61–63], or no differences in activity between PN and PS exposed animals [17,64–66]. The findings of the present experiment

demonstrate that adult PN offspring exhibit greater spontaneous hyperactivity and also habituate to the contextual cues at a slower rate than PS controls.

Following acute METH (0.3 mg/kg) injection, animals in the PN group, however, showed an enhanced response to METH, relative to PS rats. This finding provides support for the hypothesis proposed by Kandel et al., [10], which states that PN exposure renders organisms more sensitive to the effects of abused drugs at later stages of development. Further support for this hypothesis has also been reported in experiments showing that IV PN offspring self-administered lower doses of IV METH compared to PS rats when fixed-ratio schedules of reinforcement were used [55]. Interestingly, experiments investigating the effect of IV and continuous PN on adolescent offspring report no differences between PN and PS rats in the acute effects of METH [17] or cocaine [64] on locomotor activity, respectively. The latter findings with adolescents suggest that the effects of PN on neural development are manifest differently during the period of adolescence and adulthood. A developmental study, which investigates both adolescents and adults from the same PN and PS litters, is needed to determine the relative effects of PN at these two ages. Nonetheless, in the present experiments, acute exposure to METH produced a greater behavioral response in adult PN rats.

Repeated METH exposure produced behavioral sensitization of horizontal activity and center distance travelled in PN and PS animals; however, the magnitude of behavioral sensitization was not different between PN- and PS-exposed rats during the induction phase, despite PN's enhancement of METH-induced activity. That is, the relative percent increase in horizontal activity and center distance travelled from the acute exposure to that of the final day of sensitization testing was similar between the PN and PS rats. Thus, although PN rats exhibited increased sensitivity to the hyperactive effects of METH, they did not show a greater magnitude of behavioral sensitization relative to controls.

Regarding the expression of METH sensitization, which was tested 14 days after the final injection of the induction phase, the magnitude of the sensitized horizontal activity response did not differ as a function of Prenatal Treatment. Animals exposed to PN showed greater distance travelled in the center relative to PS controls, and moreover, animals in the PN-M group exhibited more distance travelled than any other group, according to the Prenatal Treatment × Adult Treatment interaction. A comparison of the final day of induction and the expression test was also conducted to determine if the magnitude of the sensitized response changed over the 14-day rest period, and the performance on the expression day was not more or less than that on the final day of induction, for either measure.

These findings differ from previous studies that investigated PN and behavioral sensitization in offspring. For example, a greater magnitude of nicotine-induced sensitization was reported in adult offspring if the dose of gestational nicotine was 5.0, but not 2.0 mg/kg/day [67]. In a different study, adolescent female PN offspring exhibited more cocaine-induced activity than PS controls following five daily injections [15 mg/kg; 64]. The most obvious difference between the present study and these experiments are different routes of PN administration. The continuous delivery of nicotine via the osmotic minipump may produce neurodevelopmental changes that potentiate the magnitude of behavioral sensitization

acquired by animals prenatally exposed to nicotine. Although 3×/day IV PN (0.05 mg/kg/injection) did not enhance behavioral sensitization, it did yield offspring that exhibited enhanced spontaneous locomotor activity as well as METH-induced hyperactivity. Testing a higher dose of IV PN may increase the likelihood that offspring in this model will develop a greater magnitude of behavioral sensitization to METH or other psychostimulant drugs.

A second baseline test, with injection of saline vehicle following the expression test day, was used to assess if animals exhibited METH-induced conditioned hyperactivity [38,39]. There are three notable findings from this test-day. First, the controls (e.g., PN-S and PS-S) exhibited a stable baseline of horizontal activity and center distance travelled on the first and second assessments. Second, offspring previously treated with METH (PN-M and PS-M) exhibited increased horizontal activity and center distance travelled compared to controls during the saline baseline 2 assessment. Third, animals prenatally exposed to nicotine and adult METH treatment (PN-M) exhibited the most center distance travelled during the day of testing. These results are consistent with a Pavlovian conditioning interpretation. The defining characteristic of Pavlovian learning is that the conditional stimulus (CS) elicits a conditional response from the animal when the stimulus is presented alone, in the absence of the unconditional stimulus (US) [68]. The form of the CR may be the same or the opposite of the unconditional response (UR) produced by the drug [38,47,69]. In the present experiment, exposure to the locomotor activity chambers (i.e., the CS) elicited increased activity and center distance travelled (CR) in the absence of METH (i.e., the US). Animals that were injected with saline throughout the experiment for control purposes (PN-S and PS-S) did not exhibit the CR. Overall, PN rats exhibited an enhanced magnitude of conditioned responding, which indicates that drug conditioning can be enhanced in animals that were prenatally exposed to nicotine. This finding is of importance because conditional cues that are acquired during drug exposures are hypothesized to influence drug seeking behavior, particularly during periods of abstinence [28]. In this regard, a prediction based on the present findings is that the offspring of maternal tobacco smoke exposure will acquire a greater magnitude of conditioned responding when the US is elicited by METH, or possibly other amphetamines. Given that a CS may serve as a strong motivating cue when experienced during a period of abstinence, further research that determines why animals prenatally exposed to nicotine also acquire relatively greater magnitudes of Pavlovian conditioned responding will be important.

Overall, the behavioral experiments demonstrate that PN exposure results in offspring that exhibit altered spontaneous locomotor activity, enhanced locomotor effects of acute METH, and a greater magnitude of METH-induced conditioned hyperactivity during adulthood.

The second experiment determined if BDNF protein levels in the mesocorticolimbic system differed between animals treated with nicotine during gestation (PN-S), rats treated with METH during adulthood (PS-M), and those that received exposure to both compounds (PN-M). PN and METH exposure each modulated BDNF protein levels in NAc, dSTR, and PFC, and the direction of change was different in each region tested. BDNF protein levels in the NAc were higher in PN-S and PS-M animals, and most interestingly, the combination of both treatments produced an overall increase in NAc BDNF protein. In the dSTR, PN treatment alone increased BDNF, but METH produced a decrease in the protein.

Interestingly, METH exposure reversed the effect of PN to increase dSTR BDNF levels in the PN-M animals. And both treatments reduced BDNF protein levels in the PFC, and the PN-M offspring exhibited the lowest levels of BDNF protein relative to the other groups. These data add to a growing literature on the role of BDNF in drug abuse. Particularly, increased BDNF levels in the NAc have been shown to be associated with increased drug abuse [seen mostly clearly in the PN-M group; 70]. While increased cortical BDNF may serve to protect against drug abuse; BDNF in the PFC was effectively reduced by METH and suggests greater susceptibility to illicit compounds in those groups [34].

The present findings are in support of previous research showing that continuous or IV PN altered BDNF in mesocorticolimbic regions of adolescent offspring. Our laboratory previously determined if IV PN- or PS-exposed rats that were repeatedly administered METH during adolescence, also exhibited changes in BDNF protein levels. We reported that IV PN exposure increased BDNF levels in the NAc, striatum, and PFC; however, the PN-induced increase in BDNF in the PFC was attenuated by adolescent methamphetamine treatment [17]. Using a mouse model of continuous PN exposure, Wei et al., [16] also demonstrated that adolescent offspring of PN exposure exhibited modulation of BDNF mRNA in the NAc and striatum of adolescent mice. Taken together, these experiments demonstrate that PN exposure, administered via two different routes of administration, produce enduring effects on protein levels of BDNF in the mesocorticolimbic system and thus, changes plasticity in the dopamine system which could have important implications on responses to rewarding stimuli.

These findings also have important implications for understanding how maternal smoking may modulate motivational states in the offspring. First, BDNF is integral in the formation and maintenance of mesocorticolimbic DA neurons [29,30] and is important for the expression of NAc DA D_3 receptors [32], which are known to play a key role in drug self-administration [71]. The long-lasting PN-induced changes in BDNF thus suggest functional alterations to the dopamine system that is critical to motivated behavior. To date, no studies have investigated BDNF-mediated changes in the function of mesocorticolimbic neurons using PN-induced offspring. The hypothesis that PN-induced changes in BDNF underlie changes in motivation and behavior is particularly relevant given that adult PN rats, derived from different nicotine exposure models, exhibit alterations in drug-maintained responding [15,55,56,72]. For example, adult rats exposed to IV PN exhibit increased sensitivity to detect IV METH when a FR schedule was used to define an inverted U-shaped dose response curve [55]. The reinforcing efficacy of sucrose and METH are greater in IV PN exposed animals compared to controls [22,56]. Likewise, two studies using continuous PN exposure have shown that adolescent rats exhibited more fixed-ratio responding for nicotine following a period of forced abstinence [72], and self-administered more cocaine on a fixed-ratio schedule than PS controls [15]. These findings indicate that PN exposure alters drug-maintained responding at various ages of offspring development.

Determining if alterations in BDNF levels functionally change goal-directed behavior in PN animals will be important in order to understand whether changes in this neurotrophic factor contribute to the increased vulnerability for drug abuse documented in individuals exposed to maternal tobacco smoke *in utero*. The findings of this experiment provide a foundation for

a novel hypothesis that PN-induced changes in mesocorticolimbic BDNF contribute to changes in the reinforcing efficacy of METH [56]. Preliminary support for this hypothesis is provided by the subsequent multivariate analysis of the BDNF and the Saline Baseline 2 data. Overall, the BDNF protein levels in the mesocorticolimbic system significantly predicted activity in both the PN and PS animals, regardless of prior experience; however, BDNF protein was a better predictor of the behavior for PN animals relative to PS offspring. This finding indicates that BDNF protein levels, obtained 24-h after the saline baseline 2 test, accounts for a significant amount of variance in the conditioned behavior. This predictive relationship indicates that PN-mediated changes in BDNF contribute to the enhanced Pavlovian conditioning observed when METH was repeatedly administered in the locomotor activity chambers.

Finally, there were several sex differences observed in the behavioral assessments of this study. The sex differences reported here are in accord with previous reports showing that females exhibited more spontaneous and stimulant-induced activity than males [53,73,74]. The sex difference was observed for the measure of horizontal activity from the habituation, initiation, expression and baseline test days. The factor of sex, however, did not interact with PN treatment on any of these test days, which indicates that the locomotor behavior of females and males was not differentially modulated by *in utero* nicotine exposure.

In conclusion, the present data add to a growing number of reports that substantiate the significant impact of PN exposure on neurodevelopmental outcomes in offspring. It is important to note that the overall amount of nicotine exposure in the present IV PN model is significantly lower than other contemporary methods. Specifically, the methods used in the present study expose pregnant dams to a total of 0.15 mg/kg/day. Other models, such as the OMP and oral exposure, can subject animals to 2+ mg/kg/day. The IV model is particularly relevant given the similarities in pharmacokinetic profile between IV injection and tobacco smoke inhalation. One experiment that would be important to assess using the IV model would be aimed at characterizing a dose-response relationship between IV nicotine and potential neurodevelopmental deficits. Lastly, the data from this study and others utilizing the IV model support the notion that pregnant mothers who smoke even a reduced number of cigarettes are imparting enduring, neurodevelopmental effects that may increase the liability for drug abuse in offspring.

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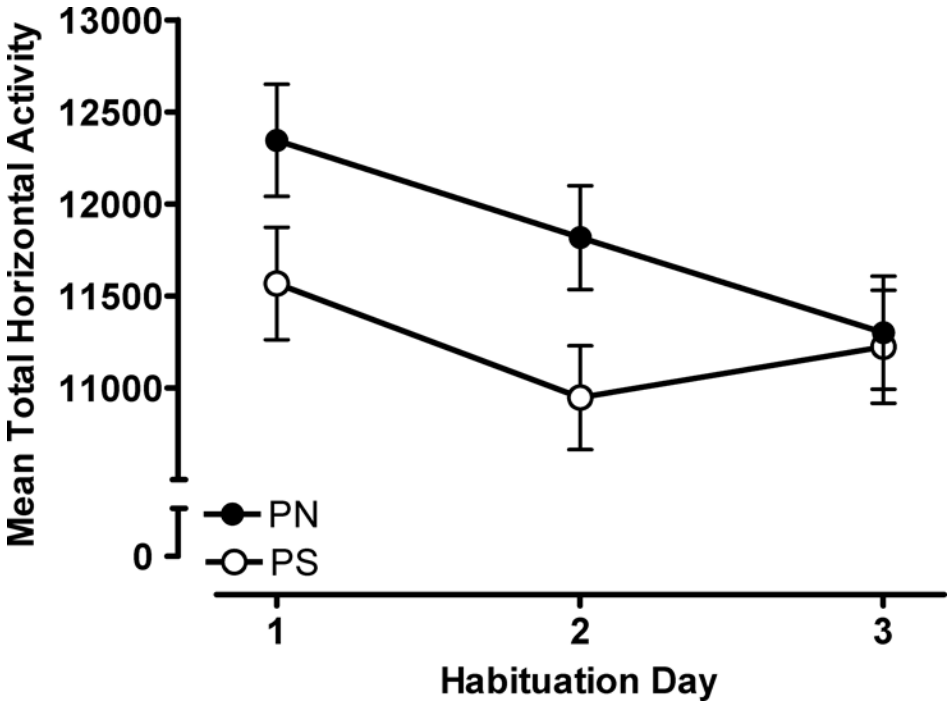

Figure 1.

The mean total horizontal activity (± SEM) across three days of habituation data is presented by treatment group. The analyses revealed a significant interaction of Day × Prenatal Treatment with PN animals exhibiting more locomotor activity than PS animals.

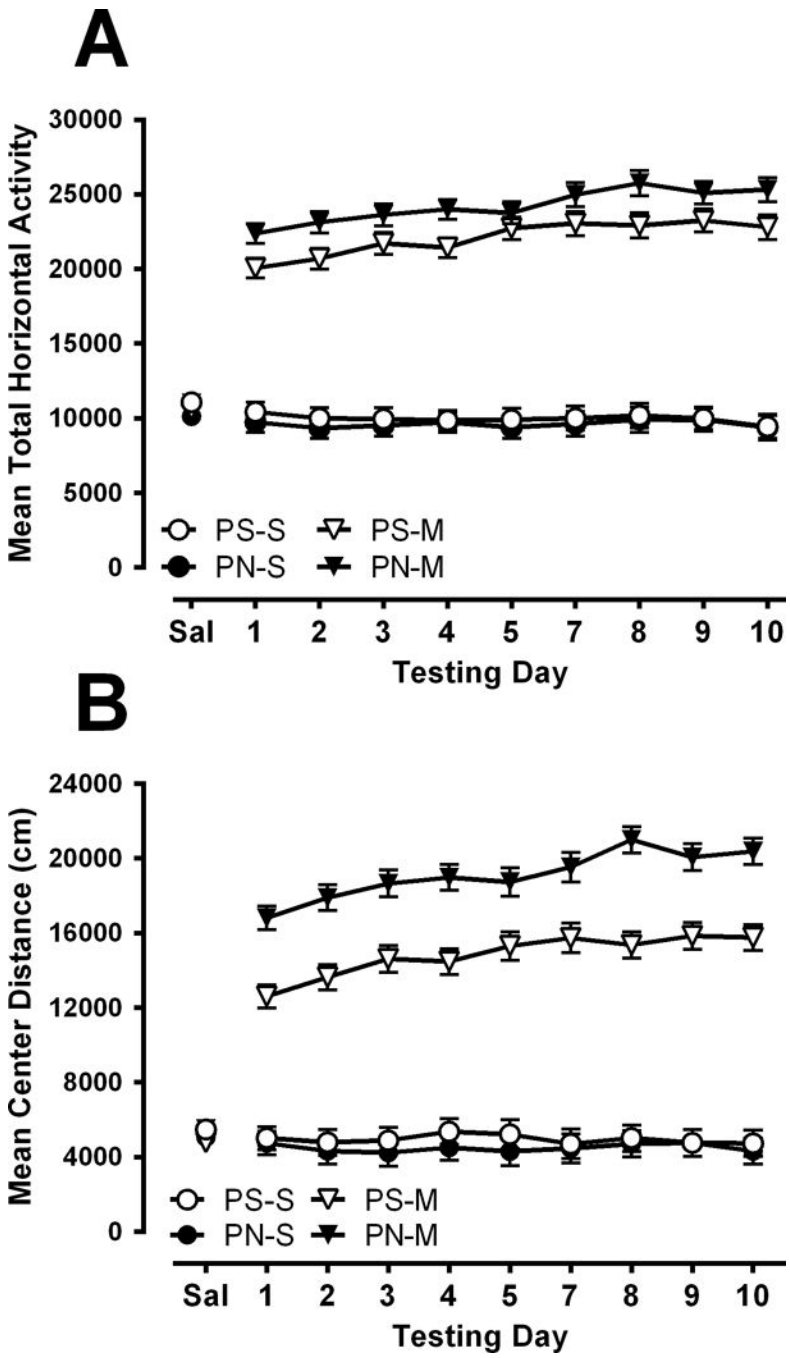

Figure 2.

(A) The mean total horizontal activity data (± SEM) are presented by Prenatal Treatment and Adult Treatment for saline baseline and sensitization testing days. The interaction of Prenatal Treatment × Adult Treatment approached significance suggesting increased responsivity to METH in PN-exposed animals. (B) The mean center distance (± SEM) data are presented by Prenatal Treatment and Adult Treatment for saline baseline and sensitization testing days. Analysis revealed a significant main effect of Prenatal Treatment

and a significant interaction of Prenatal Treatment × Adult Treatment; PN and METH exposure increased center distance travelled.

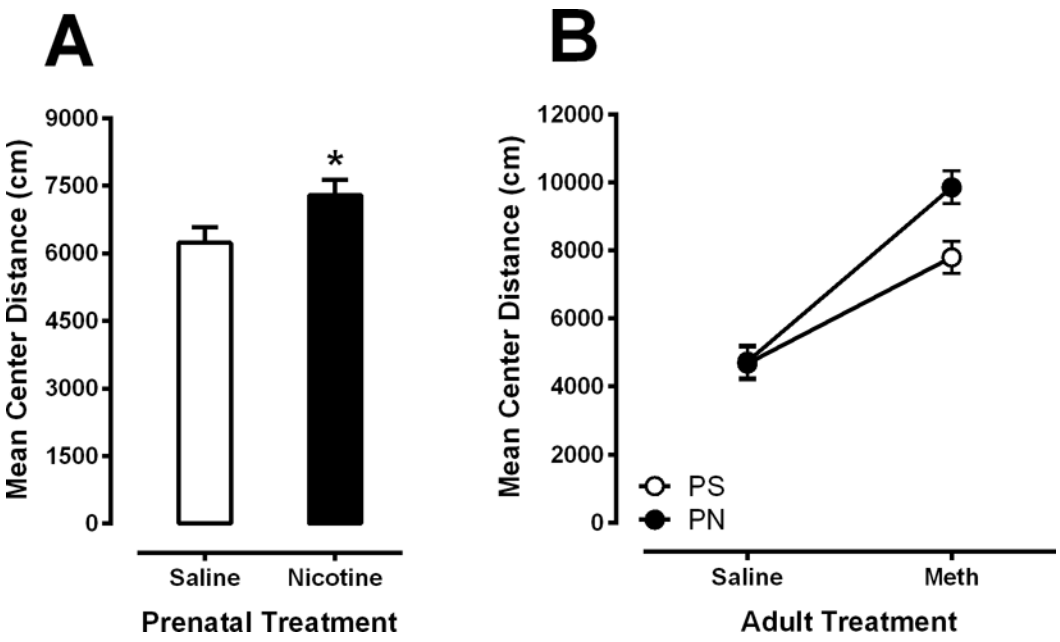

Figure 3.

(A) The significant main effect of Prenatal Treatment on center distance travelled is presented as a function of gestational treatment on the test of context conditioning: PN animals travelled significantly further than PS animals indicating differential context conditioning. (B) The significant interaction of Prenatal Treatment × Adult Treatment is presented for the test of context-conditioned hyperactivity. The results indicate that PN-M animals travelled farther in the central zone than PS-M. [* < 0.05]

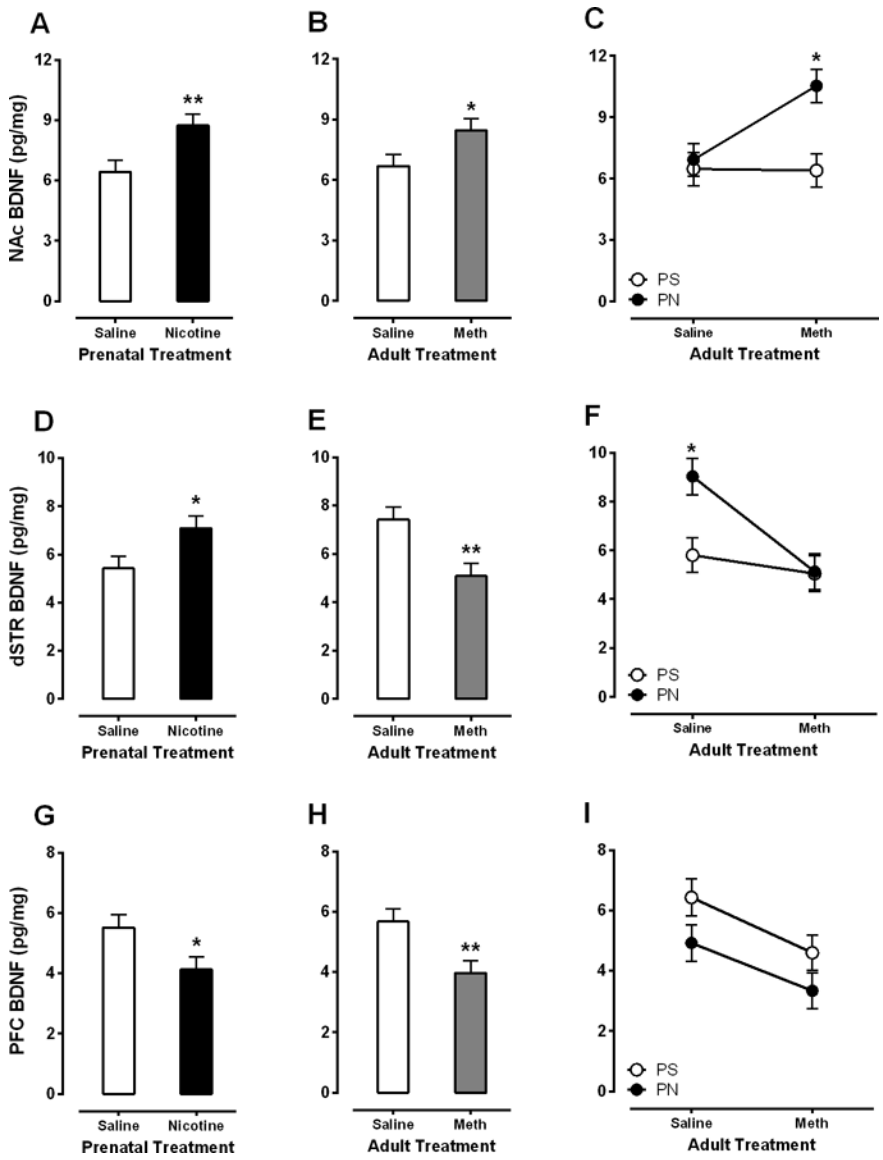

Figure 4.

The main effect of Prenatal Treatment, Adult Treatment, and the interaction of Prenatal Treatment × Adult Treatment for NAc BDNF protein levels are shown in panels A, B, and C, respectively. The main effect of Prenatal Treatment, Adult Treatment, and the interaction of Prenatal Treatment × Adult Treatment for striatal BDNF levels are shown in panels D, E, and F, respectively. The main effect of Prenatal Treatment, Adult Treatment, and the Prenatal Treatment × Adult Treatment data for PFC BDNF levels are shown in panels G, H, and I respectively. The interaction of was not significant [$p > 0.05$] for BDNF levels in the PFC. [* < 0.05; ** < 0.01].

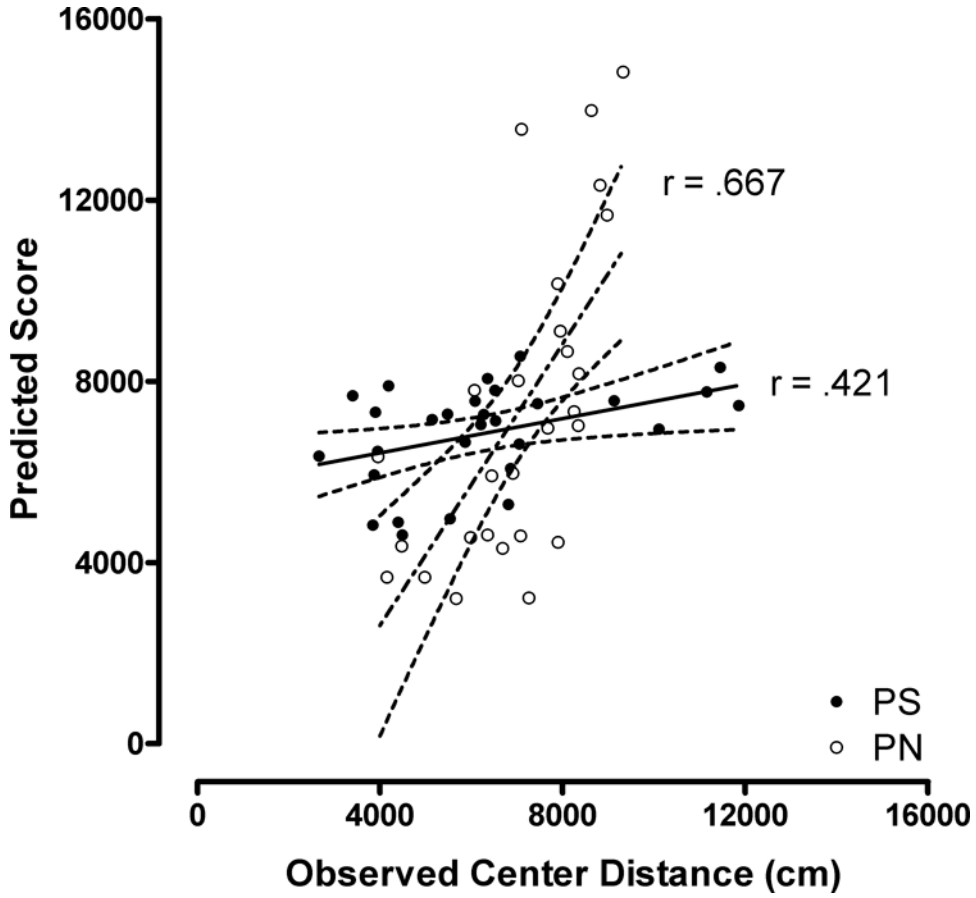

Figure 5.

The multiple regression analysis of BDNF levels and context-conditioned center distance scores are presented by gestational treatment. Analyses revealed a significant correlation for both PN (hashed line) and PS animals (solid line) between observed center distance and predicted center distance based on BDNF levels.

Table 1

Dam and pup weight gain, litter composition, and developmental milestones

Dam Weight Gain, g			Pups, n			Pup Weight Gain, g		
GD	**PS**	**PN**	**Sex**	**PS**	**PN**	**PND**	**PS**	**PN**
7	21.33±1.57	23.01±1.75	Male	4.31±0.49	4.61±0.54	7	8.66±0.13	8.66±0.15
14	47.06±2.46	41.75±2.73	Female	5.18±0.41	4.77±0.45	14	22.90±0.20	22.90±0.23
21	130.46±4.98	122.04±5.52	Total	9.50±0.68	9.38±0.76	21	42.38±0.40	42.85±0.43
Righting Reflex, s			**Negative Geotaxis, s**			**Eye Opening (0–3 ratings)**		
PND	**PS**	**PN**	**PND**	**PS**	**PN**	**PND**	**PS**	**PN**
3	5.30±0.47	4.76±0.52	8	7.91±0.69	9.85±0.76	13	0	0
4	3.41±0.32	2.73±0.36	9	7.45±0.46	6.94±0.51	14	0.14±0.04	0.24±0.04
5	2.16±0.13	1.74±0.15	10	5.76±0.67	6.68±0.74	15	1.11±0.06	1.16±0.07
						16	2.33±0.05	2.38±0.05
						17	2.92±0.02	2.87±0.03

Litter parameter data (mean ±SEM) from all dams and pups used in the present study. None of the analyses were significant, which suggests no gross alterations or delays of postnatal development in PN-exposed animals. The first data points on the Dam and Pup weight gain tables are 0 because these were the weights from which weight gain was calculated and thus are not included. Abbreviations: GD-gestational day; PN-prenatal nicotine; PS-prenatal saline; PND-postnatal day.

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