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Sex-Specific Programming of Offspring Emotionality Following Stress Early in Pregnancy

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Abstract

Prenatal stress is associated with an increased vulnerability to neurodevelopmental disorders, including autism and schizophrenia. To determine the critical time window when fetal antecedents may induce a disease predisposition, we examined behavioral responses in offspring exposed to stress during early, mid, and late gestation. We found that **male offspring exposed to stress early in gestation displayed maladaptive behavioral stress-responsivity, anhedonia, and an increased sensitivity to SSRI treatment.** Long-term alterations in central corticotropin-releasing factor (CRF) and glucocorticoid receptor (GR) expression, as well as increased HPA axis responsivity were present in these mice and likely contributed to an **elevated stress-sensitivity.** Changes in CRF and GR gene methylation correlated with altered gene expression, providing important evidence of **epigenetic programming during early prenatal stress.** In addition, we found the core mechanism underlying male vulnerability may involve sex-specific placenta responsivity, where stress early in pregnancy significantly increased expression of PPAR α , IGFBP-1, HIF3 α , and GLUT4 in male placentas but not females. Examination of placental epigenetic machinery revealed basal sex-differences, providing further evidence that sex-specific programming begins very early in pregnancy, and may contribute to the timing and vulnerability of the developing fetus to maternal perturbations. Overall, these results indicate that stress experience early in pregnancy may contribute to male neurodevelopmental disorders through impacts on placental function and fetal development.

Keywords

Placenta; Neurodevelopment; DNA methylation; Prenatal stress; Depression; Affective disorders

Introduction

Stress experience during gestation is associated with an increased incidence of numerous neuropsychiatric disorders, including depression, anxiety, schizophrenia, and autism (J. I. Koenig et al., 2002; E. J. Nestler et al., 2002; A. Gillott and P. J. Standen, 2007; E. Walker, 2007). The mechanism through which fetal antecedents contribute to disease development is not known, though likely involves a complex interaction between environment and genetics. As many of the diseases associated with prenatal stress exhibit a sex bias in presentation, determining how sex-specific susceptibility arises may provide critical mechanistic insight, leading to the identification of novel targets for therapeutic development.

While prenatal stress has been broadly associated with offspring disease, the developing nervous system is unlikely to show uniform vulnerability to perturbations over the course of gestation. We have recently established that the influence of prenatal stress on hippocampal

dependent learning and memory was specific to the timing of stress exposure and sex of the offspring (B. R. Mueller and T. L. Bale, 2006, 2007a). In addition, a recent study revealed an association between maternal stress experience during the first trimester and an increased risk of schizophrenia in males (A. S. Khashan et al., 2008). Therefore, we hypothesized that temporal specificity may contribute to the increased incidence of disease in males exposed to prenatal stress.

Although the specific mechanism whereby maternal stress contributes to disease risk remains unclear, several key contributors have been suggested involving changes in the maternal hormonal milieu. Elevated glucocorticoids *in utero* can alter early life 'programming' (Seckl, 2000; Meaney, 2004). However, the placenta inactivates a significant percentage of maternal glucocorticoids via 11 β -hydroxysteroid dehydrogenase type 2 (11 β HSD2), protecting the fetus through the late stages of pregnancy (Seckl, 2004). Despite the placenta's critical role in regulating the exchange of hormones, nutrients, and waste products between the maternal and fetal circulatory systems, very little is known regarding either the effects of maternal stress or the influence of fetal sex on placental function.

One potential mechanism whereby stress can influence fetal development and programming is through epigenetics, including DNA methylation (J. R. Seckl and M. J. Meaney, 2004; I. C. Weaver et al., 2004b; N. Tsankova et al., 2007). While recent emphasis has been placed on the involvement of epigenetic machinery in the programming of early life events, the influence of fetal antecedents on the epigenome remains unknown (M. J. Meaney et al., 2007). Central stress components, corticotrophin-releasing factor (CRF) and the glucocorticoid receptor, have been previously shown to be regulated by methylation and programmed early in life (I. C. Weaver et al., 2004b; B. E. McGill et al., 2006). Serotonin neurotransmission and disease development are associated with stress pathway dysregulation (R. J. Valentino and A. L. Curtis, 1991; E. J. Nestler et al., 2002). As such, we hypothesized that fetal antecedant-mediated alterations in programming of these circuits may be an underlying factor in neurodevelopmental disorders.

Materials and Methods

Animals

Mice used in these studies were on a mixed C57Bl/6:129 background. Virgin female mice (n = 62) were mated at 6–8 weeks of age. Presence of a copulation plug denoted gestation day 1. The pregnant female was individually housed, given a cotton nestlet, and assigned to a stress treatment group (see below).

All mice were housed under a 12-h light/dark cycle (lights on at 7:00 am) with ambient temperature of 22° C, and relative humidity of 42%. Food (Purina Rodent Chow; 28.1% protein, 59.8% carbohydrate, 12.1% fat) and water was provided throughout the study *ad libitum*. All studies were done according to experimental protocols approved by the University of Pennsylvania Institutional Animal Care and Use Committee.

Prenatal Stress (PS)

Administration of chronic, variable stress was performed as previously described (B. R. Mueller and T. L. Bale, 2006). Briefly, dams were randomly assigned to treatment groups to receive stress during one of the three weeks, or to a control non-stressed group. Pregnant mice assigned to the stress groups experienced a different stressor on each of seven days during early (days 1–7), mid (days 8–14), or late (days 15–21) gestation. Chronic variable stressors included: 36 hrs of constant light, 15 minutes (1000h) of fox odor exposure (1:5000 2,4,5-Trimethylthiazole; Acros Organics) exposure, novel object (8 marbles of similar shape and

color) exposure overnight, 5 min restraint stress (1000 – 1100 h) in a 50 mL conical tube, novel noise (White Noise/Nature Sound-Sleep Machine®, Brookstone) overnight, multiple cage changes throughout the light cycle, and saturated bedding (700 mL, 23° C water) overnight. These mild stressors were selected to be non-habituating and for not inducing pain or directly influencing maternal food intake or weight gain (B. R. Mueller and T. L. Bale, 2006).

Establishment of sex and temporal specificity of prenatal stress on behavioral stress coping responses

Offspring—In order to examine the sex and gestational timing vulnerability to the influence of prenatal stress, we compared the behavioral stress response of male and female adult offspring that were exposed to maternal stress during early, mid, and late gestation. At birth, litters were culled to 8 pups and litters containing fewer than 6 pups were excluded from analysis. During postnatal week 4 (postnatal days 25 – 27) pups were weaned, group housed with same sex littermates, and ear-tagged to provide a method of permanent identification. From 10–16 weeks of age, 1–2 males and 1–2 females per litter were tested in behavioral assays of emotionality. The performance of same-sex siblings was averaged to avoid litter effects (male control = 5 animals / 4 litters, female control = 4 animals / 4 litters, male early stress = 8 animals / 6 litters, female early stress = 7 animals / 6 litters, male mid stress = 6 animals / 4 litters, female mid stress = 6 animals / 4 litters, male late stress = 7 / 4 litters, female late stress = 6 animals / 4 litters). These offspring were tested in the tail suspension, forced swim, and open field test, as described below, with a 10 day recovery period between tests.

Behavioral Analysis

Tail Suspension Test: To examine maladaptive behaviors of adult offspring, mice were examined using the tail suspension test (TST) as previously described (L. Steru et al., 1985). Mice are secured to a suspended glass rod by adhesive tape placed approximately 1 cm from the tip of the tail and are suspended 50 cm from the bench-top within a visually isolated area. Immobility time, defined as lack of all movement except for whisker movement and respiration, was recorded throughout a 6-min test session

Forced Swim Test: Mice were tested in a modified version of the forced swim test (FST) as previously described (I. Lucki, 1997; J. F. Cryan et al., 2005). All animals were placed in the cylinder for a pre-swim for 5 min on day 1 and then monitored during a 5 min test 24 hr after the pre-swim (J. F. Cryan and I. Lucki, 2000). Time spent swimming, climbing, and immobile was determined by an investigator blind to treatment and sex. Immobility was defined as time spent still or only using righting movements to remain afloat. Swimming was defined as any movement horizontal in nature that involved at least two limbs. Climbing was defined as any vertical movement in which the bottom of the front paws touched the sides of the cylinder.

Open-Field Test: Mice were exposed to the open field test during the dark cycle one week following completion of the FST to determine the effect of PS on anxiety-like behavior and locomotor activity. The open-field apparatus consisted of a white Plexiglas box 50 × 50 × 22 cm with sixteen 12 × 12-cm squares drawn on the floor. Testing was conducted with 120 lux in the center of the box, 2–4 hours following lights out (9pm – 12pm). Each mouse was placed in the center of the box to initiate the 5-min test. Mice were scored for total line crossings and center crosses by an investigator blind to treatment group. As treatment with anxiolytic drugs increases crosses into center squares, performance in the open field test is a validated measure of anxiety-like behavior (T. L. Bale et al., 2000).

Mechanistic determination of the influence of early prenatal stress on male offspring

As male offspring stressed early in gestation showed a significant increase in maladaptive behaviors in the TST and FST, we examined anhedonic sensitivity in a sucrose preference test. To assess possible mechanisms contributing to these sex-specific long-term outcomes, comparisons were made by placental PCR array and epigenetic analyses.

Sucrose-Preference Test—Tail Suspension and Forced Swim Tests revealed early prenatal stress (E-PS) produced a depressive-like phenotype in adult male offspring. To confirm these results, E-PS adult males were examined in a sucrose preference test, a measure of anhedonia, a core symptom of clinical depression (J. L. Moreau, 1997).

8 wk old E-PS and control male offspring ($n = 16$) were housed in a single cage, received standard chow *ad libitum*, and were initially trained to consume water in the two-bottle-choice paradigm for 3 days to habituate the mice to the free choice. A bottle of water was then replaced by a bottle of 1% sucrose solution during weeks 1 and 2 of sucrose access. During week 3, sucrose concentration was increased to 5%. During weeks 4 and 5, concentration was increased to 10% (J. N. Weatherly and E. I. Arthur, 2006). The bottles were reversed twice weekly to control for side preference. Fluid intake was measured daily and replaced weekly. The results of sucrose consumption were expressed as a preference ratio for sucrose. Preference ratios were calculated as the percentage of the volume of sucrose consumed (per 24 h) divided by the total fluid intake (per 24 h). Food intake, water intake, and body weight were monitored throughout the sucrose-preference test.

Sucrose Stress Test—At the conclusion of sucrose preference (day 36), sucrose was removed for 24 hours in E-PS and control offspring. 2-hour and 24-hour sucrose intake was compared between animals exposed to an acute predator odor stressor (1:5000 2,4,5-Trimethylthiazole; Fischer Scientific), or animals that were left undisturbed ($n = 4$). A similar restriction-stress paradigm has been previously shown to induce binge-like behavior in rats (M. M. Hagan et al., 2003). Animals received standard chow and water *ad libitum* during the sucrose restriction period. Food and water intake was monitored during sucrose restriction and the 24 hours following sucrose return. To control for restriction, a second experiment was conducted comparing 2-hour and 24-hour sucrose intake between animals exposed to a 15-min restraint stress and animals that were left undisturbed ($n = 4$). These animals were sacrificed following completion of the study. No tissue was saved.

SSRI Treatment—To determine if the maladaptive stress responsive behaviors found in EPS males were responsive to an acute selective serotonin reuptake inhibitor (SSRI), citalopram was injected 30 min prior to testing on the tail suspension test ($n = 8$), as previously described (J. J. Crowley et al., 2005). All mice were behaviorally naïve. The drug was dissolved in 0.9% saline and injected at two doses: 2.5 mg/kg and 10mg/kg in a volume of 100ul. Vehicle injection was 100 ul of 0.9% saline. These animals were sacrificed following completion of the study. No tissue was saved.

Adult Brain Tissue Collection—To examine biochemical and molecular changes within the brain of adult males exposed to early prenatal stress, behaviorally naïve E-PS and control adult male offspring were sacrificed at 4 months of age ($n = 4 - 5$). Brains were removed and divided sagittally into two hemispheres. For all of our gene expression and autoradiographical analyses, tissue was obtained from or analyzed in the same hemisphere across all animals. For autoradiography and in situ hybridization studies, measurements for CRF and GR were from brain tissue sections from the left hemisphere. For real-time PCR studies, micropunches were made from tissue from the right hemisphere. For DNA methylation studies, bilateral

micropunches were obtained for DNA isolation. For adult western blot analysis, cortical tissue was taken from the right hemisphere. All brain tissue was stored at -80°C .

5-HT Transporter Autoradiography—Assessment of serotonin transporter (SERT) in E-PS and control males was performed with ^3H -Citalopram autoradiographic methods. Fresh frozen, atlas-matched brain sections were selected from control and E-PS mice ($n = 4 - 5$). Brain sections (20 μm) were incubated in Tris buffer (pH 7.6) at 37°C for 10 minutes. Sections were then incubated in buffer containing ^3H -citalopram (1.4 nM, Perkin Elmer) for 1 hour at 37°C and washed in cold ($0 - 4^{\circ}\text{C}$) incubation buffer (2×10 min). Finally, slides were rinsed in cold deionized distilled water, and dried under a stream of cold air. Brain sections were apposed to Kodak Hyperfilm (Eastman Kodak, Rochester, NY) for 21 days. Film images representing atlas-matched tissue sections were selected for analysis. Region of interest outlines were designed for standardized analysis of dorsal hippocampus, lateral septum, and hypothalamus as determined by atlas reference (Paxinos and Franklin 2001). Optical density was measured within each region of interest and averaged after subtracting background film density. Images were captured using a 10-bit cooled QICam digital camera (QImaging, British Columbia). Optical density measurements were made by IPLabs for Macintosh software (BD Biosciences/Scanalytics, Rockville, MD).

Dorsal Raphe TPH2, SERT and 5HT1A Expression Analysis—Sections through the dorsal raphe -4.04 to -4.96 mm caudal to Bregma were cut at a thickness of 300 μm (F. K. Paxinos G, 2001). The location of micropunched region corresponds to Figures 64–72 of the Paxinos and Franklin mouse atlas. The area of the dorsal raphe was determined using the dorsal hippocampus and cerebral aqueduct as landmarks. Micropunches with a diameter of 1 mm were then taken using a hollow needle (Ted Pella, Redding, CA). RNA was isolated using RNeasy (Qiagen, Valencia, California) and was resuspended in RNase free water and concentration measured on a spectrophotometer (Eppendorf AG, Hamburg, Germany). cDNA was transcribed using the SuperScript First-Strand Synthesis System for RT-PCR with random primers (Invitrogen, Calsbad, California). Changes in serotonin receptor 1A (5Htr1a; NM_008308.4); *Mm00434106_s1*; tryptophan hydroxylase (Tph2; NM_173391.2); *Mm00557717_m1*, and serotonin transporter (*Slc6a4*; NM_010484.1); *Mm00439391_m1* were measured using quantitative real-time PCR and TaqMan Gene Expression Assay (Applied Biosystems, Forster City, CA). In addition, β -actin (*Actb*; NM_007393.3); *Mm00607939_s1* was also measured for each sample as an endogenous control and the cycle threshold subtracted from the target threshold value. All samples and controls were prepared in duplicate wells of a 96-well plate and analyzed using the Applied Biosystems 7500 Fast Real-Time PCR System (Applied Biosystems). The cycle number at threshold (CT value) was used for calculations of relative amount of mRNA molecules. The CT value of each target gene was normalized by subtraction of the CT value from β -actin. This value is defined as the ΔCT . Relative quantitative change was calculated using the formula: $2^{-(\Delta\text{CT E-PS} - \Delta\text{CT control})}$ ($n = 4 - 5$).

Corticotropin-releasing Factor (CRF) and Glucocorticoid Receptor (GR) in situ hybridization—Expression of genes important in stress neurocircuitry was measured by *in situ* hybridization. E-PS and control males were sacrificed 2 weeks following last behavior test. Brains were cut on a cryostat at 20- μm coronal sections and mounted on slides (SuperFrost Plus, Fisher Scientific, Hampton, New Hampshire). Sections were post-fixed in 4% paraformaldehyde and processed as previously described (B. S. Teegarden SL, 2007). Atlas-matched tissue was hybridized with ^3S -UTP-labeled antisense GR (generous gift from Dr. Audrey Seasholtz, University of Michigan, Ann Arbor, Michigan) or CRF probes as previously described (T. L. Bale et al., 2000). Slides were incubated overnight at 55°C , washed at 65°C , dehydrated in ethanol and air-dried.

For silver grain analyses, slides were dipped in NTB liquid nuclear emulsion (Eastman Kodak) for 17 days (GR) and 21 days (CRF). Slides were developed, counter-stained with hematoxylin (Fisher), and coverslipped. The level of expression was quantified by analyzing optical density using IPLab version 3.9 imaging system for Macintosh Computer (Scanalytics, Rockville, Maryland). Under bright-field illumination, appropriate regions of interest were drawn as determined by atlas reference (F. K. Paxinos G, 2001). Brain sections were anatomically matched prior to analyses. GR expression within CA1, CA2, CA3, and DG were analyzed within the same brain section in four adjacent sections and averaged for each animal prior to statistical analysis ($n = 4 - 5$). CeA CRF expression was analyzed in two adjacent sections and averaged for each animal ($n = 4 - 5$). The same region-of-interest tool was used for each section. Background was subtracted from each density measurement by using a region just outside each tissue section. Microscopic light intensity was held constant in all analyses. Measurements were completed by an investigator blinded to treatment group ($n = 4 - 5$).

HPA Axis Stress Response—To determine if E-PS males showed altered HPA axis response to stress, corticosterone levels in 10-week old adult male offspring ($n = 6$) following a 15-min restraint were compared. Restraint was conducted in a 50-ml conical tube. Tail blood was collected immediately prior to stress start (0 min), immediately following the stress exposure (15 min), and 15 and 45 min following end of stress (30 and 60 min respectively). The blood collection procedure lasted less than 1 min. Samples were immediately centrifuged and stored at -80°C until the assay was conducted. Corticosterone levels were determined by radioimmune assay (ICN Biomedicals). ($n = 6$). The intraassay coefficient of variation was less than 5%.

Analysis of DNA Methylation of CRF, GR, and BDNF—Methylation status of specific CpG dinucleotides within the promoter region of mouse corticotropin-releasing factor (CRF) (B. E. McGill et al., 2006), glucocorticoid receptor type II (GR) (I. C. Weaver et al., 2004b) and brain-derived neurotrophic factor (BDNF) were analyzed (B. E. McGill et al., 2006) ($n = 4 - 5$). Genomic DNA from the hypothalamus and central nucleus of the amygdala of E-PS and control males were extracted from cell pellets with the DNeasy Tissue kit (Qiagen, Valencia, CA), according to the manufacturer's instructions. Pyrosequencing was performed by Epigendx, as previously described (H. R. Kim et al., 2007; T. Liu et al., 2007). Universally methylated and unmethylated DNA samples (Chemicon International, Temecula, CA) were used as controls in these experiments.

Placenta Tissue Collection—To determine placental gene expression profiles following prenatal stress, dams exposed to stress during the first 7 days of gestation and dams left undisturbed were sacrificed 12 days following presence of copulation plug. This time point was chosen because at embryonic day 12 placenta formation is complete. Embryo tissue was saved for DNA isolation and placentas were immediately frozen in liquid nitrogen for RNA isolation. As previously described, RT-PCR using primers specific for Sry (5'-GAGTACAGGTGTGCAGCTCTA-3' and 5'-CAGCCCTACAGCCACATGAT-3') were used to identify sex of individual embryos (T. Zwingman et al., 1993). Thermal cycling conditions were 98°C for 30 seconds; 54°C for 40 seconds; 72°C for 50 seconds for 30 cycles.

Placenta RNA Isolation—From each litter, one placenta from a male embryo, and one placenta from a female embryo were added to TRIzol reagent (Invitrogen) and briefly sonicated prior to total isolation with chloroform and precipitation with isopropanol. RNA was resuspended in RNase free water and concentration measured on a spectrophotometer (Eppendorf AG, Hamburg, Germany.)

PCR Array Analysis of Placental Nutrient and Oxygen Transport Gene

Expression—750 ng of RNA was transcribed to cDNA using the RT² first strand synthesis kit (C-03, Bioscience Corp, Fredrick, MD) and analyzed using the mouse hypoxia signaling pathway PCR array (PAMM.032, Bioscience Corp, Fredrick, MD) and the RT² SYBR green PCR master mix (Bioscience Corp). Each array consists of 84 genes known to be involved in the hypoxic response, cell differentiation, and metabolism, as well as 12 sequences to control for loading and cDNA quality (PUC18 plasmid DNA as negative control; actin, PPIA, and glyceraldehydes 3 phosphate dehydrogenase (GAPDH) for loading). The cycle number at threshold (CT value) was used for calculations of relative amount of mRNA molecules. The CT value of each target gene was normalized by subtraction of the CT value from average of 5 housekeeping genes. This value is defined as the Δ CT. Relative quantitative change was calculated using the formula: $2^{-(\Delta\text{CT E-PS} - \Delta\text{CT control})}$ (n = 4).

Placental Inflammation and Epigenetic Machinery Expression Analysis

750 ng of RNA was transcribed to cDNA using the SuperScript® III First-Strand Synthesis System reagent (Invitrogen, Calsbad, CA). Changes in tumor necrosis factor- α (TNF α ; NM_013693.2): *Mm99999068_m1*, interleukin-6 (IL-6; NM_031168.1): *Mm01210733_m1*, DNA methyl transferase 1 (DNMT1; NM_0107883.3): *Mm01151062_g1*, DNA methyl transferase 3a (DNMT3a; NM_007872.4): *Mm00432870_m1*, and methyl-CpG-binding protein-2 (MeCP2; NM_010788.3): *Mm00465017_m1* were assessed using TaqMan Gene Expression Assay (Applied Biosystems, Forster City, CA) (n = 4 – 5). In addition, β -actin (*Actb*; NM_007393.3): *Mm00607939_s1* was measured for each sample as an endogenous control and the cycle threshold subtracted from the target threshold value. All samples and controls were prepared in duplicate wells of a 96-well plate and analyzed using the Applied Biosystems 7500 Fast Real-Time PCR System (Applied Biosystems). Data was normalized to β -actin and analyzed by Δ CT based fold-change calculations taken from raw threshold cycle data, as described above.

Western Blot Analysis—As a gross assessment of possible sex-specific changes in brain development following prenatal stress early in gestation, levels of neurofilament-1 (NF-1) were compared between E-PS and control, male and female pups on the morning (0900–1000 h) of postnatal day 1 (the day on which a litter was present). One male and one female pup were sacrificed and whole brain rapidly removed and frozen. In adult offspring (4 mo old), 500 mg of tissue encompassing the right prefrontal cortex was excised with the aid of the mouse brain atlas reference (Paxinos and Franklin 2001) (approximately bregma 4.20 - 3.20 mm) and frozen in liquid nitrogen. Biochemical analyses were performed as described elsewhere (T. L. Bale et al., 2003). Briefly, tissue was homogenized and protein extracted. All protein was run at 30 μ g per lane. Antibodies used were neurofilament-1 (150 kD, 1:1000) (Chemicon International, Temecula, California), and vascular endothelial growth factor (42 kD, 1:1000) (Santa Cruz Biotechnology, Santa Cruz, California). All blots were stripped and re-incubated with bactin (Sigma, St. Louis, Missouri) to control for any transfer or sample-loading errors. Densitometric analyses were performed using IPLab (BD Biosciences/Scanalytics, Rockville, MD). A region of interest was drawn around one band on the film, and an identical region was used to measure densities for each subsequent band on that blot. Raw data comparisons were made only within each blot.

Statistical Analysis—All studies were conducted by an investigator blind to sex and prenatal stress (PS) treatment groups. To determine sex and temporal specific influence of PS, data were analyzed using two-way ANOVA for offspring sex and PS. For the sucrose preference and corticosterone studies, data was analyzed with day as a repeated-measures factor. A two-way ANOVA for PS and acute stress exposure determined significance of sucrose-stress test data. The effect of citalopram on offspring behavior in the tail suspension

test was analyzed by a two-way ANOVA for PS and drug treatment. Bonferroni multiple comparisons test determined post hoc to control for litter effects. For biochemical, molecular, and methylation studies, one male and female per litter were randomly chosen for analysis and Fisher's PLSD post hoc test determined influence of PS. For PCR array data, genes displaying ± 2 fold changes were analyzed by a two-way ANOVA for sex and PS to determine main effects. A Fisher's PLSD post hoc test with a reduced p value of 0.01 was used to control for multiple comparisons. Fold changes were computed relative to unstressed controls for male and female placentas. All statistical analysis was performed using StatView SE+ (Abacus Concepts, Berkeley, CA).

Results

Behavioral Analyses

To determine potential temporal and sex-specific effects of prenatal stress (PS) on adult offspring depression- and anxiety-like behaviors, male and female offspring stressed during early (E), mid (M), late (L) gestation, or unstressed controls (C) were examined for behavioral stress responses in the tail suspension test (TST), forced swim test (FST), and open-field test. There was a significant main effect of prenatal stress (PS) ($F_{(3,33)} = 3.9$, $p < 0.05$, Fig. 1A) and a significant interaction between sex and PS treatment ($F_{(3,33)} = 2.95$, $p < 0.05$, Fig. 1A) on time spent immobile in the TST. Males exposed to PS early in gestation (E-PS) spent significantly more time immobile compared to control males ($p < 0.01$, Fig. 1A). No significant effect of PS was evident in females ($F_{(3,17)} = 0.8$, $p = 0.49$).

In the FST, there was a significant main effect of PS on time spent immobile ($F_{(3,26)} = 5.2$, $p < 0.05$) and a significant interaction between PS and sex ($F_{(3,26)} = 2.8$, $p < 0.05$). E-PS male offspring spent significantly more time immobile than control males ($p < 0.01$; Fig. 1B). In this test, the increased time spent immobile resulted from a significant decrease in time spent climbing ($F_{(3,26)} = 4.25$, $p < 0.01$, Fig. 1C) and a trend for decreased time spent swimming ($F_{(3,26)} = 3.1$, $p = 0.06$, Fig. 1D) indicating a sex-specific effect of early PS on the catecholamine and serotonin neurotransmitter systems. No significant effect of PS on time spent immobile was present in female offspring ($F_{(3,13)} = 0.7$, $p = 0.51$, Fig. 1B).

Analysis of center crossings in the open field test revealed no effect of PS ($F_{(3,31)} = 0.14$, $p = 0.92$) or sex ($F_{(1,31)} = 1.1$, $p = 0.31$, Fig. 1E). To determine if TST and FST results were affected by changes in locomotor ability, total line crosses during the open field test were measured. There were no significant effects of PS treatment ($F_{(3,31)} = .164$, $p = 0.03$, Fig. 1F) or sex ($F_{(1,31)} = 1.3$, $p = 0.27$, Fig. 1F).

Mechanistic determination of the influence of early prenatal stress on male offspring

Sucrose Preference Test—To determine if maladaptive stress-coping behaviors displayed by E-PS males in the TST and FST reflect a depressive-like phenotype, the hedonic state of these offspring was assessed in a sucrose preference task. E-PS males displayed a decreased intake of 1% sucrose compared to control male offspring ($F_{(1,179)} = 8.2$, $p < 0.05$, Fig. 2A). Increasing sucrose concentration to 5% or 10% ameliorated the diminished preference of E-PS males ($F_{(1,179)} = 1.5$, $p > 0.85$, Fig. 2A).

Body weight, food intake, and water consumption were monitored weekly during sucrose preference testing (Table 1). Prenatal stress did not significantly affect body weight ($F_{(1,168)} = 3.5$, $p = 0.05$, Table 1a), food ($F_{(1,168)} = 1.3$, $p > 0.05$, Table 1b), or water ($F_{(1,168)} = 1.2$, $p > 0.05$ Table 1c) intake. Over the five week period, body weights for both groups increased compared to start weights ($F_{(5,168)} = 13.5$, $p < 0.05$, Table 1a), while food ($F_{(5,168)} = 18.5$, $p < 0.05$, Table 1b), and water ($F_{(5,168)} = 2.4$, $p < 0.05$, Table 1c) intake decreased.

Sucrose Stress Test—To assess the effect of E-PS and acute stress on reward seeking behavior, sucrose was removed for 24 hours. Mice were exposed to an acute stressor or left undisturbed prior to sucrose return. Sucrose intake was measured over 2 and 24 hours. There was a significant main effect of acute stress exposure ($F_{(1,27)} = 13.2$, $p < 0.05$, Fig. 2B) and an interaction between E-PS and acute stress exposure on 2-hour sucrose intake ($F_{(1,27)} = 5.3$, $p < 0.05$, Fig. 2B) and 24-hour intake ($F_{(1,27)} = 4.1$, $p < 0.05$, Fig. 2C) following overnight sucrose restriction. During the 2 hours following acute stress, E-PS males consumed significantly more sucrose than control males ($p < 0.05$, Fig. 2B) and undisturbed E-PS males ($p < 0.05$, Fig. 2B). Over 24 hours, intake of stress exposed E-PS males remained significantly elevated compared to controls ($p < 0.05$, Fig. 2C).

To control for the effects of stress during overnight sucrose restriction, we compared intake 2 hours and 24 hours following acute stress exposure under continuous access conditions. E-PS ($F_{2 \text{ hr } (1,27)} = 1.2$, $p = 0.3$; $F_{24 \text{ hr } (1,27)} = 0.9$, $p = 0.5$) and acute stress ($F_{2 \text{ hr } (1,27)} = 1.8$, $p = 0.3$; $F_{24 \text{ hr } (1,27)} = 0.9$, $p = 0.5$) did not influence 2-hour (Fig. 2D) and 24-hour sucrose intake (Fig. 2E).

SSRI Treatment—As E-PS males exhibit depression-like behaviors in the TST, FST, and sucrose tests, we compared behavioral responses to an acute SSRI. A main effect of E-PS was present ($F_{(1,29)} = 4.3$, $p < 0.05$) indicating vehicle treated E-PS males spent significantly more time immobile than vehicle treated controls ($p < 0.01$, Fig. 3A). There was also a main effect of citalopram treatment on time spent immobile ($F_{(2,29)} = 3.9$, $p < 0.05$, Fig. 3A). Acute administration of 2.5 mg/kg of citalopram 30 minutes before testing ameliorated the significant difference in time spent immobile between vehicle treated E-PS and control males ($p = 0.7$, Fig. 3A), while administration of 10 mg/kg significantly decreased time spent immobile for control and E-PS mice ($p < 0.05$, Fig. 3A). A main effect of citalopram ($F_{(2,29)} = 8.2$, $p < 0.05$, Fig. 3B) and a significant interaction between citalopram and E-PS ($F_{(2,29)} = 4.4$, $p < 0.05$, Fig. 3B) influenced number of immobility bouts. Vehicle treated E-PS males exhibited significantly more bouts of immobility than vehicle treated controls ($p < 0.01$, Fig. 3B). Acute SSRI administration at 2.5 mg/kg significantly decreased bouts of immobility displayed by E-PS males ($p < 0.05$, Fig. 3B), but did not affect control males ($p = 0.8$, Fig. 3B). Administration of 10 mg/kg significantly decreased bouts of immobility for both control and E-PS males ($p < 0.05$, Fig. 3B). Latency to first bout of immobility was also measured. There was a main effect of E-PS ($F_{(2,29)} = 15.5$, $p < 0.05$, Fig. 3C) and a significant main effect of citalopram treatment ($F_{(2,29)} = 4.4$, $p < 0.05$, Fig. 3C) on latency to first bout of immobility. Supporting our previous findings, vehicle treated E-PS males displayed a shorter latency to the first bout of immobility ($p < 0.01$, Fig. 3C). For E-PS males, both 2.5 mg/kg of citalopram ($p < 0.05$, Fig. 3C) and 10 mg/kg significantly increased latency to first bout of immobility ($p < 0.05$, Fig. 3B). For control males, there was no effect of 2.5 mg/kg of citalopram ($p = 0.3$, Fig. 3C) or 10 mg/kg of citalopram ($p = 0.2$, Fig. 3C) on latency to first bout of immobility.

Serotonin Transporter Autoradiography—To examine possible effects of E-PS on serotonin transporter (SERT) levels that may be contributing to the increased SSRI sensitivity in males, SERT levels were measured by autoradiography. E-PS males showed a significant reduction in SERT levels in the CA1 ($F_{(1,7)} = 25.6$, $p < 0.05$) and CA2 ($F_{(1,7)} = 7.6$, $p = 0.04$, Fig. 4A,B) region of the hippocampus compared to control males. No differences were present in the hypothalamus ($F_{(1,7)} = 0.8$, $p = 0.4$), lateral septum ($F_{(1,7)} = 2.7$, $p = 0.16$), or CA3 regions of the hippocampus ($F_{(1,7)} = 2.0$, $p = 0.2$).

Dorsal Raphe TPH2, SERT and 5HT1A Expression Analysis—As the dorsal raphe provides serotonergic input to the hippocampus, expression levels of tryptophan hydroxylase-2 (TPH2), serotonin 1a receptor (5HT1A), and serotonin transporter (SERT) were measured on dorsal raphe punches by quantitative real-time PCR. Gene expression analysis showed an

apparent increase in tryptophan hydroxylase-2 (TPH2) expression ($F_{(1,7)} = 5.7$, $p = 0.06$, Fig. 4B). No differences in SERT ($F_{(1,7)} = 0.9$, $p = 0.4$, Fig. 4C) or 5-HT1A ($F_{(1,7)} = 0.3$, $p = 0.7$, Fig. 4D) expression were present.

CRF In Situ Hybridization—As stress responsivity can be regulated by CRF, we examined CRF expression by in situ hybridization. In the central nucleus of the amygdala, E-PS males showing a significant elevation in expression compared to controls ($p < 0.01$, Fig. 5A,B).

HPA Axis Stress Response—As increased stress sensitivity is a core component of affective disorders, corticosterone levels were compared between E-PS and control adult male offspring following a 15-min stress exposure. Early prenatal stress treatment ($F_{(1,18)} = 9.2$, $p < 0.05$) and 15-min stress exposure ($F_{(3,20)} = 14.2$, $p < 0.05$) significantly altered corticosterone levels. E-PS did not influence baseline corticosterone levels. E-PS males have significantly elevated corticosterone levels following 15 minutes of restraint (time 15, $p < 0.05$), and 15 minutes following stress end (time 30 min, $p < 0.05$, Fig. 5C). The recovery of E-PS males appeared to be delayed, with corticosterone levels 45 minutes after stress end remaining elevated ($p = 0.07$, Fig. 5C).

GR In Situ Hybridization—E-PS significantly decreased glucocorticoid receptor (GR) expression in the CA3 ($p < 0.05$, Fig. 5D,E) and dentate gyrus (DG) regions of the hippocampus ($p < 0.05$, Fig. 5D,F). A non-significant reduction was also seen in CA1 following early prenatal stress ($p = 0.06$, Fig. 5D,G). No differences were detected in the CA2 region ($p = 0.4$, Fig. 5D,G).

Analysis of DNA Methylation of CRF, GR, and BDNF genes—As previous studies have shown that early life experience can alter the epigenome, we investigated if changes in methylation were driving altered expression of CRF and GR (Weaver, 2004a). In the hypothalamus, cytosine-methylation analysis within the CRF promoter revealed main effects of base pair position ($F_{(8,51)} = 44.25$, $p < 0.05$) and early prenatal stress exposure ($F_{(1,51)} = 4.35$, $p < 0.05$) with reduced methylation in E-PS males ($p < 0.05$, Fig. 6A). No differences in methylation between prenatal stress treatments were evident within analyzed region of the CRF intron ($F_{(1,51)} = 1.35$, $p = 0.4$, Fig. 6B). Analysis of methylation levels within the exon 1₇ region of the GR promoter revealed significant main effects of base pair position ($F_{(8,51)} = 10.5$, $p < 0.05$) and E-PS ($F_{(1,51)} = 4.35$, $p < 0.05$) with increased methylation in E-PS males ($p < 0.05$, Fig. 6C). No effect of E-PS was evident in the promoter region of brain derived neurotrophic factor (BDNF) ($F_{(1,51)} = 0.75$, $p > 0.7$, Fig. 6D). Similar to effects in hypothalamic tissue, in DNA isolated from the central nucleus of the amygdala, cytosine-methylation analysis within the CRF promoter revealed main effects of base pair position ($F_{(8,50)} = 34.5$, $p < 0.05$) and early prenatal stress exposure ($F_{(1,50)} = 3.65$, $p < 0.05$) with reduced methylation in E-PS males (−336 bp: Ctrl = 67.2 ± 2.8 , E-PS = 62.1 ± 2.5 ; −232 bp: Ctrl = 58.2 ± 3.3 , E-PS = 62.9 ± 4.8 ; −226 bp: Ctrl = 52.5 ± 2.4 , E-PS = 52.4 ± 3.8 ; −212 bp: Ctrl = 73.6 ± 3.1 , E-PS = 64.9 ± 5.4 ; −147 bp: Ctrl = 45.1 ± 7.0 , E-PS = 36.2 ± 5.49 ; −101 bp: Ctrl = 42.2 ± 8.0 , E-PS = 37.8 ± 4.98 ; −95 bp: Ctrl = 35.8 ± 9.6 , E-PS = 19.7 ± 3.25 ; −79 bp: Ctrl = 42.8 ± 9.0 , E-PS = 37.5 ± 5.3 ; −55 bp: Ctrl = 28.8 ± 3.6 , E-PS = 24.5 ± 1.3 ; −36 bp: Ctrl = 24.9 ± 2.6 , E-PS = 18.8 ± 2.6).

PCR Array Analysis of Placental Gene Expression—To determine the underlying mechanism driving the sex-specific effects of early prenatal stress, control and E-PS day 12 placentas of male and female embryos were examined using a focused PCR array. E-PS, offspring sex, and an interaction between offspring sex and E-PS significantly influenced placental expression of peroxisome proliferator-activated receptor alpha (PPAR α), insulin-like growth factor binding protein 1 (IGFBP-1), glucose transporter 4 (GLUT4), and hypoxia-inducible factor 3a (HIF3a) (Table 2). Compared to control male placentas, E-PS male

placentas showed significant *upregulation* of PPAR α (4.7-fold, $p < 0.01$, Fig. 7A), IGFBP-1 (8.1-fold, $p < 0.01$, Fig. 7A), GLUT4 (3.6-fold, $p < 0.01$, Fig. 7), and HIF3 α (2.9-fold, $p < 0.01$, Fig. 7A). In contrast, E-PS female placentas showed significant *downregulation* of PPAR α (3.2-fold, $p < 0.01$), and a trending downregulation of IGFBP-1 (4.7-fold, $p = 0.06$) compared to female control placentas. *For complete list of genes and fold changes, see supplementary tables 1 and 2.*

Placental TNF α and IL-6 Expression Analysis—Tumor necrosis factor-alpha (TNF α) expression was not altered by offspring sex ($p = 0.8$) or E-PS ($p = 0.13$, Fig. 7B). Interleukin-6 (IL-6) expression was not altered by sex ($p = 0.4$) or E-PS ($p = 0.07$, Fig. 7C).

Placental DNMT1, DNMT3a, and MeCP2 Expression Analysis—DNMT1 expression was altered by offspring sex ($F_{(1,11)} = 5.4$, $p < 0.05$) and E-PS ($F_{(1,11)} = 4.8$, $p < 0.05$). Control male placentas had significantly lower expression of DNMT1 than control female placentas ($p < 0.01$, Fig. 7D). Further, E-PS significantly increased DNMT1 expression in female placentas ($p < 0.01$, Fig. 7D), but not male placentas ($p = 0.15$, Fig. 7D). DNMT3a expression was not altered by sex ($p = 0.1$) or E-PS ($p = 0.1$, Fig. 7E). MeCP2 expression was not altered by sex ($p = 0.9$) or E-PS ($p = 0.2$, Fig. 7F).

Western Blot Analysis—As a gross assessment of possible sex-specific delays in brain development following prenatal stress early in gestation, levels of neurofilament-1 (NF-1) were compared in postnatal day 1 brains of E-PS male and female pups. Additionally, vascular endothelial growth factor (VEGF) was examined to determine the influence of prenatal stress on the developing vasculature. E-PS males showed a significant decrease in whole-brain NF-1 ($p < 0.05$, Fig. 8A). No effect of early prenatal stress was seen in female NF-1 levels ($p = 0.7$, Fig. 8B). No differences were found between groups at 4 mo in NF-1 in prefrontal cortex ($p = 0.8$, Fig. 8C). Early prenatal stress did not significantly influence postnatal day 1 male ($p = 0.7$, Fig. 8D) or female ($p = 0.9$, Fig. 8E) VEGF levels.

Discussion

The mortality and morbidity due to neurodevelopmental and mood disorders has remained largely unchanged over the last fifty years (T. R. Insel and E. M. Scolnick, 2006). Given the difficulty in treating these diseases, a cross-disciplinary approach that identifies the developmental origin of disease and vulnerable populations is needed to create novel prevention and treatment strategies. The predisposition toward sex-biased disorders, including schizophrenia, autism, and affective disorders has been associated with stress-related fetal antecedents (A. J. Ward, 1991; J. B. Watson et al., 1999; C. A. Pardo and C. G. Eberhart, 2007). However, the mechanism underlying this influence of prenatal stress (PS) remains unclear. In our studies, behavioral coping responses of male and female offspring exposed to stress across gestation were initially examined to determine if temporal specificity may underlie a sex-dependent vulnerability to prenatal stress.

Male offspring exposed to stress early in gestation (E-PS) exhibited **maladaptive behaviors** in both the tail suspension and forced swim tests. While female controls generally showed increased immobility when compared to males, prenatal stress did not further **alter stress coping responses**. Because of the existing sex-differences in these tests, a ceiling effect may have precluded prenatal stress from further altering female outcomes. Interestingly, our data correlate with the recent clinical findings that show **stress early in pregnancy increased the presence of schizophrenia in males**, supporting a sex-specific risk early in development (A. S. Khashan et al., 2008). While our previous studies have reported effects of stress during additional gestational time points on parameters of learning and memory and energy balance

regulation (B. R. Mueller and T. L. Bale, 2006, 2007b), our current results reveal a vulnerability of stress pathway programming specific to early pregnancy.

As the male E-PS increased immobility in the tail suspension and forced swim tests was suggestive of a **depression**-like phenotype, we also examined hedonic sensitivity in these mice using a sucrose preference test (J. L. Moreau, 1997). E-PS males exhibited a diminished preference for a 1% sucrose solution, suggesting a diminished basal sensitivity to hedonic rewards. However, an acute stress exposure induced E-PS males to consume 100% more sucrose in the two hours following a sucrose deprivation compared to continuous access mice. The majority of the difference between control and E-PS 24-hour sucrose consumption resulted from this two-hour intake, indicative of stress-induced bingeing responses (W. J. Lynch and J. R. Taylor, 2005; N. M. Avena et al., 2006).

Given the depression-like phenotype of E-PS males, behavioral responses to an acute SSRI, citalopram, were measured. E-PS males exhibited a greater sensitivity to a sub-threshold dose of citalopram in a tail suspension test. As this test measures active coping behaviors that are responsive to acute changes in synaptic serotonin (5-HT), the greater sensitivity detected in E-PS males may indicate a dysregulation in 5-HT pathways. Further examination of the 5-HT system in these mice revealed reduced 5-HT transporter levels in the hippocampus and a trend for increased tryptophan hydroxylase-2 expression in the dorsal raphe. Thus, potential increased 5-HT output and decreased reuptake may underlie the increased sensitivity to acute SSRI treatment in these mice.

Dysregulation of the serotonin system has been classically linked to the etiology of affective disorders. Corticotropin-releasing factor (CRF) is a known regulator of 5-HT neurotransmission and a key determinant of stress responsivity (R. J. Valentino et al., 1991; T. L. Bale and W. W. Vale, 2004; H. Tan et al., 2004). Therefore, dysregulation of CRF and stress pathway components could be an upstream contributor to abnormalities in 5-HT transmission and a stress-sensitive phenotype. In the CeA, E-PS males showed a significant increase in CRF expression. As an additional indication of increased stress responsiveness, E-PS males also exhibited higher corticosterone levels following an acute restraint stress, and decreased hippocampal glucocorticoid receptors (GR). While previous studies have reported similar changes in GR levels following stress experienced late in gestation, our results indicate an earlier developmental window during which stress pathways display a sex-specific vulnerability (M. S. Cratty et al., 1995; M. Darnaudery and S. Maccari, 2007).

The long-term impact of prenatal stress on regulation of central gene expression may occur via effects on the epigenome. In our examination of the methylation pattern of CRF, we report reductions in methylation at specific cytosines within the identified CRF promoter CpG island in both the hypothalamus and CeA that may contribute to the increased CRF detected in these mice. In our examination of the exon 1₇ region of the GR promoter, E-PS males showed a site-specific increase in methylation that occurred in the region containing the consensus sequence of NGFI-A where methylation status has previously been linked to early life experience and decreased expression (I. C. Weaver et al., 2004b; I. C. Weaver et al., 2004a).

As the fetal brain is not present during this period of early stress, we hypothesized that sex-specific changes in fetal development and long-term sensitivity may occur through actions on the placenta as it is derived from the developing blastocyst and undergoes critical development during this period of E-PS (M. Hemberger, 2007). The placenta is the intermediary by which the maternal hormonal milieu influences the developing embryo, and therefore is poised to be influenced by perturbations occurring early in pregnancy that could affect fetal development throughout pregnancy by altering 1) nutrient and oxygen transport, 2) inflammatory responses, and 3) epigenetic programming machinery. To examine this hypothesis, we examined effects

of E-PS on male and female placental gene expression patterns utilizing a focused PCR Array for growth factors and nutrient transport genes. E-PS male placentas exhibited significant *increases* in PPAR α , IGFBP-1, GLUT4, and HIF3 α . Surprisingly, E-PS female placentas showed a *reduction* in PPAR α and IGFBP-1 expression. As glucocorticoids increase expression of PPAR α (T. Lemberger et al., 1994), and PPAR α in turn increases expression of IGFBP-1 (T. Degenhardt et al., 2006), our results support a potential mechanism whereby maternal stress could directly affect placental gene expression patterns. Reductions in growth factors have been linked to affective and neurodevelopmental disorders (J. E. Malberg et al., 2007). Thus, an elevation in placental IGFBP-1, and a consequent decrease in available growth factors during critical developmental periods may play a role in male fetal programming.

Alterations in oxygen and nutrient availability have been associated with inflammation (T. Altay et al., 2004), and as recent studies have shown an important link between inflammatory events and the onset of major depression (R. Dantzer et al., 2008) additional comparisons of placental inflammatory markers were conducted. While no significant differences were detected for levels of TNF α and IL-6 at this time point, it is interesting that male placentas showed a directional change suggestive of possible inflammation following the E-PS that may be more robust at different pregnancy time points.

To further investigate potential mechanisms underlying the sex-specific effect of E-PS on placenta gene expression, we examined DNA methylation machinery in male and female placentas. Interestingly, expression of DNMT1, the enzyme responsible for methylation maintenance, was significantly lower in male control placentas as compared to female control placentas, providing support for our hypothesis that the influence of E-PS may begin at the level of the placenta (H. Leonhardt et al., 1992). In addition, while E-PS appeared to increase DNMT1 expression in both sexes, this change was only significant in females. As previous studies have shown that the maintenance of methylation patterns are critical in neurodevelopment, and perturbations in placental DNA methylation patterns are strong predictors of fetal changes, a stress-induced elevation of DNMT1 may indicate that females are able to circumvent the effects of stress by strengthening the maintenance of methylation during this perturbation (R. M. Rivera et al., 2008; H. Wang et al., 2008).

These changes in placental gene expression related to growth factors, nutrient transport, and epigenetics may impact the developing central nervous system. As a gross marker of brain development we compared neurofilament-1 (NF-1) levels from postnatal day 1 male and female offspring. E-PS males but not females showed a significant reduction in NF-1, supporting a possible delay in brain development. As neuronal abnormalities and delayed development have been associated with major depressive disorder and schizophrenia, such changes in central NF-1 may be predictive of future disease (A. Reines et al., 2004; J. J. Miguel-Hidalgo et al., 2005). No effects of E-PS were detected in NF-1 levels in adult males, indicating that E-PS males eventually catch up with control males.

In summary, as it is unlikely that any one specific gene produces the difference in behaviors detected between control and E-PS males, an array of genetic, hormonal, and epigenetic factors were examined in these studies. Our results support the hypothesis that E-PS dysregulation of CRF and stress pathway programming may be a key contributor to the stress-sensitive phenotype detected in these male mice, including the increased immobility in the tail suspension and forced swim tests, heightened HPA axis responsivity, and influences on the 5-HT system. These studies provide critical mechanistic insight regarding the underlying programming of sex-biased neurodevelopmental disorders.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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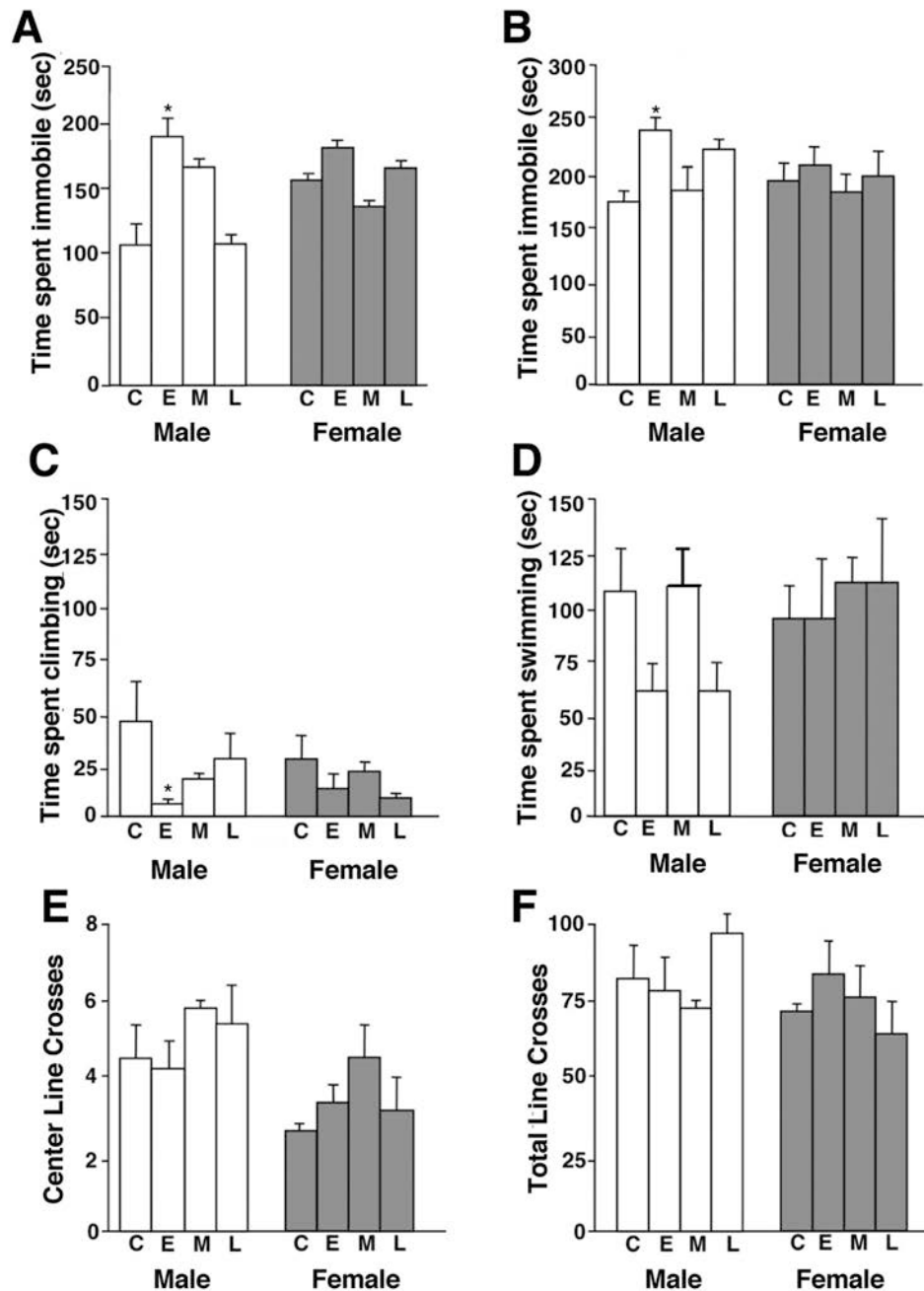


Fig. 1. E-PS males showed maladaptive behavioral stress responsivity in tail suspension and forced swim tests

Male and female offspring born to unstressed dams (control, C), or dams exposed to prenatal stress (PS) during early (E), mid (M), or late (L) gestation were compared. E-PS males spent significantly more time immobile in a (A) tail suspension test and a (B) forced swim test compared to control males (*, $p < 0.05$). (C) E-PS males spent significantly less time climbing compared to control males in the forced swim test (*, $p < 0.05$). (D) No significant differences in time spent swimming were detected between control and prenatally stressed male or female offspring. No differences were found in an open-field test for (E) crosses into the center arena or (F) total number of crossed lines. Data are mean $\pm$ SEM. $n = 4 - 6$.

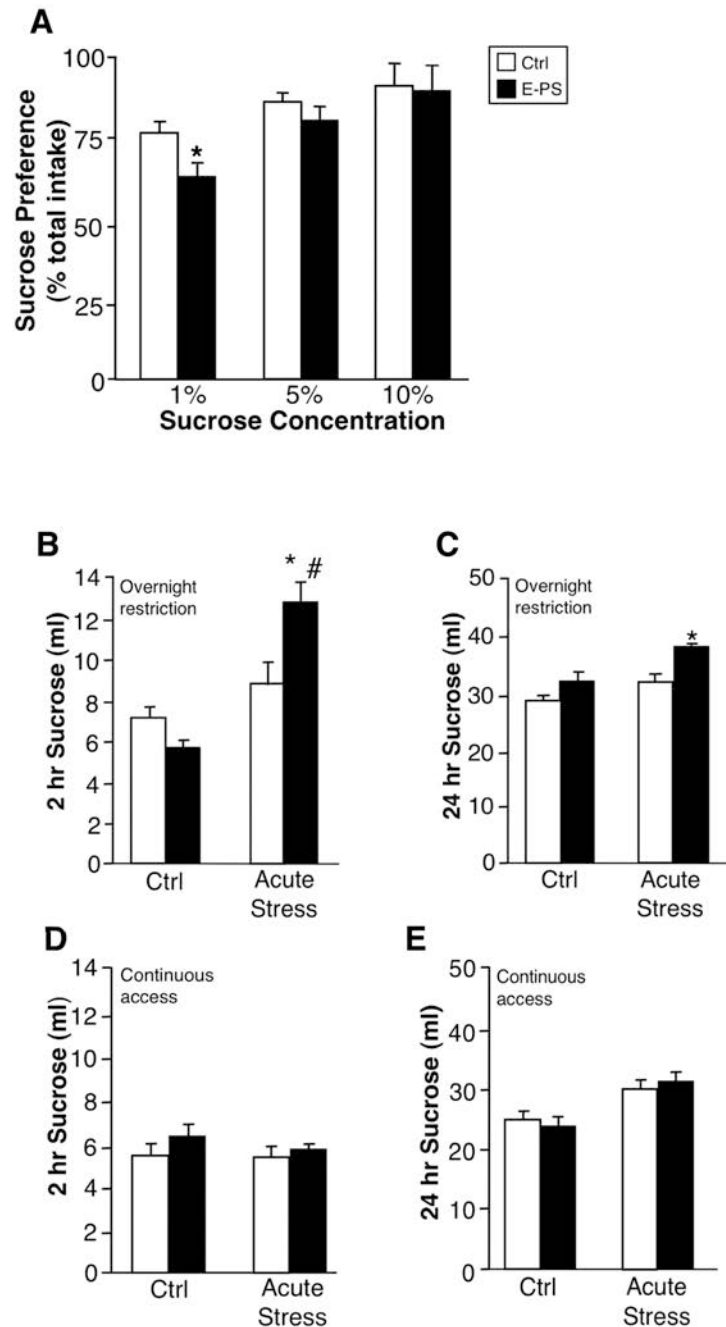


Fig. 2. E-PS males showed decreased basal hedonic behavior and increased stress-induced sucrose binging

Anhedonia of E-PS males was examined in a sucrose preference test. **A)** E-PS males displayed a significant decrease in sucrose preference at a low concentration when compared to control (ctrl, $n = 16$) male offspring (*, $p < 0.05$) ($n = 16$). **B)** 2 hrs and **(C)** 24 hrs following an acute stress E-PS males consumed significantly more 10% sucrose than control (*, $p < 0.05$) and undisturbed E-PS males (#, $p < 0.05$) following an overnight sucrose restriction ($n = 8$). During continuous sucrose access, an acute stress exposure did not influence **(D)** 2 hr or **(E)** 24 hr 10% sucrose intake for control or E-PS males ($n = 4$). Data are mean $\pm$ SEM.

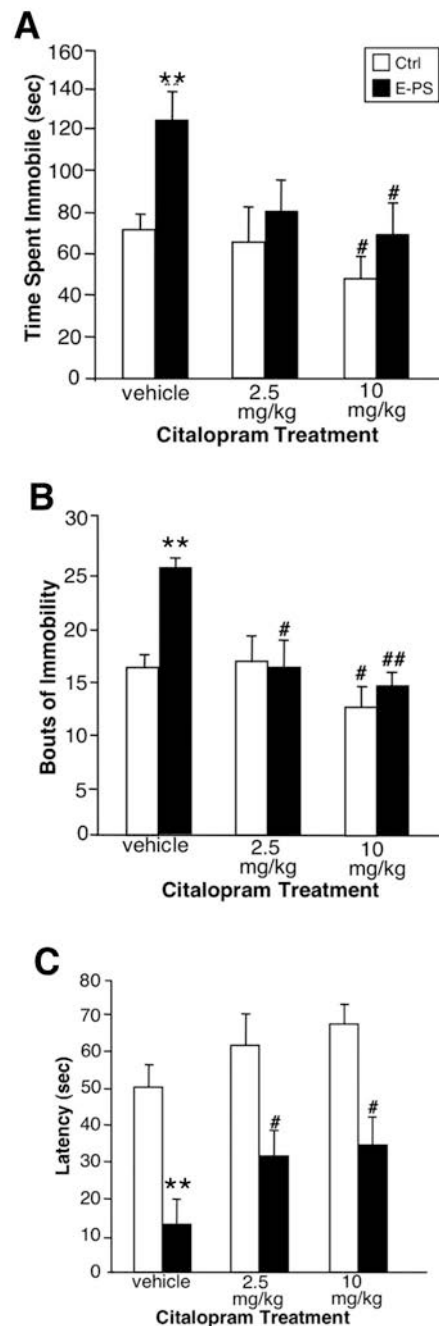


Fig. 3. E-PS males showed an increased sensitivity to acute SSRI treatment

In a tail suspension test, E-PS males spent more (A) time immobile, displayed an increased number of (B) immobility bouts, and showed a decreased (C) latency to first bout of immobility compared to control males (**, $p < 0.01$). For E-PS males, a subthreshold dose of 2.5 mg/kg of citalopram significantly reduced (B) bouts of immobility and (C) latency to first bout of immobility (#, $p < 0.05$) compared to vehicle. 10 mg/kg of citalopram significantly reduced time spent immobile and bouts of immobility for control and E-PS males (#, $p < 0.05$; ##, $p < 0.01$) compared to vehicle. C) 10 mg/kg of citalopram also significantly increased latency to first bout of immobility for E-PS males (#, $p < 0.05$). Data are mean $\pm$ SEM. $n = 8$.

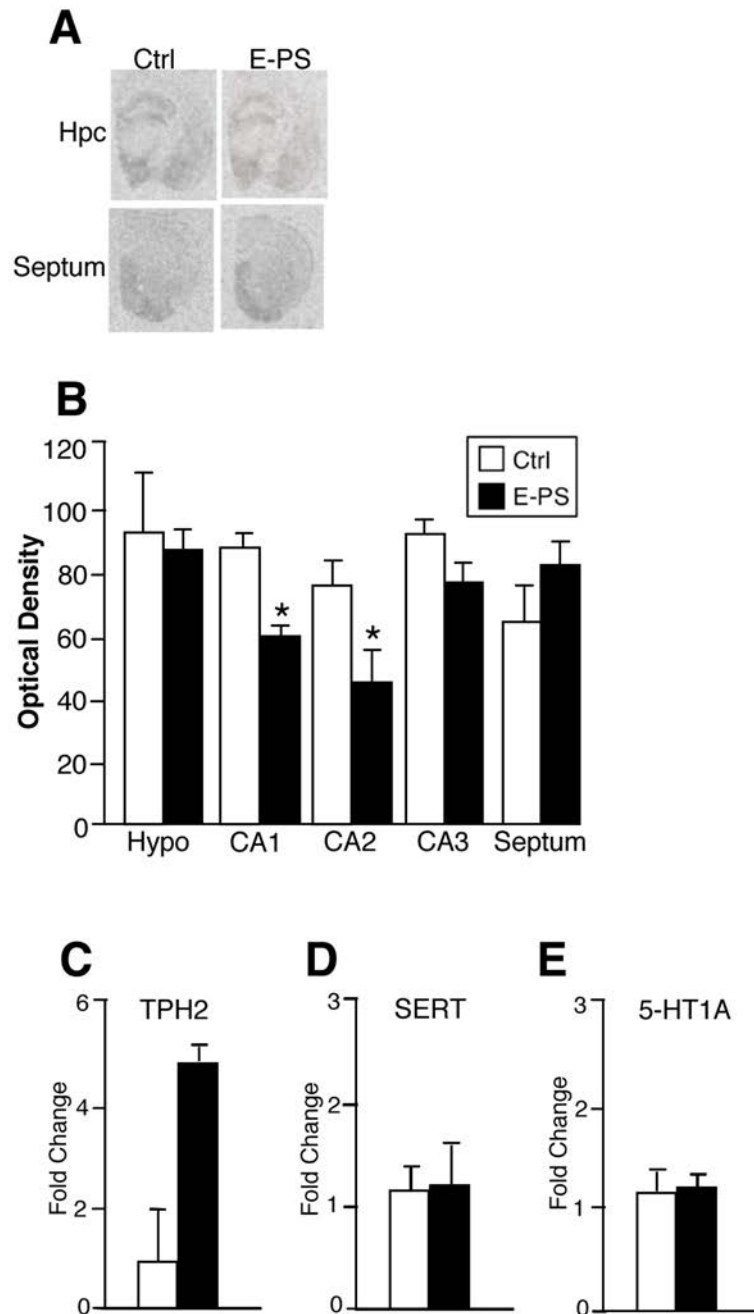


Fig. 4. E-PS males showed altered serotonin transporter levels

A & B) E-PS males showed a significant reduction in serotonin transporter (SERT) levels in the CA1 region of the hippocampus as compared to control males (*, $p < 0.05$). **C)** Real time PCR performed on dorsal raphe micropunches revealed no significant effect of E-PS on tryptophan hydroxylase-2 (TPH2) expression ($p = 0.06$). No differences were detected in **(D)** SERT or **(E)** 5-HT1A expression in the dorsal raphe. Data are mean $\pm$ SEM. $n = 4 - 5$.

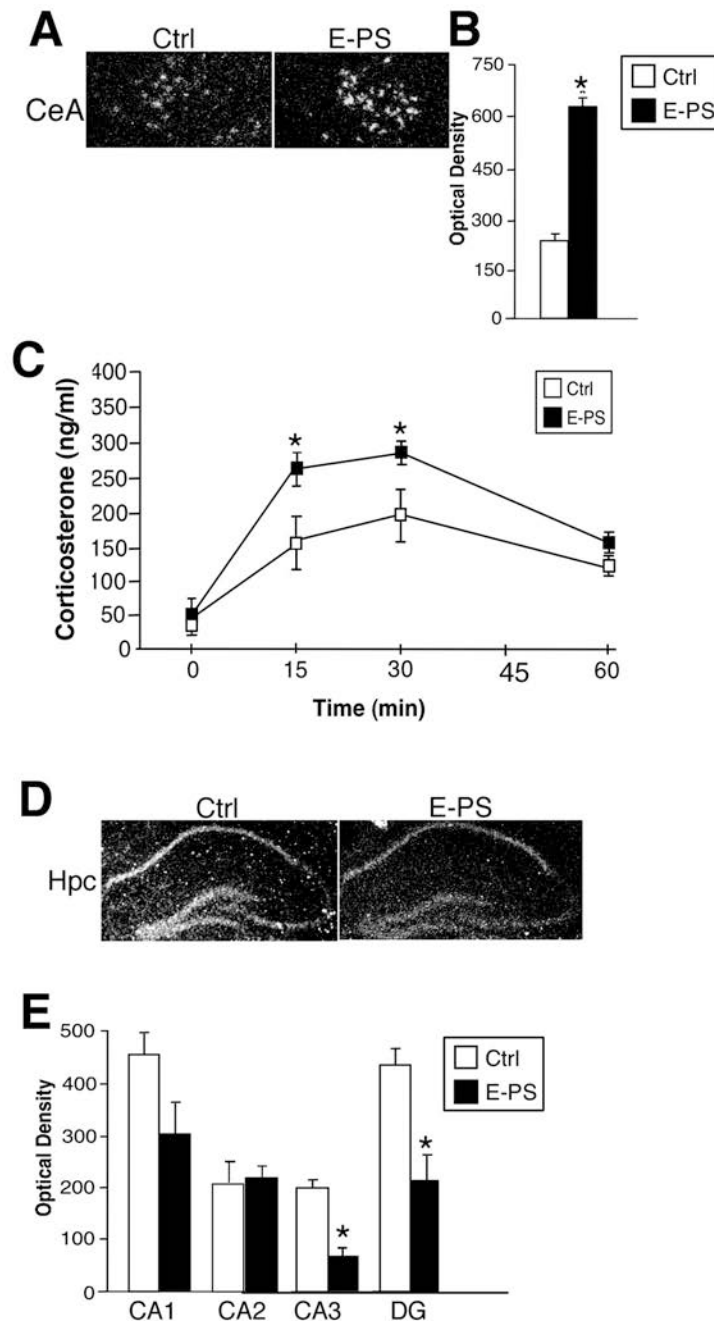


Fig. 5. E-PS males displayed stress pathway dysregulation including increased CeA CRF, decreased hpc GR, and an increased HPA axis stress response

A & B) E-PS significantly increased CRF expression in the central nucleus of the amygdala compared to controls (*, $p < 0.05$) ($n = 4$). **C)** Corticosterone levels following a 15-min restraint stress were significantly higher in E-PS males compared to controls (*, $p < 0.05$) ($n = 6$). **D–G)** Glucocorticoid receptor (GR) expression was significantly lower in E-PS males compared to controls in the **(E)** CA3 and dentate gyrus (DG) of the dorsal hippocampus (*, $p < 0.05$). The apparent decrease in CA1 GR expression was not significant ($p = 0.07$) ($n = 5$). No effect of E-PS was seen in CA2. Data are mean $\pm$ SEM.

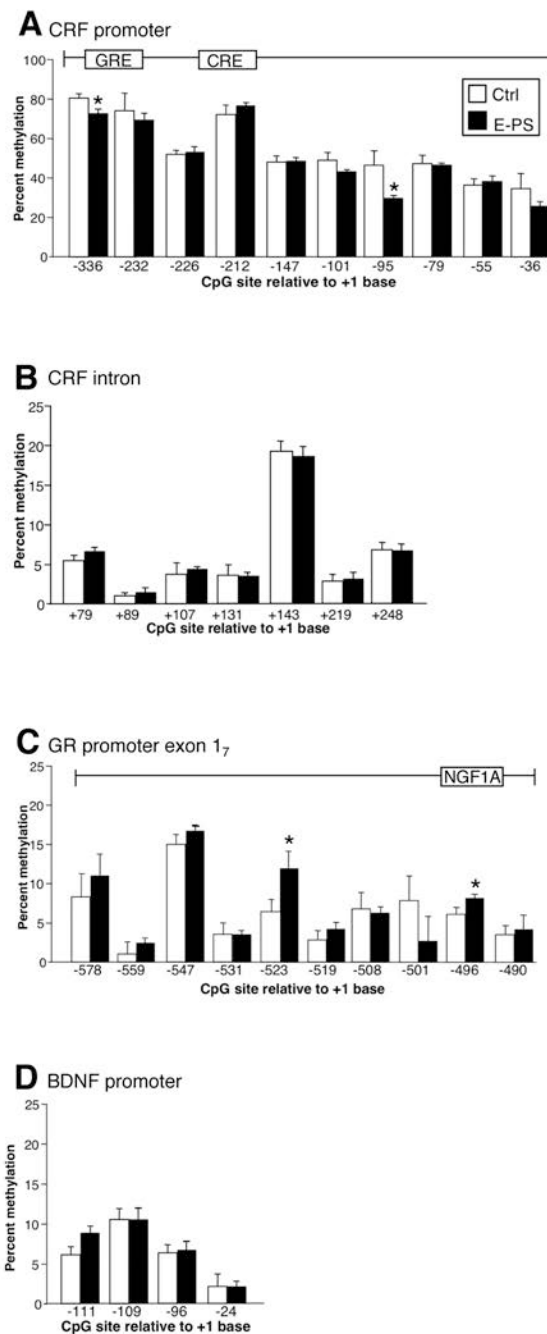


Fig. 6. Alterations in DNA methylation of CRF and GR in E-PS males correlated with directional changes in gene expression patterns

A) Methylation analysis of the 10 CpG dinucleotides within the CRF promoter (–336 to –36 bp) revealed a significant decrease in methylation at specific cytosines in E-PS males as compared to control males (*, $p < 0.05$). **B)** No change in methylation was evident in the CRF intronic region (79kb to 248kb). **C)** A significant decrease in methylation at specific cytosines in E-PS males as compared to control males was detected in the GR promoter region (–578 to –490 bp) (*, $p < 0.05$). **D)** No changes in methylation were detected in the promoter region (–111 to –24bp) of brain derived neurotrophic factor (BDNF). Data are mean $\pm$ SEM. $n = 4$.

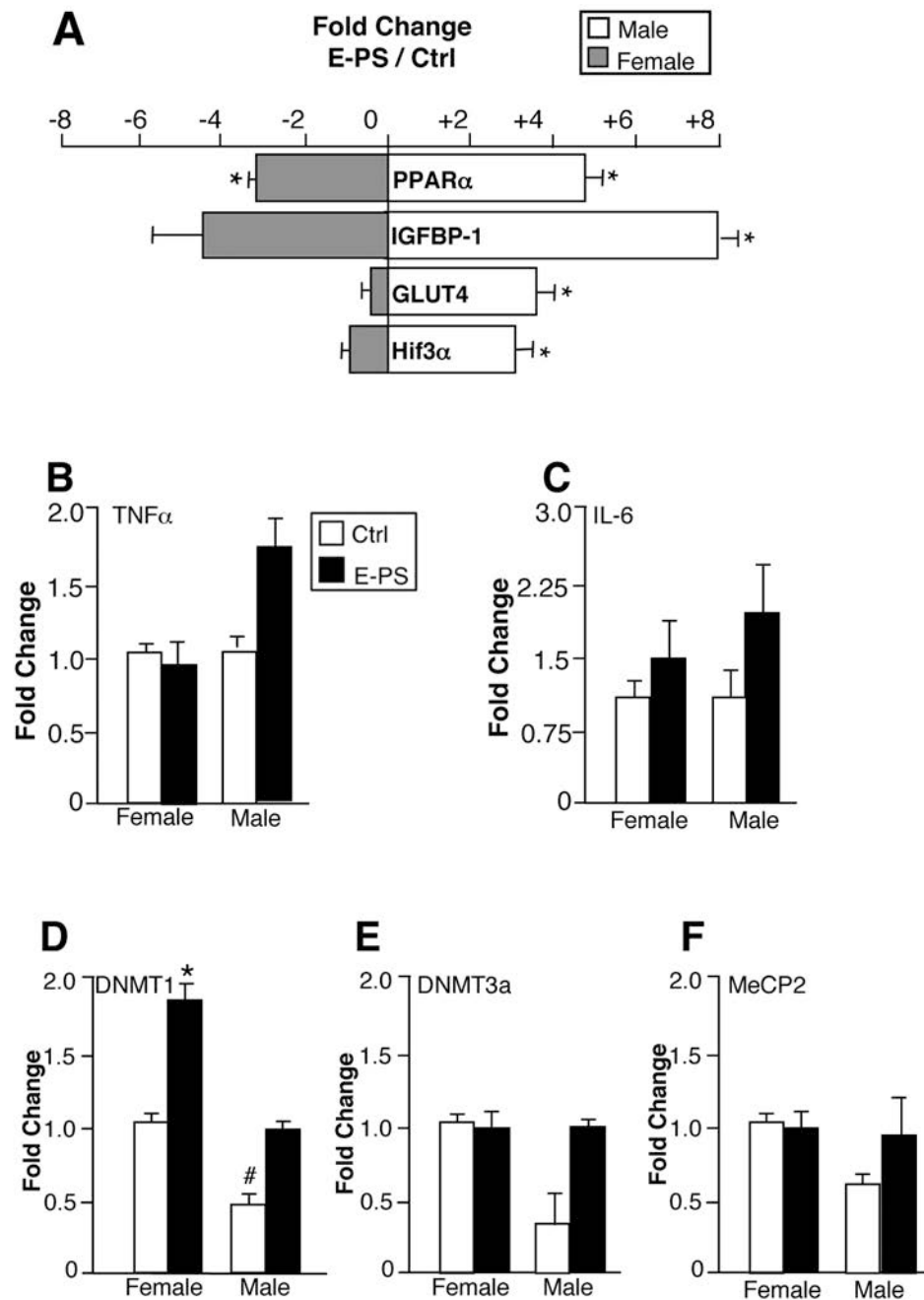


Fig. 7. E-PS male placentas showed robust changes in expression of genes critical for growth and development signals

A) E-PS male placentas showed significant upregulation of PPAR α , IGFBP-1, GLUT4, and HIF3 α compared to male control placentas (*, $p < 0.01$). In contrast, E-PS female placentas showed significant downregulation of PPAR α (*, $p < 0.01$), and a trending downregulation of IGFBP-1 ($p = 0.06$) when compared to female control placentas. **B)** TNF α and **(C)** IL-6 expression levels were not significantly altered by sex or E-PS ($n = 5$). **D)** In our analysis of sex differences in placental epigenetic machinery, control male placentas showed significantly lower expression of DNMT1 when compared to control female placentas (#, $p < 0.01$). E-PS increased expression of DNMT1 in female placentas (*, $p < 0.05$). No effect of E-PS was

detected in male DNMT1 levels. **E)** DNMT3a and **(F)** MeCP2 expression levels were not significantly altered by sex or E-PS (n = 4). Data are mean $\pm$ SEM.

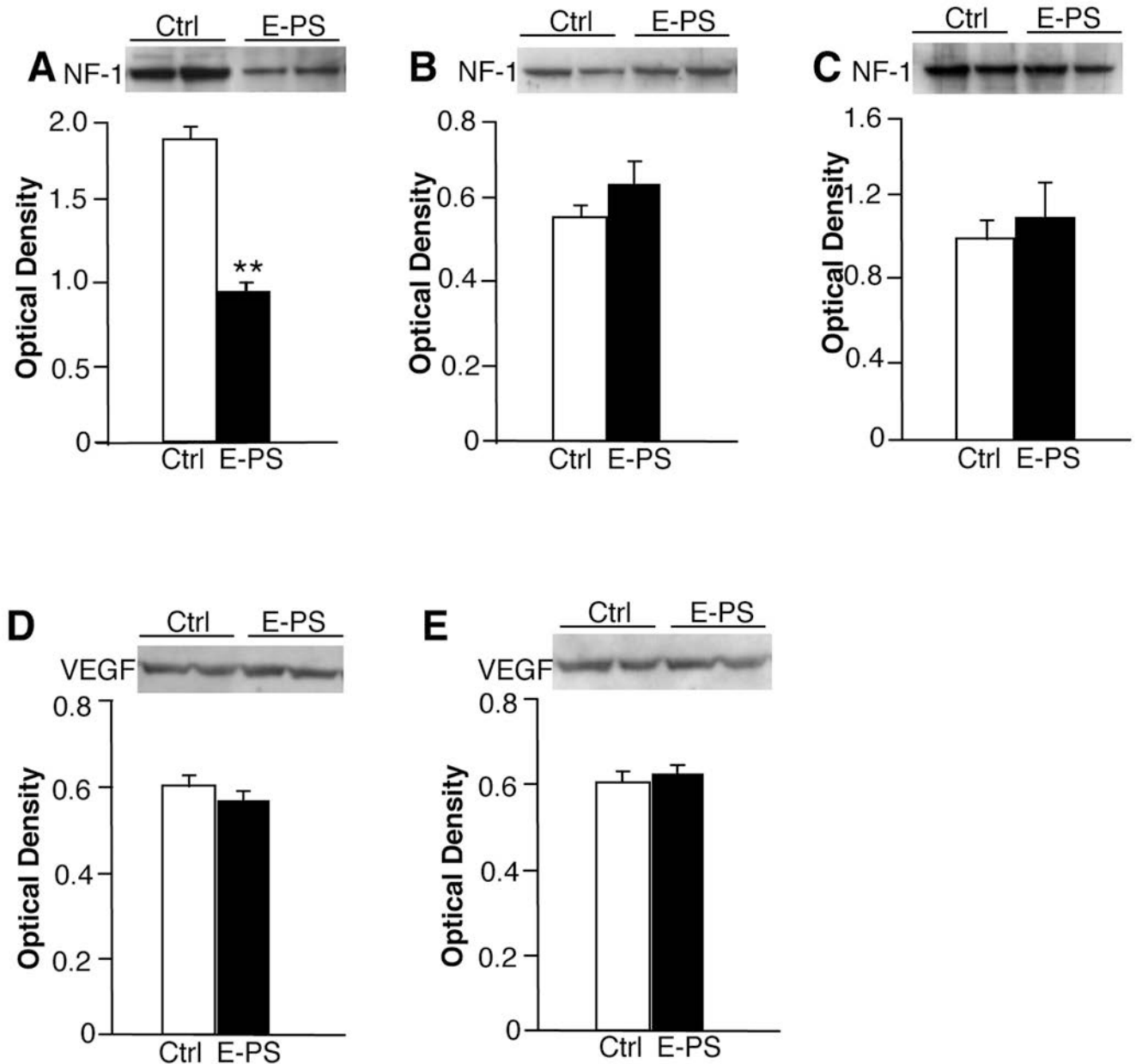


Fig. 8. Significant decreases in brain NF-1 levels in E-PS males support delayed CNS development
 As a gross marker of brain development, we compared neurofilament (NF-1) levels from postnatal day 1 male and female offspring. **A)** Whole brain levels of NF-1 were significantly decreased in E-PS males compared to control male levels at postnatal day 1 (**, $p < 0.01$). **B)** NF-1 levels were not different between E-PS and ctrl females at postnatal day 1. **C)** Pre-frontal cortex levels of NF-1 levels were not different between E-PS and control male adults (16 weeks). **D & E)** There was no significant effect of E-PS on vascular endothelial growth factor (VEGF) in **(C)** male or **(D)** female postnatal day 1 brains. Data are mean $\pm$ SEM. $n = 3 - 4$.