

Prenatal Stress-Induced Increases in Placental Inflammation and Offspring Hyperactivity Are Male-Specific and Ameliorated by Maternal Antiinflammatory Treatment

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Adverse experiences during gestation such as maternal stress and infection are known risk factors for neurodevelopmental disorders, including schizophrenia, autism, and attention deficit/hyperactivity disorder. The mechanisms by which these distinct exposures may confer similar psychiatric vulnerability remain unclear, although likely involve pathways common to both stress and immune responses at the maternal-fetal interface. We hypothesized that maternal stress-induced activation of immune pathways within the placenta, the sex-specific maternal-fetal intermediary, may contribute to prenatal stress programming effects on the offspring. Therefore, we assessed for markers indicative of stress-induced placental inflammation, and examined the ability of maternal nonsteroidal antiinflammatory drug (NSAID) treatment to ameliorate placental effects and thereby rescue the stress-dysregulation phenotype observed in our established mouse model of early prenatal stress (EPS). As expected, placental gene expression analyses revealed increased levels of immune response genes, including the proinflammatory cytokines IL-6 and IL-1 β , specifically in male placentas. NSAID treatment partially ameliorated these EPS effects. Similarly, in adult offspring, males displayed stress-induced locomotor hyperactivity, a hallmark of dopaminergic dysregulation, which was ameliorated by maternal NSAID treatment. Fitting with these outcomes and supportive of dopamine pathway involvement, expression of dopamine D1 and D2 receptors was altered by EPS in males. These studies support an important interaction between maternal stress and a proinflammatory state in the long-term programming effects of maternal stress. (*Endocrinology* 155: 2635–2646, 2014)

Epidemiological studies establish gestation as a period of vulnerability to environmental perturbations. Fetal antecedents such as maternal stress or infection are associated with an increased risk for neurodevelopmental disorders, including schizophrenia, autism spectrum disorder (ASD), and attention deficit hyperactivity disorder (ADHD) (1–7). Gender also appears to be a contributing factor, as such disorders present with known sex biases in onset, symptomology, and treatment. For instance, boys present with ASD at 4–5 times the rate of girls, and ADHD at 2–3 times the rate of girls (8–11). The contributing mechanisms by which distinct exposures such as stress and

infection program disease vulnerability are not clear, although likely involve pathways common to both at the maternal-fetal interface.

Studies in humans and animal models support the critical involvement of the developing placenta in the long-term programming effects and transmission of the maternal milieu to the fetus (12–14). Derived from the blastocyst, the placenta reflects the fetal genetic sex, and sex differences in patterns of gene expression, signaling, and responses to maternal stimuli have been established in human and rodent tissue (15–24). Differential placental responses to prenatal perturbations such as maternal

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Abbreviations: ADHD, attention deficit hyperactivity disorder; ASD, autism spectrum disorder; CCL2, chemokine ligand 2; CCR7, chemokine receptor 7; D1 and D2, dopamine receptors type 1 and type 2; EPS, early prenatal stress; FASL, Fas ligand; GAD1, glutamic acid decarboxylase 1; Nac, nucleus accumbens; NSAID, nonsteroidal antiinflammatory drug; PFC, prefrontal cortex; PPI, prepulse inhibition; TH, tyrosine hydroxylase.

stress may lead to sex-specific fetal programming ultimately contributing to disease risk.

In our early prenatal stress (EPS) mouse model, we have previously shown that male offspring present with heightened stress sensitivity and impaired cognitive function (25–27). Similar outcomes have been reported in guinea pigs as well (28, 29). Further, we have established that the mouse placenta responds to EPS with sex-specific changes in gene expression and function, supporting a placental mechanism whereby stress experienced early in gestation can influence embryo development throughout gestation (18, 26).

Reciprocal interactions between neuroendocrine stress response pathways and the immune system are well documented, and evidence suggests that inflammation occurs downstream of stress in pregnancy (30–37). Furthermore, maternal immune activation has been similarly linked to neurodevelopmental disorder risk, and in animal models, leads to offspring outcomes that are analogous to maternal stress effects (1, 2, 12, 38–42). Thus, examination of placental inflammation as a potential contributing mechanism is warranted. Therefore, we hypothesized that offspring programming effects of maternal stress early in pregnancy involve, in part, sex-specific changes in placental immune pathway activation. To test this, we examined markers indicative of placental inflammation in our EPS model and the potential amelioration of the EPS offspring phenotype by maternal antiinflammatory treatment concomitant with prenatal stress.

Materials and Methods

Animals

Male and nulliparous female C57/Bl6:129 F₁ hybrid mice generated in house were bred at 10–12 weeks of age as described elsewhere (18). Detection of a copulation plug denoted embryonic day (E) 1. Females were individually housed and randomly assigned to stress or control groups, with or without antiinflammatory treatment. Following completion of maternal treatments, dams were either sacrificed at E12.5 for placental analyses (6–8 dams/group) or allowed to give birth (8–10 dams/group) and remain with litters until weaned on postnatal day (P) 28. For all embryonic and postnatal studies, only 1 male and female per litter were used in each assay to avoid litter effects. Mice were maintained on a 12-hour light, 12-hour 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 were provided ad libitum. All animal procedures were conducted in agreement with the Guide for the Care and Use of Laboratory Animals in accordance with NIH guidelines and were approved by the University of Pennsylvania Institutional Animal Care and Use Committee.

Maternal treatments

EPS. Chronic variable stress was administered to dams on embryonic days E1–E7 as described previously (43). Dams experienced 1 novel stressor per day as follows: multiple cage changes, 100 dB white noise overnight (Sleep Machine; Brookstone), 15-minute restraint in a 50-mL conical tube, 36-hour constant light, novel objects in the home cage overnight (8 marbles similar in size and color), 1-hour exposure to fox odor (1:5000; 2,4,5-trimethylthiazole; Acros Organics), and wet bedding overnight. Nonstressed controls remained undisturbed with the exception of weekly cage changes.

Nonsteroidal antiinflammatory drug (NSAID)

administration. Dams assigned to receive antiinflammatory treatment were administered acetylsalicylic acid (aspirin; 81-mg tablet; CVS Pharmacy) at 162 mg/L in drinking water on E1–E7 as described previously (44). NSAID-treated water was replaced every 48 hours. Normal drinking water was resumed upon light-phase onset on E8. Acetylsalicylic acid was selected due to its cyclooxygenase-inhibitory action, oral bioavailability, known efficacy across diverse species, and previously established dosing paradigm (45, 46). Duration of NSAID treatment was selected in order to encompass the period of maternal stress and avoid the dysmasculinizing effects resulting from cyclooxygenase inhibition during late gestation (45). NSAID treatment did not alter maternal food or water intake; however, the potential influence on postnatal maternal care was not examined.

Embryonic analyses

Placenta and embryonic tissue collection. To determine the effects of EPS on placental gene expression, dams designated for embryonic analyses were killed on E12.5 as described previously (26). The E12.5 placenta is used for analysis because the mouse chorioallantoic placenta is fully differentiated at this time (21). All dissections were completed between 10:00 AM and 2:00 PM. Conceptuses were isolated from the uterine wall, rinsed in dH₂O, blotted dry, and placed on Parafilm (Fisher Scientific). The amniotic sac was pierced with a needle and discarded. The umbilical cord was removed using forceps. Placentas were placed fetal side down on a petri dish, hemisected in the transverse plane, submerged in 500 μ L RNA Later (Ambion), and stored at –80 C until RNA isolation. Intrauterine position and observed resorptions were recorded. Embryonic tissue was retained for determination of sex by genotyping using primers specific to Jarid1 (5'-TGAAGCTTTTGGCTTTGAG-3' and 5'-CCGCTGCCAA ATTCTTTGG-3') as described elsewhere (47). One male and one female placenta per litter were selected for gene expression analyses. To limit effects of intrauterine position, we selected placentas from the first third of the embryos from the cervical end and excluded female placentas flanked by two males (2M females) (48). Because the steroidogenic cells of the fetal gonad do not differentiate until E12.5, placentas have not yet been influenced by gonadal hormones of neighboring embryos (49).

TaqMan low-density quantitative RT-PCR immune

array. Placentas (n = 6–8 per group) were submerged in 2 mL TRIzol reagent (Invitrogen), probe sonicated, and RNA isolated. Total RNA (150 ng) was reverse transcribed to cDNA using the High Capacity cDNA Reverse Transcriptase Kit (Applied Biosystems). Expression levels of 90 immune response genes and 6

candidate endogenous controls were determined using preconfigured 384-well microfluidic cards (catalog no. 4367786; Applied Biosystems) run on a 7900-HT fast real-time PCR Machine (Applied Biosystems). Data were analyzed using the comparative Ct method (50). For each sample, raw Ct values for each target gene were normalized to the average of the endogenous controls, β -actin (*Actb*) and glyceraldehyde-3-phosphate dehydrogenase (*Gapdh*). Gene expression relative to nonstressed, vehicle-treated controls was determined. Ct values greater than 35 and genes with expression in less than 50% of samples were excluded from analysis.

Offspring behavior analyses

Offspring were weaned on P28 and housed 3–4 per cage by sex and treatment. Littermates were distributed into cohorts for testing as follows: 1) light-dark exploration test; 2) prepulse inhibition of the acoustic startle response; 3) Barnes maze test of spatial learning; and 4) postmortem brain analyses. Cohorts comprised 1 male and 1 female per litter in order to avoid litter effects. Mice were habituated to handling 1 week prior to testing. The potential impact of estrous cycle stage was not examined.

Light-dark exploration test. The light-dark exploration test was performed as described previously (51). The apparatus comprised a Plexiglas enclosure divided into a brightly illuminated chamber (300 lux) and a dark chamber (5 lux). Experimentally naïve adult offspring (10–12 weeks old; $n = 8$ –9 per group) were placed in the center of the light chamber, and their activity was video monitored for the 10-minute test duration. Testing was conducted between 9:00 PM and 2:00 AM, 2–5 hours after lights off. Time spent in the light compartment, total distance traveled, and light-dark transitions were quantified by ANY-maze version 4.75 (Stoelting Co).

Prepulse inhibition (PPI) of acoustic startle. PPI was recorded in SR-LAB startle chambers (San Diego Instruments) as described previously (52, 53). All test sessions were conducted 2–6 hours after lights off. A session began with 5 minutes acclimation to background noise (65 dB) followed by 5 consecutive acute habituation tones (40 milliseconds duration; 120 dB). Ten repetitions of each of the following types were then presented pseudorandomly: startle pulse only (40 milliseconds; 120 dB), no stimulus (65 dB background), and prepulses (20 milliseconds; +4, +8, or +16 dB above background) preceding the startle pulse (40 milliseconds; 120 dB) by 100 milliseconds. Variable intertrial intervals averaged 15 seconds. Acoustic startle response was defined as the peak startle magnitude recorded during 65 consecutive 1-millisecond readings following the startle pulse onset. The percentage PPI for each prepulse intensity was calculated for each animal as $[1 - (\text{average response to prepulse} + \text{startle}) / (\text{average response to startle only})] \times 100$ before group averages were compared. Animals with average response in startle-only trials of less than or equal to 50 mV were excluded from analysis.

Barnes maze test. The Barnes maze test of spatial learning and memory was performed as described previously (25). The apparatus consisted of a white circular platform with 24 holes evenly dispersed around the perimeter. A target escape box was located in the same hole throughout the test. Distinct visual cues re-

mained in a fixed position around the maze perimeter. Mice (11–14 weeks old; $n = 7$ –9 per group) were trained for 2 trials per day for 3 days. A trial ended upon entry into the target box or after 4 minutes of elapsed time. If the mouse failed to locate the target, the investigator guided the mouse into the target box, and a latency of 240 seconds was assigned. Trials were video recorded and scored by a trained experimenter blinded to treatment. Latency to nose-poke into the target box was determined for each trial. Mice that failed to locate the target in at least 1 of the 6 trials were excluded from analysis ($n = 15$).

Adult brain tissue collection. Experimentally naïve male offspring ($n = 7$ –9/group for mRNA; $n = 4$ /group for protein) were anesthetized and decapitated at 4 months of age. Brains were removed, rapidly frozen on dry ice, and stored at -80°C . Whole brains were cryosectioned at -20°C and micropunched using 0.75-mm Harris Uni-Core tissue punchers (Ted Pella), according to the Paxinos and Franklin mouse brain atlas (54). For the prefrontal cortex (PFC), bilateral punches were collected from 2 successive 300- μm sections from +1.98 to +1.38 mm anterior to bregma. For the nucleus accumbens (NAc), bilateral punches from 2 successive 300- μm sections from +1.68 to +1.08 anterior to bregma, with punchers placed immediately ventral to the anterior commissure tracts. Micropunches for gene expression assays were immediately submerged in 100 μL TRIzol reagent (Invitrogen), frozen on dry ice, and stored at -80°C until RNA isolation. Micropunches for Western blots were submerged in 50 μL radioimmune precipitation assay buffer, protein extracted, and stored at -80°C .

Prefrontal cortical, nucleus accumbens, and ventral tegmental area quantitative RT-PCR. Total RNA (150 ng) was reversed transcribed to cDNA as described above. PFC and NAc gene expression was analyzed by TaqMan Gene Expression Assays (Life Technologies) for the target genes glutamic acid decarboxylase 1 (*Gad1*; Mm00725661_s1), dopamine receptor type I (*Drd1a*; Mm01353211_m1), and dopamine receptor type II (*Drd2*; Mm00438545_m1). Raw Ct values for each sample were normalized to glyceraldehyde-3-phosphate dehydrogenase (*Gapdh*; Mm99999915_g1) and analyzed using the comparative Ct method as described above.

Western blot. Following quantification by Pierce BCA Protein Assay (Thermo Scientific), 20 μg total protein was loaded per lane of NuPAGE 10% Bis-Tris gels (Life Technologies). Western blots were completed as described (55). Blots were probed for tyrosine hydroxylase (TH) (1:1000; Pel-Freeze), stripped, and reprobed for β -actin (1:1000; EMD Millipore). Densitometric analyses were performed in ImageJ (NIH). Within each sample, TH values were normalized to β -actin for quantitative analysis.

Statistical analyses

Statistical analyses were performed using JMP10 (SAS Institute) and R for Mac version 3.0.1 (R Foundation for Statistical Computing). Statistical significance was set at $P < .05$. Data are shown as mean \pm SEM. Data were analyzed by two-way ANOVA within sex for prenatal stress (control, EPS) and anti-inflammatory treatment (vehicle, NSAID), with repeated measures where appropriate (trials 1–6 in the Barnes maze test; prepulse intensity for PPI). Upon detection of significant EPS \times

NSAID interactions, the ability of NSAID treatment to rescue EPS effects was assessed by post hoc Tukey HSD test. Statistically significant rescue was defined a priori by the co-occurrence of an EPS effect (control/vehicle vs EPS/vehicle, $P < .05$) that was no longer present in offspring administered concomitant NSAID (control/vehicle vs EPS/NSAID; $P > .05$). Western blots were analyzed by two-tailed Student's t test.

Results

Litter size, sex ratio, and resorption rate

Because early gestation manipulations can result in fetal lethality, we assessed for treatment effects on litter size and sex ratio (56). Consistent with our previous studies using this mild-stress paradigm, EPS did not alter litter size [$F(1, 33) = 0.0027, P = .96$] or sex ratio [$F(1, 33) = 0.032, P = .86$] (43). There were no effects of maternal NSAID treatment on litter size [$F(1, 33) = 0.14, P = .72$] or sex ratio [$F(1, 33) = 0.09, P = .77$]. In agreement with these results, neither EPS [$F(1, 33) = 1.7; P = .20$] nor maternal NSAID [$F(1, 33) = 0.035; P = .85$] altered the number of fetal resorptions observed on E12.5. No significant interactions between EPS or NSAID treatment were detected. Mean litter size, male percentage, and resorption rates are summarized in Supplemental Table 1.

EPS induces sex-dependent changes in placental gene expression

To test the hypothesis that early-gestation maternal stress induces placental inflammation, expression of immune response genes in placentas from E12.5 males and females was analyzed by TaqMan Low Density Mouse Immune Array. Of the 90 target genes assayed, 81 were detectable in the placenta. Only one gene, the proapoptotic factor, Fas ligand (FASL), was affected in placentas of both sexes (Figure 1, A and B). EPS significantly increased FASL [males: $F(1, 24) = 8.19, P = .0086$; females: $F(1, 26) = 5.18, P = .031$], an effect that did not interact with NSAID treatment [males: $F(1, 24) = 0.001, P = .97$; females: $F(1, 26) = 0.19; P = .67$].

In male placentas, 12 genes were up-regulated and ranged in magnitude from 1.25- to 1.75-fold relative to nonstressed, vehicle-treated controls (Figure 1A). EPS increased proinflammatory cytokines, [IL-6; $F(1, 24) = 4.3; P = .049$] and IL-1 β [$F(1, 24) = 4.71; P = .04$], cytokine receptor IL-2 receptor α [$F(1, 24) = 5.51; P = .027$], and the cyclooxygenase, prostaglandin-endoperoxide synthase 2 [$F(1, 24) = 4.53, P = .044$]. EPS also up-regulated genes involved in chemokine signaling, including chemokine ligand 5 [CCL5; $F(1, 24) = 5.86; P = .023$] and chemokine ligand 10 [CXCL10; $F(1, 24) = 9.19; P = .0058$]. Cell surface antigens, histocompatibility 2 class II

antigen E β [$F(1, 24) = 8.2; P = .0086$] and protein tyrosine phosphatase receptor C [$F(1, 24) = 7.56; P = .011$], were significantly increased. Finally, EPS increased expression of the endothelial molecules, endothelin 1 [$F(1, 24) = 5.21; P = .032$] and P-selectin [$F(1, 24) = 4.93; P = .036$]. Although the magnitude of change often appears decreased by concomitant maternal antiinflammatory treatment, NSAID rescued EPS effects on IL-6 and chemokine receptor 7 (CCR7) only, as evidenced by significant interactions between EPS and NSAID [IL-6: $F(1, 24) = 6.11, P = .021$; CCR7: $F(1, 24) = 4.64; P = .041$]. Post hoc comparisons revealed increased IL-6 ($P = .019$) and CCR7 ($P = .048$) in stressed male placentas relative to nonstressed males (Figure 2, B and C). Maternal NSAID treatment rescued these EPS effects, because placental IL6 ($P = .64$) and CCR7 ($P = .73$) of EPS/NSAID males did not differ from controls. No main effects of NSAID treatment were detected. ANOVAs for all expressed genes are detailed in Supplemental Tables 2 and 3.

In females (Figure 1B), EPS decreased chemokine ligand 2 [CCL2; $F(1, 26) = 4.41; P = .046$], an effect that did not interact with maternal NSAID treatment [$F(1, 26) = 0.002; P = .96$]. Notably, proinflammatory cytokine expression was unaffected in female placentas (Supplemental Table 3).

Offspring behavioral assessment

Maternal antiinflammatory treatment rescues locomotor hyperactivity in prenatally stressed males

To test the hypothesis that inflammatory processes mediate behavioral dysregulation by EPS, offspring were examined for the potential rescue of disease-relevant endophenotypes. We identified significant EPS effects in the light-dark exploration test that interacted with NSAID treatment. In males (Figure 2, A–C), there was a significant effect of EPS [$F(1, 30) = 6.94; P = .013$] and a significant interaction between the effects of EPS and NSAID on time in light [$F(1, 30) = 6.64; P = .015$]. As shown (Figure 2A), prenatally stressed males spent more time in the light chamber relative to nonstressed controls ($P = .0047$). Maternal NSAID treatment rescued EPS effects on light time, as offspring of stressed dams treated with concomitant NSAID did not differ from controls ($P = .093$). To determine whether changes in activity accounted for the increased light time, distance traveled and number of chamber transitions were analyzed. There was a significant effect of EPS on distance traveled [$F(1, 30) = 5.27; P = .023$], and a significant interaction between the effects of EPS and NSAID on distance traveled [$F(1, 30) = 7.96; P = .0084$] and number of light-dark transitions [$F(1, 30) = 6.75; P = .014$]. Post hoc comparisons revealed that pre-

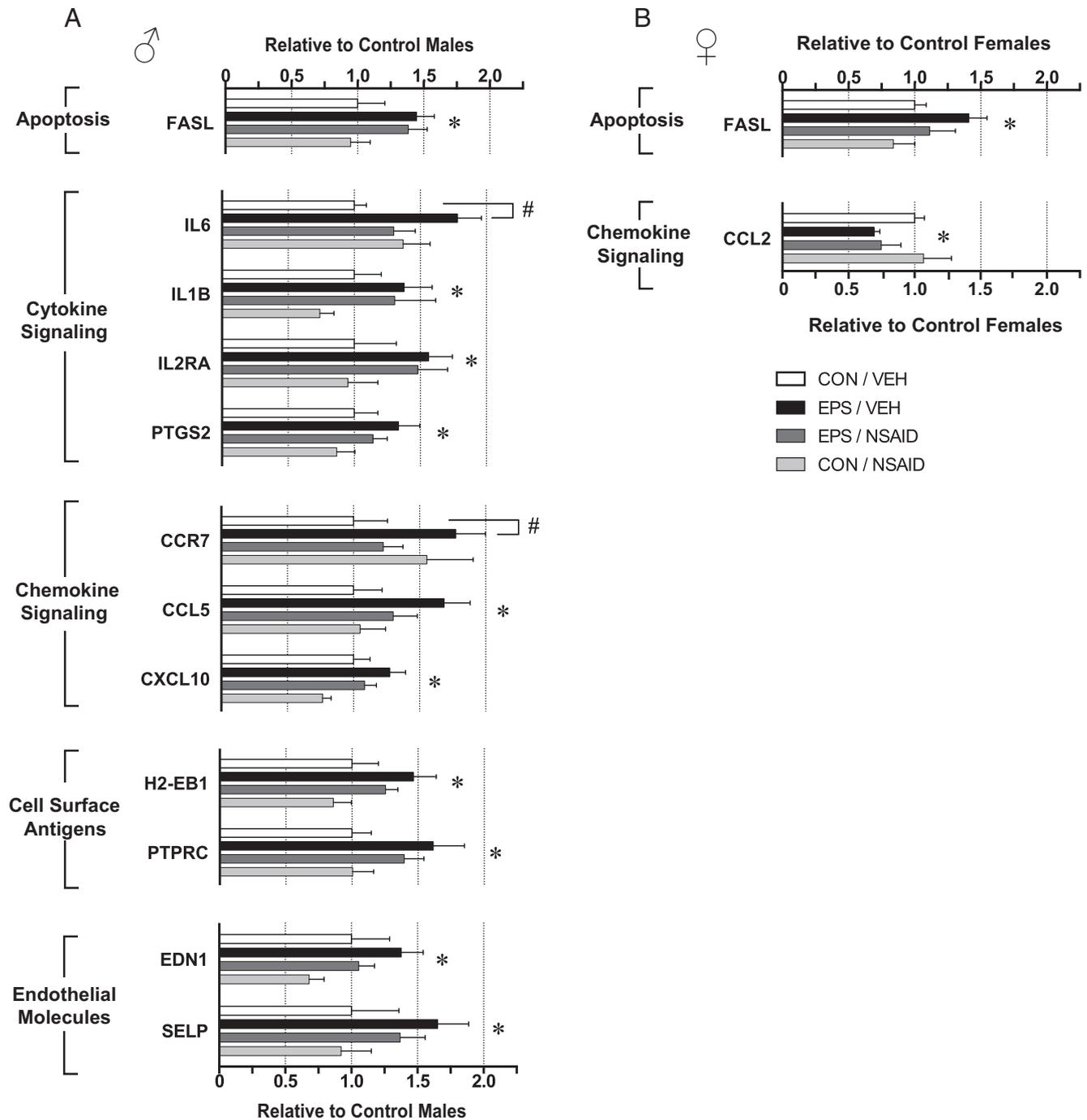


Figure 1. EPS altered expression of immune response genes in E12.5 placentas. Analyses of TaqMan Low Density Mouse Immune Array revealed significant main effects of EPS. Differentially expressed genes in males (A) and females (B) are organized into functional clusters, and their relative expression values are shown. The proapoptotic factor, FASL, was up-regulated in prenatally stressed males and females. In males, 11 additional genes were up-regulated by prenatal stress, including genes involved in cytokine signaling, chemokine signaling, cell surface antigens, and endothelial molecules. Significant interactions between EPS and NSAID treatment were detected for IL-6 and CCR7. Post hoc comparisons revealed increased IL-6 and CCR7 in stressed male placentas relative to nonstressed males. Maternal NSAID treatment rescued these EPS effects, as placental IL6 and CCR7 of EPS/NSAID males did not differ from controls. EPS decreased CCL2 in females (B). No main effects of NSAID were observed. Data are mean \pm SEM. *, $P < .05$ main effect of EPS by two-way ANOVA; #, $P < .05$ vs CON/VEH by Tukey HSD. CON, control; EDN1, endothelin 1; PTPRC, protein tyrosine phosphatase receptor C; SELP, P-selectin; VEH, vehicle.

nately stressed males exhibited increased distance traveled ($P = .0056$) and transitions ($P = .048$) relative to nonstressed males (Figure 2, B and C). Maternal NSAID treatment rescued these EPS effects, because distance trav-

eled ($P = .17$) and transitions ($P = .1$) of EPS/NSAID males did not differ from controls. There were no main effects of NSAID on light time [$F(1, 30) = 0.82$; $P = .37$], distance traveled [$F(1, 30) = 0.023$; $P = .87$], or transitions [$F(1,$

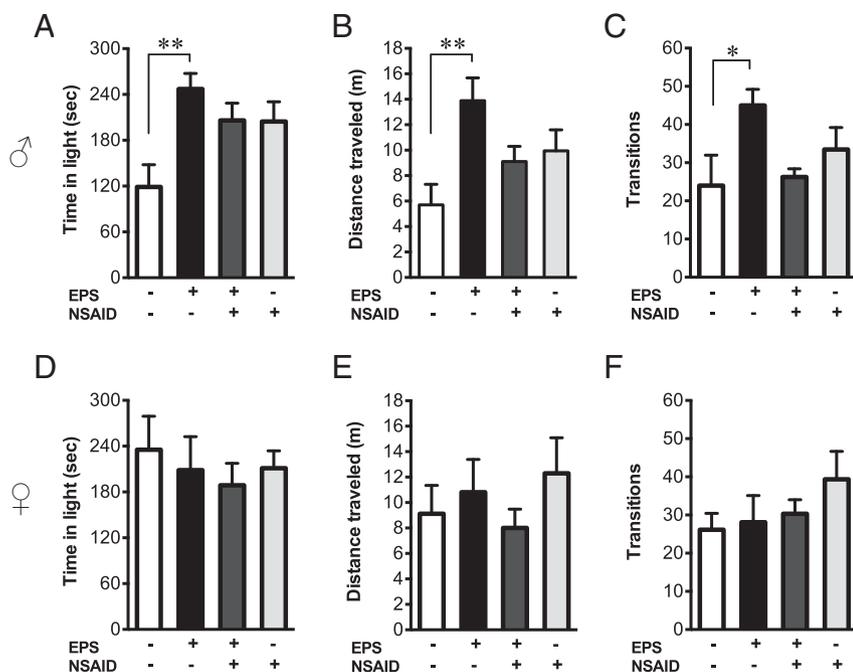


Figure 2. Prenatal stress elicited locomotor hyperactivity in adult males that was prevented by maternal antiinflammatory treatment. In the light-dark exploration test, time in light, distance traveled, and zone transitions were assessed in male (A–C) and female (D–F) offspring. Prenatally stressed males exhibited increased time in the light compartment (A), due to general hyperactivity as assessed by distance traveled (B) and zone transitions (C). NSAID treatment concomitant with maternal stress prevented hyperactivity in males, because exploration of EPS/NSAID males did not differ from controls. Light-dark box exploration in females was unaffected by EPS and NSAID (D–F). *, $P < .05$; **, $P < .01$. sec, seconds.

30) = 0.73; $P = .4$]. In females (Figure 2, D–F), neither EPS nor NSAID affected time spent in light [EPS: $F(1, 31) = 0.54$; $P = .47$; NSAID: $F(1, 31) = 0.44$; $P = .51$; EPS*NSAID: $F(1, 31) = 0.0043$; $P = .95$], distance traveled [EPS: $F(1, 31) = 0.26$; $P = .61$; NSAID: $F(1, 31) = 0.0057$; $P = .94$; EPS*NSAID: $F(1, 31) = 1.38$; $P = .25$], or light-dark transitions [EPS: $F(1, 31) = 0.29$; $P = .59$; NSAID: $F(1, 31) = 1.42$; $P = .24$; EPS*NSAID: $F(1, 31) = 0.72$; $P = .40$].

EPS does not alter sensorimotor gating or spatial learning

ASR and PPI. Acoustic startle to 120 dB was unaffected in males (Supplemental Table 4). As shown (Figure 3, A and B), expected main effects of prepulse intensity on PPI were observed in males [$F(2, 27) = 3.63$; $P < .0001$] and females [$F(2, 20) = 5.54$; $P < .0001$]. In males, PPI was not significantly altered by EPS [$F(1, 28) = 0.57$; $P = .46$] or NSAID [$F(1, 28) = 1.69$; $P = .20$]. In females, there was a significant effect of NSAID on PPI [$F(1, 21) = 4.45$; $P = .047$] that did not interact with prepulse intensity [$F(2, 20) = 0.063$; $P = .54$] or EPS [$F(1, 21) = 1.73$; $P = .20$], and there was no effect of EPS [$F(1, 21) = 2.1$; $P = .16$].

Barnes maze. Spatial learning in the Barnes maze was unaffected by EPS (Figure 3, C and D). Although latency to

find the target decreased for all groups [males: $F(5, 19) = 2.38$; $P = .0002$; females: $F(5, 30) = 3.74$; $P < .0001$], there were no effects of EPS [males: $F(5, 19) = 0.34$; $P = .30$; females: $F(5, 30) = 0.022$; $P = .98$] or NSAID [males: $F(5, 19) = 0.11$; $P = .83$; females: $F(5, 30) = 0.37$; $P = .078$], and no interactions [males: $F(5, 19) = 0.49$; $P = .15$; females: $F(5, 30) = 0.15$; $P = .46$].

Mechanistic evaluation of male-specific hyperactivity

To identify neural circuits mediating male-specific hyperactivity in the light-dark exploration test, we examined gene expression of experimentally naïve male littermates in brain regions related to locomotor activity and novelty responsiveness. Specifically, we assessed dopamine receptor type 1 (D1), dopamine receptor type 2 (D2), and glutamic acid decarboxylase 1 (GAD1) expression in the PFC and NAc, circuits known to mediate locomotor activity and that are disrupted after prenatal stress and prenatal infection (57–68). In the NAc (Figure 4, A–C), there was no effect of EPS on D1 expression [$F(1, 25) = 3.019$; $P = .095$] and no interaction with NSAID treatment [$F(1, 25) = 1.16$; $P = .29$]. There was a significant effect of EPS on NAc D2 [$F(1, 24) = 8.53$; $P = .0075$], but no effect of NSAID [$F(1, 24) = 2.044$; $P = .17$] and no interaction [$F(1, 24) = 0.12$; $P = .73$]. NAc D2 was decreased in prenatally stressed males relative to nonstressed controls with ($P = .0052$) and without ($P = .029$) maternal NSAID treatment (Figure 4C). In the PFC (Figure 4, D–F), there was a main effect of EPS on D1 [$F(1, 24) = 9.211$; $P = .0057$], that did not interact with NSAID [$F(1, 24) = 1.22$; $P = .28$]. Post hoc comparisons revealed that PFC D1 was significantly increased only in EPS/NSAID males relative to nonstressed controls ($P = .035$). PFC D1 in EPS/vehicle offspring did not differ from controls ($P = .53$). There was no effect of EPS [$F(1, 23) = 2.28$; $P = .14$] or NSAID [$F(1, 23) = 0.068$; $P = .80$] on PFC D2, and no interaction [$F(1, 23) = 0.084$; $P = .77$]. No main effects of NSAID on dopamine receptor expression were observed. GAD1 was not affected by EPS [NAc: $F(1, 25) = 0.071$; $P = .79$; PFC: $F(1, 24) = 0.12$; $P = .72$] or NSAIDs [NAc: $F(1, 25) = 1.098$; $P = .95$; PFC: $F(1, 24) = 0.22$; $P = .65$].

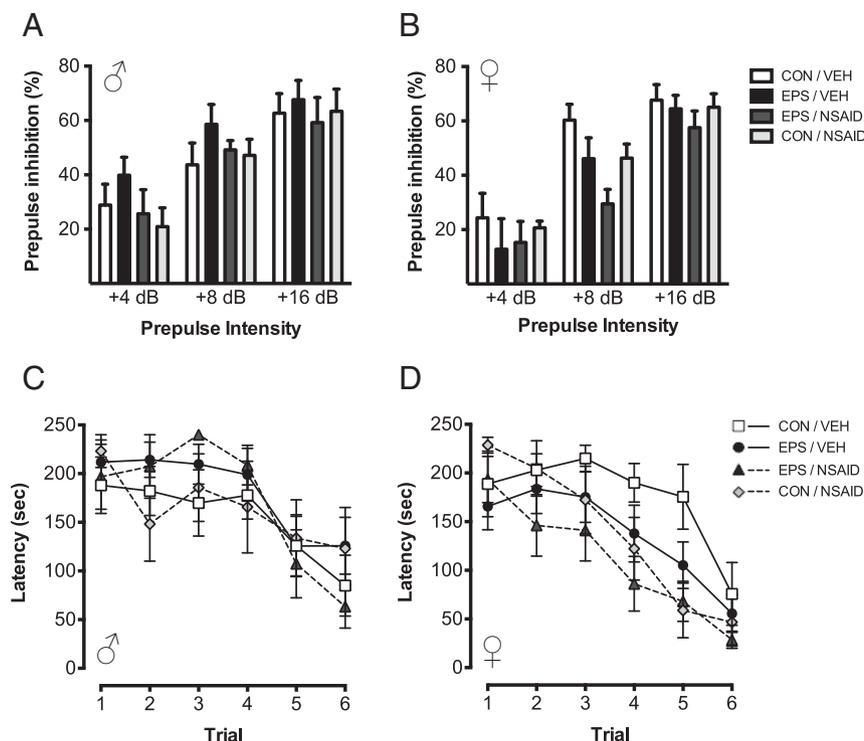


Figure 3. EPS did not alter sensorimotor gating or spatial learning. Neither EPS nor NSAID altered PPI of the acoustic startle response in male (A) or female (B) offspring. In the Barnes maze test of spatial learning, the latency to find the target improved for all groups. No effects of EPS or NSAIDs on target latency were observed (C and D). Data are mean \pm SEM. CON, control; VEH, vehicle.

Based on EPS effects on dopamine receptor expression, we quantified TH protein, the rate-limiting enzyme for dopamine synthesis, in the NAc and PFC by Western blot. EPS did not impact TH, because there was no difference between control/vehicle and EPS/vehicle [NAc: $t(6) = 0.19$, $P = .85$; PFC: $t(6) = 0.16$; $P = .88$] (Figure 5, A and C). In prenatally stressed males (Figure 5, B and D), NSAID treatment did not impact TH [NAc: $t(6) = 1.14$; $P = .30$; PFC: $t(6) = 0.24$; $P = .82$].

Discussion

Maternal stress during pregnancy is associated with increased risk of neurodevelopmental disorders, including schizophrenia, ASD, and ADHD (5–7, 69–72). Although substantial evidence from animal models demonstrates that prenatal stress elicits sex-dependent endophenotypes relevant to these disorders, the mechanisms by which these programming effects occur are not clear (25, 26, 64, 73, 74). Similar outcomes arise following maternal immune activation, suggesting that the detrimental processes of maternal stress and infection may converge upon common pathways (2, 12, 38–40, 42). Certainly, elevated immune system activation has been increasingly identified as a risk factor for neuropsychiatric disease (75–78). To investigate

the hypothesis that stress-induced immune activation contributes, in part, to prenatal stress programming, pregnant dams were NSAID-treated during chronic stress exposure early in gestation (EPS), and offspring were examined for the potential rescue of disease-relevant endophenotypes.

Because the placenta is the sex-specific intermediary at the maternal-fetal interface that is responsive to a changing maternal milieu, we hypothesized that sex-dependent EPS effects may arise via actions on the developing placenta (13, 17). Prenatal stress produced significant changes in gene expression patterns, where immune-related genes including proinflammatory cytokines (IL-1 β , IL-6) and chemokines (CCL5, chemokine ligand 10) were up-regulated by EPS specifically in males. In addition, EPS increased expression of genes that are positively regulated by inflammatory stimuli (IL-2 receptor α , prostaglandin-endoperoxide synthase 2, protein tyrosine phosphatase receptor C, P-selectin, and endothelin 1) in males. Such changes are indicative of a placental proinflammatory state in prenatally stressed males (79).

As predicted, maternal NSAID treatment during EPS ameliorated, in part, these placental changes, including normalizing IL-6 levels in male tissue. These data suggest that cyclooxygenase-dependent processes occurring during chronic stress exposure contribute to placental effects. These data are in agreement with previous studies indicating that early gestation psychogenic stress in mice triggers inflammatory responses in the decidua (56, 80). It is well established that maternal immune stimulation in rodents elevates proinflammatory cytokines, especially IL-6, in the placenta (81–83). Moreover, this cytokine elevation is necessary and sufficient to induce the long-term programming effects of maternal immune activation on sensorimotor gating, open field exploration, social preference, and frontal cortex gene expression in mice (84). Although maternal NSAID treatment appeared to reduce most EPS effects, a statistically significant rescue was limited to IL-6 and CCR7. Because these genes had the greatest magnitude of up-regulation by EPS, it is conceivable that statistical power to detect significant interactions upon more modestly affected genes was insufficient. Fu-

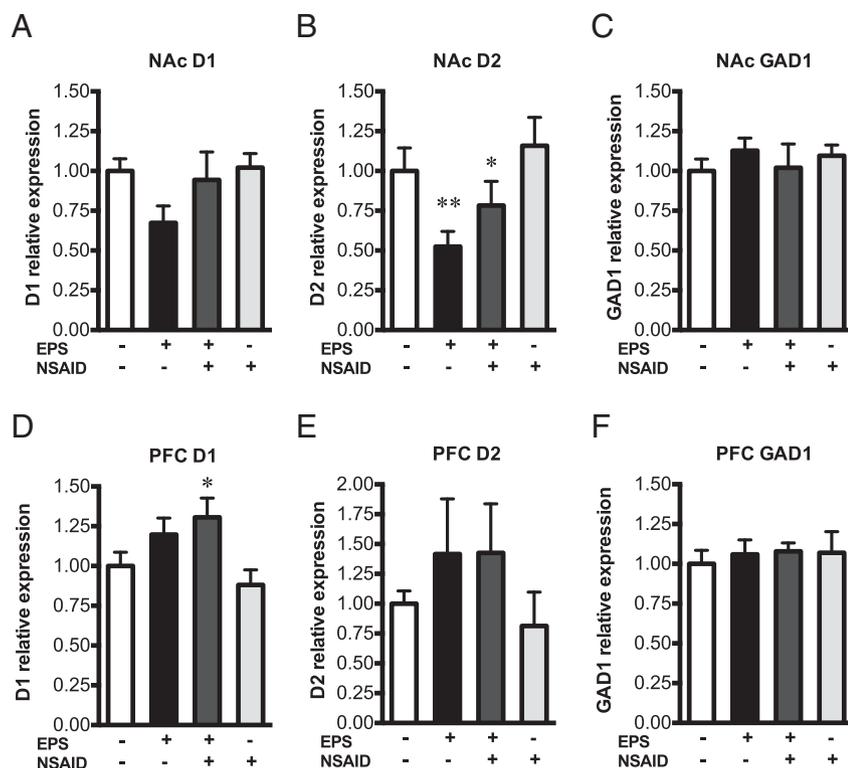


Figure 4. EPS programmed changes in dopamine receptor gene expression in males. Real-time PCR performed on NAc (A–C) and PFC (D–F) micropunches from experimentally naïve male offspring revealed significant main effects of EPS on D1 and D2 dopamine receptor expression. EPS significantly decreased D2 in the NAc (B). Post hoc comparisons revealed a significant reduction of NAc D2 in prenatally stressed males with and without NSAID treatment. In the PFC, a main effect was observed for EPS on D1 (D). Post hoc comparisons revealed significantly increased D1 only in offspring administered NSAID concomitant with EPS. Neither EPS nor NSAID treatment affected GAD1 expression (C and F). Bar graphs are mean expression relative control \pm SEM. *, $P < .05$; and **, $P < .01$ vs control (CON)/vehicle (VEH).

ture studies may benefit from increased NSAID dose or greater specificity in immune system targeting.

Consistent with our hypothesis, the proinflammatory gene expression pattern occurred in prenatally stressed males, but not females. In addition, EPS up-regulated the proapoptotic factor, FASL, in males and females and decreased the monocyte chemoattractant, CCL2, in females. Within the placenta, FASL is expressed by maternal macrophages and placenta trophoblasts, where it mediates immune tolerance of the embryo, vascular remodeling, and trophoblast turnover (85, 86). Increased FASL has also been associated with trophoblast apoptosis following potent immune challenge in E13 placentas (87). Thus, identifying cell type specificity in future immunohistologic studies may help to elucidate downstream effects of increased FASL.

Although treatment effects were detected for a limited number of targets analyzed (15%), the relatively specific up-regulation of proinflammatory genes is in agreement with our hypothesis that maternal stress induces placental inflammation. Placentas were collected on E12.5 (upon

full differentiation), 5 days following stress cessation. We have previously described dynamic patterns of placental gene expression across gestation; therefore, it is likely that longitudinal analyses would reveal a spatiotemporal pattern of differentially expressed genes and effect magnitudes (18). Dramatic transcriptome changes would not be expected using this mild, time-limited stress paradigm, because it does not alter fetal survival (43). Furthermore, placental gene expression was assessed using preconfigured arrays for a broad set of immune-related genes, including a subset undetectable in E12.5 placentas (as described above) and genes for which the extraembryonic function is unclear. Notably, RNA was extracted from whole-placenta homogenates comprised of the labyrinth zone, junctional zone, spongiotrophoblast giant cells, as well as maternal and fetal vasculature. Because these cell layers vary in origin (fetal or maternal) and function, region-specific treatment effects are likely but were not examined in the present study. This methodological limitation may account

for the modest relative expression changes observed.

To determine whether the normalization of placental proinflammatory gene expression in males correlated with a rescue of long-term behavioral outcomes after EPS, we examined adult offspring for evidence of behavioral stress dysregulation. In the light-dark exploration test, we observed a significant increase in overall activity of EPS males, with elevated distance traveled and light-dark transitions, suggestive of a hyperactive phenotype. As hypothesized, maternal NSAID treatment rescued this phenotype in males. Related to the increased activity of these animals, their time spent in the light compartment was also increased. Although the light-dark box task has been pharmacologically validated to assess anxiety-like behaviors, locomotor activity is a necessary control, and thus a clear interpretation of time spent in the light and dark compartments in our mice is not possible (88). However, robust effects on distance and transitions suggest that EPS influences spontaneous or stress-induced activity, in part, through activation of proinflammatory signaling, specifically in developing males. Notably, previous studies have

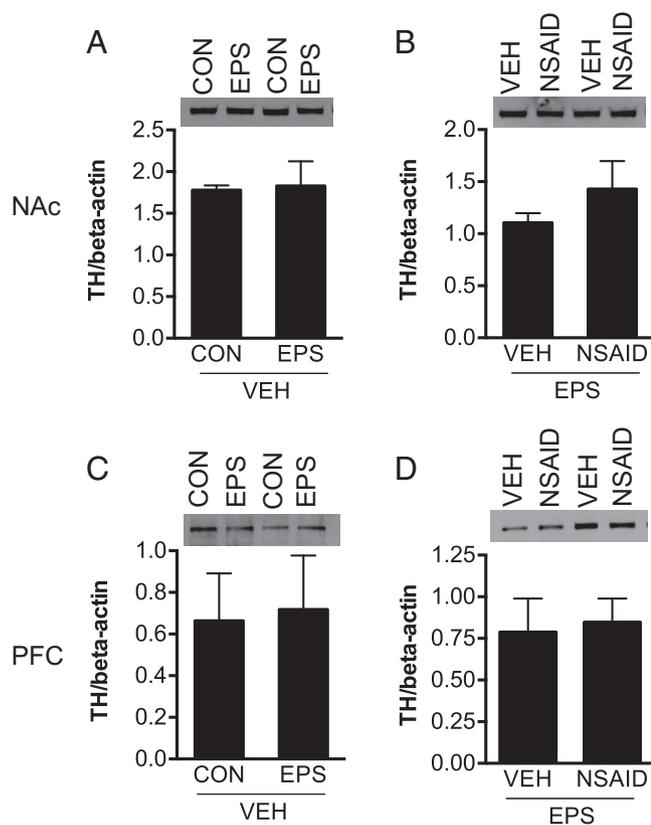


Figure 5. Mesocorticolimbic TH protein content was unaffected in males. In vehicle-treated offspring, prenatal stress did not alter TH content in the NAc (A) or PFC (C). Maternal NSAID treatment did not impact TH levels in prenatally stressed males (B and D). Representative Western blots for TH are shown in the upper panels. Protein levels were determined by densitometric analyses, normalized to β -actin, analyzed by Student's *t* test, and expressed as mean \pm SEM in lower panels.

shown that both prenatal stress and prenatal immune activation induce hyperactivity, although such locomotor effects are dependent on strain/species, gestational timing, and stressor type or duration (89–96). Of translational importance, in humans, maternal stress has been associated with behavioral hyperactivity and ADHD (6, 7).

To further assess potential phenotypic changes in these offspring relevant to neurodevelopmental disorders, mice were examined in tests for sensorimotor gating (PPI) and hippocampal-dependent spatial learning (Barnes maze). Acoustic startle appeared reduced by EPS and rescued by NSAID treatment; however, this effect did not reach significance. It is likely that a more challenging environment (eg, chronic stress) would reveal more maladaptive responses in these animals. In examination of cognitive performance in the Barnes maze, no effects of treatment were found. However, we noted that, similar to our previous reports, EPS males were slower to learn the task, and EPS females were faster than their same-sex controls (25). This outcome has been shown across many species in which

maternal stress has an interaction with offspring sex for direction of impact on cognition (64, 97). These data are in agreement with our previous investigations demonstrating increased susceptibility of male offspring to EPS (25, 26). Although similar male vulnerability has also been reported in mouse, rat, and guinea pig models of maternal stress, sex-specific effects may depend on stressor types, gestational timing, and duration (28, 29, 98). In the present study, maternal stress elicited hyperlocomotion in male offspring without significantly impacting sensorimotor gating or spatial learning. Substantive evidence supports behavioral domain specificity of maternal stress effects, for which gestational stage and stressor(s) are key factors (99). Moreover, such specificity aids in identifying underlying mechanisms of EPS because neural circuits known to mediate hyperlocomotion are well delineated (57–59).

To identify neural circuits mediating locomotor hyperactivity after EPS, we examined mesocorticolimbic dopaminergic circuitry, because it is implicated in the pathophysiology of neurodevelopmental disorders, is perturbed in models of prenatal stress and infection, and is involved in spontaneous and novelty-stimulated locomotion (57–68). In males, we detected main effects of EPS on dopamine receptor gene expression in the NAc (decreased D2) and PFC (increased D1). Because EPS affected both D1 and D2 receptors, we hypothesized that these changes may be secondary to differences in upstream dopamine synthesis and availability. However, TH levels were unaffected by EPS in both PFC and NAc. Consistent with the present work, multiple lines of evidence demonstrate that the dopamine system is sensitive to perturbation by adverse prenatal events, including prenatal stress and infection, thereby supporting the link between these early insults and neurodevelopmental disorders in which dopamine dysregulation has been strongly implicated (60, 63, 68, 100).

In the present study, aspirin was selected as a tool by which to assess the involvement of proinflammatory processes in our mouse model of prenatal stress. We are not suggesting the use of NSAIDs during pregnancy to prevent neurodevelopmental abnormalities, because adverse effects on platelet function, heart and lung development, and miscarriage incidence have been reported, and the long-term effects on fetal development have not been fully investigated (101).

In summary, our findings demonstrate that maternal stress induces placental inflammation, specifically in males, and identify inflammation as a likely contributor to the sex-specific effects of maternal stress on offspring neurobehavioral outcome. Together, these data reveal a novel mechanism underlying the heightened susceptibility of

males to early developmental insult and identify inflammation as one common factor mediating neuropsychiatric disease risk across diverse fetal antecedents known to challenge the immune system.

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