Prenatal Stress Leads to Intrauterine Dysfunction and Offspring Behavioral Deficits

Helen J. Chen, Adrienne M. Antonson, Ph.D., Therese A. Rajasekera, M.P.H., Tamar L. Gur, M.D., Ph.D.

Abstract

Prenatal stress (PNS) is associated with neuropsychiatric disorders in offspring, including anxiety, depression, and autism. There is mounting evidence that these behavioral phenotypes have origins *in utero*. Maternal microbes and inflammation have been implicated as potential mediators of the behavioral consequences of PNS; whether and how these systems interact is unclear. Here, we examine the effects of PNS *in utero* using late-gestation maternal restraint stress in wild-type (WT), germ-free (GF), and CCL2$^{-/-}$ genetic knock-out (KO) mice. In WT mice, PNS leads to placental and fetal brain inflammation, including an elevation in the chemokine CCL2. This inflammation is largely absent in GF mice, indicating the critical role of maternal microbes in mediating immune processes *in utero*. Furthermore, PNS in the absence of CCL2 failed to increase pro-inflammatory cytokine IL-6 in the fetal brain. PNS induced a pro-inflammatory phenotype in fetal microglia, which may be the source of the CCL2- and microbe-dependent inflammation in the fetal brain. Finally, PNS offspring exhibited deficits in sociability and anxiety-like behavior that were absent in CCL2$^{-/-}$ PNS offspring. Altogether, these findings suggest that a complex interaction between maternal microbes and inflammation regulates the emergence of behavioral abnormalities following PNS.

Introduction

Prenatal stress (PNS) has been linked with adverse neuropsychiatric outcomes[1,2]. Children of women who experience psychosocial stress during pregnancy are at elevated risk of

depression, anxiety, and Autism Spectrum Disorders[1–3]. This phenomenon is supported by findings from rodent models, in which PNS leads to anxiety-like behavior, social deficits, and depressive-like behavior in the offspring[4–8]. However, the mechanisms underlying possible fetal programming during PNS are still being elucidated.

Perturbation of the maternal gut microbiome has been implicated as a mechanism through which PNS can influence neurodevelopment[9–11]. There is mounting evidence that commensal microbiota intimately interact with and shape both the developing immune system and the brain[12,13]. Toll-like receptors (TLR) present on immune cells and trophoblast cells of the placenta[14,15] allow for microbes or microbial components to signal across fetal tissue, initiating an inflammatory cascade and resulting in the production of proinflammatory cytokines and chemokines[15–17]. These interactions are necessary for the maturation of the immune system, as germ free mice have diminished hematopoietic capacity and blunted cytokine production[18,19]. While PNS has been shown to upregulate expression of pro-inflammatory cytokines in the placenta and fetal brain[5,6,20], the interplay between maternal microbes and inflammation in mediating the programming effects of PNS is unclear.

C-C motif chemokine ligand 2 (CCL2) is a chemokine that primarily functions in the recruitment of leukocytes to propagate an inflammatory response[21] and recruits monocytes to the brain to elicit an anxiogenic response during stress[22,23]. CCL2 is also highly expressed in uterine and placental tissue[24–26]; whether CCL2 plays a role in fetal neurobiological and behavioral programming is unknown. Microglia, the resident innate immune cell of the brain, is one potential source of CCL2 following exposure to stress. Originating from the embryonic yolk sac[27], microglia begin colonizing the developing brain around embryonic day 8 in rodents[28] and play an important role in detecting and eliminating pathogens, initiating and propagating an

immune response, and regulating neurodevelopment[29,30]. Furthermore, PNS has been shown to lead to an activated microglial phenotype in adult offspring, both morphologically[4,31] and functionally[32]. However, the effects of PNS on microglia *in utero* have yet to be defined.

We hypothesized that an inflammatory response elicited by PNS is dependent upon maternal microbes and CCL2 signaling, culminating in adverse behavioral outcomes in adult offspring. Through the use of our chronic restraint stress model in pregnant C57BL/6, germ-free, and $CCL2^{-/-}$ mice, we demonstrate that PNS induces microbe-dependent intrauterine inflammation, and that CCL2 is critical for mediating deficits in adult offspring behavior.

Methods

Animals and Experimental Design

Nulliparous wild type (WT) C57BL/6 mice and $CCL2^{-/-}$ (knock-out; KO) mice were obtained from Jackson Laboratories (Bar Harbor, ME) and germ–free (GF) C57BL/6 mice from Taconic Biosciences (Germantown, NY) and maintained in conventional or sterile conditions at the Ohio State University. Mice were bred for 48 hours, and copulation plugs were used to determine the first gestational day (GD1) of pregnancy. Pregnant dams were randomly assigned to stressed (PNS) or non-stressed groups. As previously described[4,5], the PNS group underwent restraint stress from 9:00AM–11:00AM on GD10–GD16 using a 50 mL conical tube with perforations to allow for ventilation, while the non-stressed group was left undisturbed. The timing of the restraint stress was chosen to coincide with key processes in neurodevelopment and the establishment of yolk sac placental circulation on E9-10[33,34]. In the first set of experiments, WT, $CCL2^{-/-}$ and GF dams were euthanized on GD17 for tissue collection (Group 1). In a second set of experiments, WT and $CCL2^{-/-}$ dams went through parturition and pups were weaned at

PND 28, divided by sex, and co-housed with litter-mates, with a maximum of five mice per cage, until offspring behavioral testing was performed at 10 weeks of age (Group 2; **Fig. 1A**). Tissue was collected from, and behavior was performed, using a maximum of four offspring from each litter, balancing for sex. All experiments were conducted in accordance with the principles and procedures outlined in the National Institutes of Health Guidelines for the Care and Use of Experimental Animals and were approved by the Institutional Animal Care and Use Committee at the Ohio State University.

Tissue Collection

Dams were euthanized on GD17 using CO_2 and a sterile cesarean section was performed to remove the uterus and excise placentas and fetal brains. A first subset of fetal brains was pooled per dam and collected into PBS on ice for microglia isolation. A second subset of whole placentas and fetal brains were frozen on dry ice and stored at -80°C until processing for RNA or protein. A third set of fetal brains was post-fixed in 4% paraformaldehyde for 24 hours at 4°C, and then in 30% sucrose for 24 hours at 4°C. Fixed fetal brains were embedded in Tissue-Tek O.C.T. Compound (Sakura Finetek, Torrance, CA) and flash frozen using isopentane. Frozen fetal brains were sectioned at 12 µm using a cryostat, mounted onto slides, and stored at -20°C until staining. Fetal microdissection and examination of the reproductive structures was used to determine fetal sex, which was confirmed using genotyping of fetal tails for the SRY gene.

Fetal Brain Microglia Isolation

Fetal brains were mechanically disrupted using a Potter-Elvehjem tissue grinder (DWK Life Sciences, Millville, NJ) and myelin was removed using a 30% isotonic Percoll solution (Millipore Sigma, Burlington, MA). The cells were then washed with FACS Buffer and

incubated with purified rat anti-mouse CD16/CD32 Fc blocking antibody for 15 minutes, and then with mouse CD11b Microbeads (Miltenyi Biotec, Bergisch Gladbach, Germany) for 15 minutes. CD11b+ cells were isolated by applying the single cell suspension to MS columns (Miltenyi Biotec, Bergisch Gladbach, Germany) mounted on MiniMACS separators (Miltenyi Biotec, Bergisch Gladbach, Germany), per manufacturer's instructions. The CD11b+ cells were stored at -80°C in Trizol reagent (Invitrogen, Carlsbad, CA) until further processing for RNA.

Quantitative Real-Time PCR

For whole placental and fetal brain tissue, Trizol reagent (Invitrogen, Carlsbad, CA) was used for RNA extraction, per manufacturer's instructions. For fetal brain microglia, cells were lysed and RNA was extracted using the Direct-zol RNA Miniprep Kit (Zymo Research, Irvine, CA). cDNA was synthesized using High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA). PCR reactions were performed using the Taqman Gene Expression Master Mix protocol (Applied Biosystems, Foster City, CA) and QuantStudio 3 Real-Time PCR Systems machine (Applied Biosystems, Foster City, CA). qPCR data is presented as fold change compared to the non-stressed control group, using the $2^{-\Delta\Delta Ct}$ method. For comparing across WT and CCL2$^{-/-}$ tissue, data were normalized to the WT control group. For determining sex differences, data were compared to the female control group. *TBP* was used as an endogenous control gene for placental samples and *GAPDH* was used for fetal brain and microglial samples.

ELISA

Placentas and fetal brains were homogenized in T-PER Tissue Protein Extraction Reagent (Thermo Scientific, Waltham, MA) by sonication. CCL2 was measured in the protein lysates using the Mouse CCL2/JE/MCP-1 Duoset ELISA (R&D Systems, Minneapolis, MN).

Immunofluorescence

Fetal brain sections were washed with PBS to clear the O.C.T. compound, blocked with 5% normal donkey serum and 1% bovine serum albumin (BSA) for 1 hour, and then incubated with rabbit anti-Iba1 (1:1000; Wako Chemicals, Richmond, VA; Catalog #019-19741) overnight at 4°C. Sections were then washed with 1% BSA in PBS and incubated in Alexa Fluor 594 donkey anti-rabbit secondary antibody (1:1000; Abcam, Cambridge, MA; Catalog #R37119) overnight at 4°C. Sections were then washed with 1% BSA in PBS and nuclei were labeled with DAPI (300 nM in PBS; ThermoFisher Scientific, Waltham, MA) and the slides were coverslipped with Fluoromount G (Southern Biotec, Birmingham, AL). Images were captured using an Evos FL Auto 2 Imaging System (ThermoFisher Scientific, Waltham, MA) at 20X magnification. The hippocampus was identified according to a fetal mouse brain atlas[35]. For analysis of Iba+ microglia, cells were counted in the region of interest, and results are expressed as cells per mm^2 area.

Social Behavior Testing

Sociability was assessed using the three-chamber social behavior test, as previously described[4]. Briefly, testing was completed in a three-chamber box with metal cages placed in the center of the two end chambers. A 10-minute acclimation phase was followed by a 10-minute test phase, during which a novel object (orange Pyrex cap (Corning, NY)) and a social stimulus DBA2J mouse of the same sex and age (Jackson Laboratories, Bar Harbor, ME) were placed in

the metal cages. The chamber that contained the conspecific mouse was randomly assigned for each test mouse to control for side preference. The trials were recorded using the Noldus EthoVision (Wageningen, The Netherlands). Social approach behavior was calculated by subtracting the time spent actively investigating the novel object from the time spent with the social stimulus mouse and dividing by the total time interacting with both cages.

Light-Dark Preference Test

Light-dark preference testing was performed in a 40x40x25 cm Plexiglas box divided into equal 20x40x25 cm compartments by a black Plexiglass separator, with a 3x10 cm doorway allowing for the test mouse to transition between zones. The light compartment was illuminated at an intensity of 150 lux, while the dark compartment was enclosed by a black Plexiglass cover. The test mouse was placed into the light compartment to begin the 5 min trial, and time to enter the dark compartment, duration of time spent in each zone, and total distance traveled was measured using Fusion software (Omnitech Electronics, Inc., Columbus, OH).

Statistical Analyses

Statistical tests were performed using Graphpad Prism. Multi-factorial analysis of variance (ANOVA) followed by a Tukey's post hoc test was performed to determine possible effects of sex. For tests in which the main effect of sex and the interaction between sex and stress were not significant, data were collapsed and unpaired t-tests were used to determine statistical significance between stressed and non-stressed groups, and unpaired t tests with Welch's correction were performed for groups with statistically significant variances (F test). Differences between WT and $CCL2^{-/-}$ tissue were assessed using a two-way ANOVA followed by a Tukey's

post hoc test. The ROUT method was used to identify outliers (with Q set to 1%), which were excluded from analyses. Significance was defined as $p < 0.05$.

Results

Prenatal Stress Leads to Alterations in the CCL2-*CCR2* Axis in the Intrauterine Environment

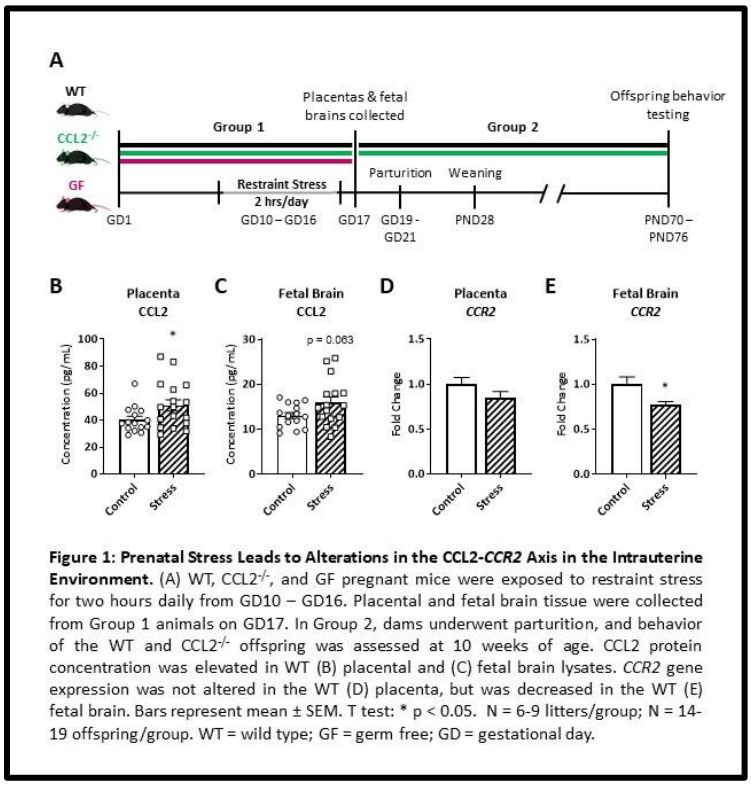

Figure 1: Prenatal Stress Leads to Alterations in the CCL2-*CCR2* Axis in the Intrauterine Environment. (A) WT, CCL2$^{-/-}$, and GF pregnant mice were exposed to restraint stress for two hours daily from GD10 – GD16. Placental and fetal brain tissue were collected from Group 1 animals on GD17. In Group 2, dams underwent parturition, and behavior of the WT and CCL2$^{-/-}$ offspring was assessed at 10 weeks of age. CCL2 protein concentration was elevated in WT (B) placental and (C) fetal brain lysates. *CCR2* gene expression was not altered in the WT (D) placenta, but was decreased in the WT (E) fetal brain. Bars represent mean ± SEM. T test: * $p < 0.05$. N = 6-9 litters/group; N = 14-19 offspring/group. WT = wild type; GF = germ free; GD = gestational day.

PNS increased CCL2 protein levels in the placenta (**Fig. 1B**; t(25.80)=2.17; p=0.039)), and resulted in a nonsignificant increase in CCL2 protein in the fetal brain (**Fig. 1C**; t(22.60)=1.96; p=0.063). Expression of C-C Motif Chemokine Receptor 2 (*CCR2*), the primary receptor for CCL2, was decreased in PNS fetal brains (**Fig. 1E**; t(17.09)=2.49; p=0.023), but not placentas (**Fig. 1D**; t(28)=1.49; p=0.148). Together, this suggests that our model of PNS results in disruption of the CCL2-*CCR2* axis in the intrauterine environment.

Prenatal Stress Increases Fetal Brain *IL6* in a CCL2-Dependent Manner

To determine the role of CCL2 in the intrauterine environment following PNS, CCL2$^{-/-}$ placentas and fetal brains were examined and compared to WT. Absence of CCL2 in CCL2$^{-/-}$ tissues was confirmed by PCR. Exposure to PNS resulted in a nonsignificant decrease in *CCR2*

expression in the CCL2$^{-/-}$ placenta (**Fig. 2A**; t(24)=2.02; p=0.054), while there was no change in the fetal brain (**Fig. 2B**; t(27)=0.53; p=0.599). If maternal microbial homeostasis is disrupted by PNS, microbes or their products may reach the intrauterine environment, which can be monitored in part through the expression of pattern recognition receptors, such as TLR4. PNS increased *TLR4* expression in the placenta (**Fig. 2C**; main effect of stress: f(1,53)=8.61; p=0.005) and decreased *TLR4* in the fetal brain (**Fig. 2D**; main effect of stress: f(1,56)=4.48; p=0.04), though CCL2$^{-/-}$ placentas had lower expression (**Fig. 2C**; main effect of genotype: f(1,53)=12.23; p=0.001) and CCL2$^{-/-}$ fetal brains had higher expression (**Fig. 2D**; main effect of genotype: f(1,56)=4.35; p=0.039) compared to WT.

Since TLR4 activation can enhance expression of pro-inflammatory cytokines, a panel of cytokines and immune-related receptors was examined in the WT placenta and fetal brain. Among the genes of interest, the most compelling stress-induced immune dysregulation was evident in the intrauterine expression of *IL6* and *TNF* following PNS. CCL2$^{-/-}$ placentas had decreased expression of *IL6* compared to WT (**Fig 2E**; main effect of genotype: f(1,53)=6.18; p=0.016), while there was a stress x genotype interaction in the fetal brain (**Fig. 2F**; f(1,53)=8.50; p=0.005), with stress increasing

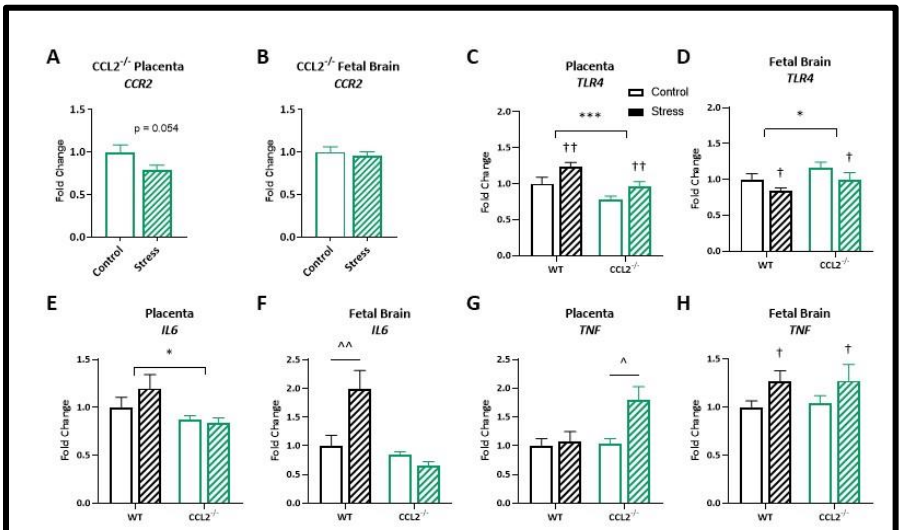

Figure 2: Prenatal Stress Increases Fetal Brain *IL6* in a CCL2-Dependent Manner. *CCR2* gene expression in CCL2$^{-/-}$ (A) placentas and (B) fetal brains following stress. Gene expression of the pattern recognition receptor *TLR4* in the WT and CCL2$^{-/-}$ placenta (C) and fetal brain (D). Gene expression of pro-inflammatory cytokines (E, F) *IL6* and (G, H) *TNF* in the WT and CCL2$^{-/-}$ placenta and fetal brain, respectively. Bars represent mean ± SEM. Two-way ANOVA: asterisks (*) represent main effect of genotype (* $p < 0.05$;*** $p < 0.001$); daggers (†) represent main effect of stress († $p < 0.05$; †† $p < 0.01$); carets (^) represent stress x genotype interaction with significant Tukey post hoc test (^ $p < 0.05$; ^^ $p < 0.01$). N = 5-9 litters/group; N = 12-16 offspring/group.

IL6 expression only in WT mice. These data indicate that PNS increases fetal brain *IL6* expression in a CCL2-dependent manner. Interestingly, PNS increased *TNF* expression in both WT and CCL2$^{-/-}$ fetal brains (**Fig. 2H**; main effect of stress: f(1,54)=5.29; p=0.025) and CCL2$^{-/-}$ placentas but not WT (**Fig. 2G**; stress x genotype interaction: f(1,55)=4.61; p=0.036).

Altogether, these data suggest that the placenta may be responding to a microbe-associated molecular pattern (possibly a TLR4 ligand) following PNS, leading to inflammation within the fetal brain due to CCL2 signaling.

In the Absence of Microbes, Prenatal Stress does not Induce Intrauterine Inflammation

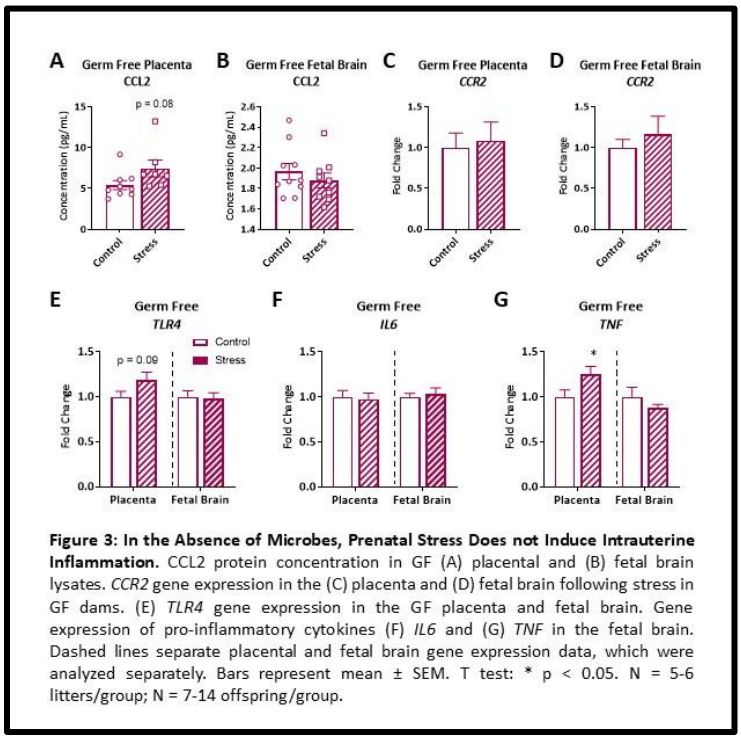

Figure 3: In the Absence of Microbes, Prenatal Stress Does not Induce Intrauterine Inflammation. CCL2 protein concentration in GF (A) placental and (B) fetal brain lysates. *CCR2* gene expression in the (C) placenta and (D) fetal brain following stress in GF dams. (E) *TLR4* gene expression in the GF placenta and fetal brain. Gene expression of pro-inflammatory cytokines (F) *IL6* and (G) *TNF* in the fetal brain. Dashed lines separate placental and fetal brain gene expression data, which were analyzed separately. Bars represent mean ± SEM. T test: * p < 0.05. N = 5-6 litters/group; N = 7-14 offspring/group.

In GF animals, PNS resulted in a nonsignificant increase in CCL2 protein in the placenta (**Fig. 3A**; t(14)=1.87; p=0.083), and no change in the fetal brain (**Fig. 3B**; t(17)=0.80; p=0.433). *CCR2* gene expression in GF tissue was not altered by PNS (placenta: **Fig. 3C**; t(25)=0.28; p=0.778; fetal brains **Fig. 3D**; t(15.36)=0.66; p=0.522). Likewise, *TLR4* gene expression in GF tissue remained unchanged (**Fig. 3E**; placenta: t(25)=1.77; p=0.089; fetal brain: t(24)=0.19; p=0.85). Finally, only GF placental *TNF* (**Fig. 3G**; t(24)=2.07; p=0.049) was altered by PNS, while *IL6* (**Fig. 3F**; placenta: t(23)=0.26; p=0.800; fetal brain: t(19.65=0.34; p=0.741) and fetal brain *TNF* (**Fig. 3G**; t(14.56)=1.10; p=0.288) did

not differ. Together, this suggests that PNS fails to initiate an inflammatory cascade within the fetal brain when microbes are not present.

Prenatal Stress Induces a Pro-Inflammatory Phenotype in Fetal Microglia

To identify a potential source for the fetal brain inflammation observed following PNS, we examined microglia in the WT fetal brain on GD17. There was a trending increase in the number of Iba-1 positive microglia in the fetal hippocampus following exposure to PNS (**Fig. 4A-B**; t(10)=1.963; p=0.08). Additionally, microglia isolated from fetal brains expressed higher levels of pro-inflammatory cytokines including *IL1B* (**Fig. 4C**; p=0.02) and *TNF* (**Fig. 4C**; p=0.02), with a trending increase in *IL6* (**Fig. 4C**; p=0.10) and no change in *CCL2* expression (**Fig. 4C**; p=0.18). Altogether, this suggests that PNS increases the number of microglia in the fetal brain and induces a pro-inflammatory microglial phenotype during development.

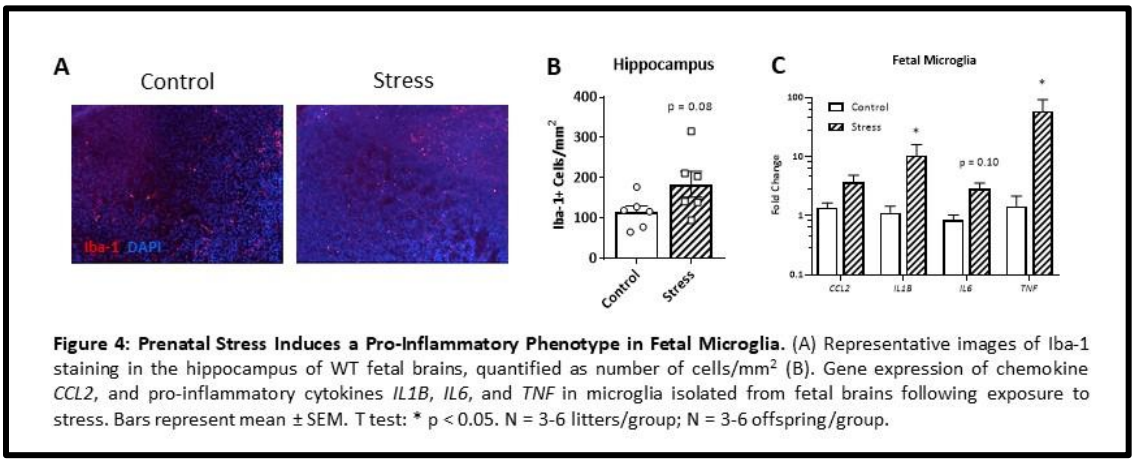

Figure 4: Prenatal Stress Induces a Pro-Inflammatory Phenotype in Fetal Microglia. (A) Representative images of Iba-1 staining in the hippocampus of WT fetal brains, quantified as number of cells/mm^2 (B). Gene expression of chemokine *CCL2*, and pro-inflammatory cytokines *IL1B*, *IL6*, and *TNF* in microglia isolated from fetal brains following exposure to stress. Bars represent mean ± SEM. T test: * $p < 0.05$. N = 3-6 litters/group; N = 3-6 offspring/group.

Prenatal Stress Leads to CCL2-Dependent Behavioral Abnormalities in Adult Offspring

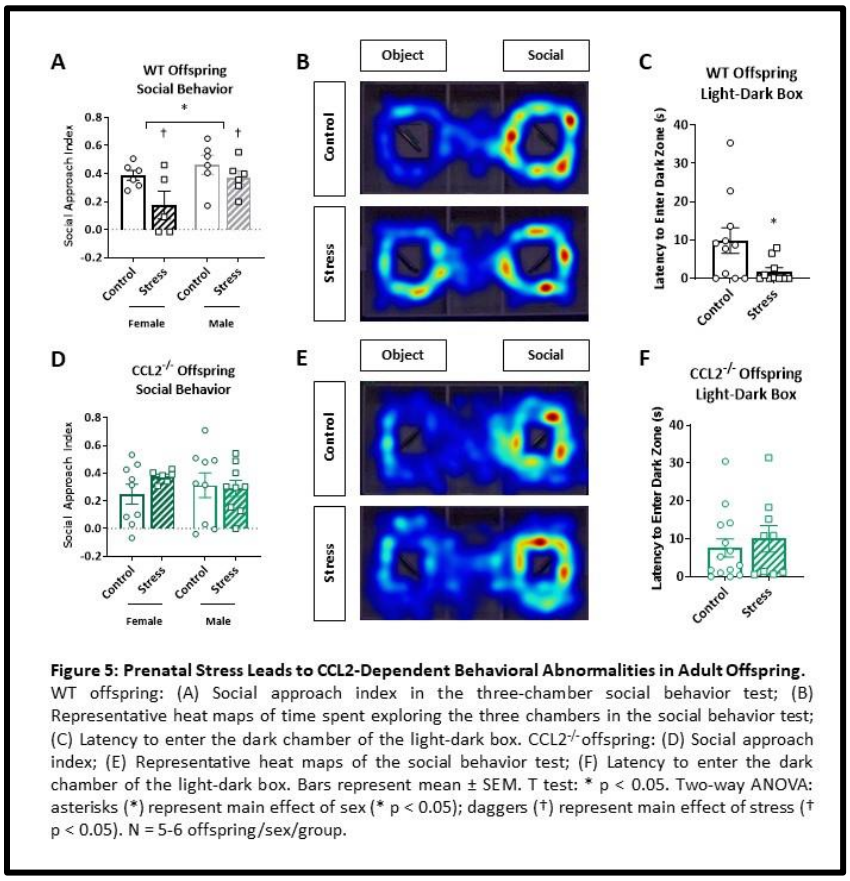

Figure 5: Prenatal Stress Leads to CCL2-Dependent Behavioral Abnormalities in Adult Offspring. WT offspring: (A) Social approach index in the three-chamber social behavior test; (B) Representative heat maps of time spent exploring the three chambers in the social behavior test; (C) Latency to enter the dark chamber of the light-dark box. $CCL2^{-/-}$ offspring: (D) Social approach index; (E) Representative heat maps of the social behavior test; (F) Latency to enter the dark chamber of the light-dark box. Bars represent mean ± SEM. T test: * $p < 0.05$. Two-way ANOVA: asterisks (*) represent main effect of sex (* $p < 0.05$); daggers (†) represent main effect of stress († $p < 0.05$). N = 5-6 offspring/sex/group.

WT PNS offspring exhibited deficits in social approach behavior (**Fig. 5A**; main effect of stress: $f(1,19)=5.61$; $p=0.029$, also reflected in representative heat maps, **Fig 5B**) that were sex-dependent (main effect of sex: $f(1,19)=4.60$; $p=0.045$) . In the light-dark preference test, PNS offspring had decreased latency to enter the dark zone (**Fig. 5C**; $t(11.62)=2.34$; $p=0.038$). Stress did not alter locomotor activity or initial side-preference within the social apparatus (data not shown). Notably, $CCL2^{-/-}$ offspring were protected from social deficits, as measured by the social approach index (**Fig. 5D-E**; stress effect: $f(1,29)=0.54$; $p = 0.470$) and did not display sex differences (sex effect: $f(1,26)=0.02$; $p=0.883$). Similarly, $CCL2^{-/-}$ offspring did not differ in their latency to enter the dark zone during light-dark preference testing (**Fig. 5F**; $t(21)=0.59$; $p=0.560$). Together, our data indicate that PNS results in social- and anxiety-related behavioral abnormalities in the offspring, which are ameliorated in the absence of CCL2.

Discussion

We provide evidence that maternal microbes and the chemokine CCL2 play critical roles in mediating the sequelae of PNS, including intrauterine inflammation and offspring behavioral

abnormalities. This is the first time, to our knowledge, that maternal microbes and CCL2 have been linked to intrauterine inflammation and that CCL2 has been implicated in mediating offspring behavioral deficits following PNS.

Recent clinical and pre-clinical studies suggest that PNS leads to inflammation in the intrauterine environment[5,6,20,36,37]. To extend these findings, we demonstrate that PNS increases CCL2 protein levels in the placenta and fetal brain. As a chemokine, CCL2 recruits circulating CCR2+ monocytes to infiltrate sites of active inflammation and mature into tissue macrophages or produce cytokines[21]. Thus, the reduced expression of *CCR2* in the fetal brain could be due to a compensatory mechanism to counteract detrimental elevations in CCL2. As expected, a decrease in fetal brain *CCR2* expression following PNS was not present in $CCL2^{-/-}$ mice.

Previously, our lab has shown that restraint stress alters the maternal gut microbiome[5]. Stress can increase permeability of the gut epithelial barrier, allowing microbes or microbial components to escape from the gut lumen and enter the bloodstream[38–40]. The presence of microbes in the intrauterine environment is highly controversial[41–43], and was not investigated in the present study. Instead, GF tissues were examined to ascertain the necessity of maternal microbes in mediating the immunomodulatory effects of PNS in the intrauterine environment. To our knowledge, this study is the first to examine the GF intrauterine environment in the context of PNS, though prior studies have shown that GF mice exhibit aberrant behavior[44,45] and an exaggerated stress response[46]. While placental CCL2 tended to increase in PNS GF animals, the GF fetal brain appeared to be protected from inflammatory signaling, with no change in CCL2 protein or *CCR2* expression.

We next investigated the contribution of CCL2 and maternal microbes to PNS-induced inflammation in the intrauterine environment. Even if viable microbes are not present in the

placenta, microbial components in the maternal bloodstream can initiate an immune response within the uterus by signaling through TLRs[47]; thus, we investigated bacterial lipopolysaccharide (LPS)-receptor TLR4 as a potential mediator of stress-induced inflammation. In our model, restraint stress increased *TLR4* gene expression in both WT and CCL2$^{-/-}$ placentas, and decreased expression in the fetal brain, suggesting that microbes or microbial components may be reaching the placenta and initiating an immune response. Furthermore, these data indicate that PNS-induced increases in *TLR4* are independent of CCL2 production in the intrauterine environment, despite lower *TLR4* expression in CCL2$^{-/-}$ placentas at baseline. Of note, PNS did not induce significant changes in placental or fetal brain *TLR4* expression under GF conditions.

In addition to the alterations in *TLR4* expression, cytokine expression was also dysregulated in the intrauterine environment following exposure to PNS. Specifically, stress enhanced expression of the pro-inflammatory cytokine *IL6* in the WT fetal brain, which was ameliorated in the CCL2$^{-/-}$ fetal brain. However, *TNF* expression was elevated in both WT and CCL2$^{-/-}$ fetal brains, suggesting that IL-6 production, in particular, might be dependent upon CCL2 signaling. These data are in line with previous reports demonstrating that recombinant CCL2 administered directly into the brain increases production of IL-6, but not TNF-α[48]. Overall, we theorize that PNS enhances CCL2 secretion in the fetal brain, which is necessary for *IL6* upregulation. PNS did not alter fetal brain *IL6* or *TNF* expression under GF conditions, suggesting that PNS does not induce fetal neuroimmune activation in the absence of maternal microbes. Since CCL2 levels are lower in GF mice compared to WT mice at baseline[49], our findings further support the idea that PNS increases *IL6* expression in the fetal brain in normal CCL2 conditions, but fails to do so when CCL2 is low or absent.

In contrast to the fetal brains, PNS did not alter placental *IL6* expression in WT or CCL2$^{-/-}$ mice. However, PNS increased *TNF* expression in CCL2$^{-/-}$ placentas, but not in WT. Interestingly, in the absence of microbes, PNS also elevated expression of *TNF* in the placenta. Together, this may indicate that CCL2 suppresses placental *TNF* expression following PNS, which is distinct from the function of CCL2 in the developing brain. This is further consistent with the finding that administration of LPS to CCL2$^{-/-}$ mice increases serum TNF-α to a greater degree than in WT mice[50,51], indicating that CCL2 may be suppressing or modulating TNF-α production in the face of an immune challenge.

One consideration in using GF and CCL2$^{-/-}$ mice is that their immune systems develop in a dramatically different fashion compared to WT mice. Indeed, exposure to commensal microbes is necessary for education and priming of immune cells, and GF mice have been shown to have deficits in function of both the innate and adaptive immune system[52,53]. However, there is evidence that GF mice are capable of producing IL-6 in response to restraint stress similar to WT mice[54], suggesting that the absence of *IL6* upregulation in GF fetal brains cannot be explained by baseline developmental differences in IL-6 production. On the other hand, CCL2$^{-/-}$ mice have impairments in recruiting monocytes to sites of infection, though the number of circulating leukocytes is similar to WT mice[55]. In our model, we observed slight but significant decreases in *IL6* expression in CCL2$^{-/-}$ tissue compared to WT at baseline, but not *TNF*. Others have also shown that CCL2$^{-/-}$ mice are capable of mounting measurable, albeit blunted, neuroimmune responses to a peripheral immune challenge[50], and that astrocytes from CCL2$^{-/-}$ brains are capable of increasing production of IL-6 and TNF in response to *in vitro* challenge[56]. These *in vivo* and *in vitro* responses are easily differentiated from baseline differences due to KO status,

suggesting that the PNS-induced alterations in cytokine expression observed in our model cannot be attributed solely to developmental differences.

Given that PNS induced changes in fetal brain cytokine expression, we next aimed to identify a potential source of the inflammation. Since microglia are the resident innate immune cell of the brain and begin to populate the developing brain around gestational day 8, we investigated the effects of PNS on fetal microglia in our restraint stress model. There was a trending increase in the number of Iba-1 positive microglia in the fetal hippocampus following exposure to PNS. Taken in conjunction with previous findings in our lab and others that PNS leads to increased Iba-1 staining in adult offspring[4,7], this indicates that PNS has long-lasting effects on microglia with origins *in utero*. In addition to increasing the number of microglia in the fetal hippocampus, PNS also resulted in increased expression of pro-inflammatory cytokines in microglia isolated from the fetal brain, including *IL6* and *TNF*, indicating that microglia are likely the source of the pro-inflammatory cytokines observed in the whole fetal brain. These data are consistent with evidence that PNS leads to increased cytokine expression and production by microglia isolated from neonatal rat brains[31]. Interestingly, *CCL2* expression in the microglia was not altered by PNS; however, CD11b is also expressed on circulating monocytes[57], which were also isolated in our magnetic bead separation protocol in addition to the resident microglia. Since microglia have been shown to recruit monocytes to the brain by releasing CCL2 in response to stress[22], it is possible that the CD11b+ fetal microglia may be producing more CCL2 in the context of PNS, but not the CD11b+ monocytes. More precise isolation techniques may be required to fully resolve the functional changes in the fetal microglia and monocytes in response to PNS.

PNS is associated with anxiety- and depressive-like behaviors in offspring, and deficits in social interaction characteristic of autism; this is evident both in rodent models[4–8,58,59] and in humans[60,61]. Here, we show that PNS indeed leads to altered social and anxiety-like behaviors in adult offspring, which aligns with our previous findings[4,5]. Furthermore, our current dataset suggests that offspring behavioral deficits can manifest regardless of sex, though male offspring demonstrated greater preference for social stimuli compared to female offspring. The etiology of behavioral disruption is complex, often case-specific, and influenced by a multitude of factors. One such factor is exposure to elevated cytokines, particularly IL-6, during gestation[62,63]. Administration of IL-6 to pregnant mice induces offspring anxiety-like behavior[64], and deletion of IL-6 receptor in trophoblast cells ameliorates deficits in sociability due to maternal inflammation[65]. Since PNS increased fetal brain IL-6, and this was mitigated in the absence of CCL2, we hypothesized that CCL2 would play an indirect role in regulating the development of aberrant behavior through the modification of IL-6. Indeed, the sociability deficits and anxiety-like behavior observed in WT adult offspring were ameliorated in $CCL2^{-/-}$ offspring. While the use of a global CCL2 KO animal in the present study indicates the necessity of CCL2 in disrupting the development of certain behavioral circuits, the exact timing and mechanism by which CCL2 acts prenatally still needs to be investigated. Conclusions about the role of microbes in behavioral regulation are limited by the baseline aberrant behavior observed GF mice, including social deficits and anxiolytic behavior[44,66–68]. Careful and meticulous dissection of behavioral outcomes in GF offspring due to PNS, not explored in the current study, represents an avenue for future investigation.

Despite the prevalence of stress in modern day society, and the deleterious effects of maternal stress exposure on neurodevelopment, options for treatment or prophylaxis are

suboptimal. Here, we provide evidence of the critical role of microbes and CCL2 in mediating fetal brain inflammation following maternal stress and leading to aberrant sociability and anxiety-like behavior in adult offspring. Sex differences among certain parameters, but not all, indicate that vulnerability to PNS is partially sex-biased. Altogether, these data suggest that the sequelae of maternal stress have prenatal origins, and that maternal microbes and CCL2 are tantalizing targets for developing novel treatments

References

1. Bale TL, Baram TZ, Brown AS, et al. Early life programming and neurodevelopmental disorders. *Biol Psychiatry*. 2010;68(4):314-319. doi:10.1016/j.biopsych.2010.05.028

2. Van den Bergh BRH, van den Heuvel MI, Lahti M, et al. Prenatal developmental origins of behavior and mental health: The influence of maternal stress in pregnancy. *Neurosci Biobehav Rev*. 2017. doi:10.1016/j.neubiorev.2017.07.003

3. Beversdorf DQ, Stevens HE, Jones KL. Prenatal Stress, Maternal Immune Dysregulation, and Their Association With Autism Spectrum Disorders. *Curr Psychiatry Rep*. 2018;20(76). doi:10.1007/s11920-018-0945-4

4. Gur TL, Palkar AV, Rajasekera T, et al. Prenatal stress disrupts social behavior, cortical neurobiology and commensal microbes in adult male offspring. *Behav Brain Res*. 2018;359:886-894. doi:10.1016/j.bbr.2018.06.025

5. Gur TL, Shay L, Palkar AV, et al. Prenatal stress affects placental cytokines and neurotrophins, commensal microbes, and anxiety-like behavior in adult female offspring. *Brain Behav Immun*. 2017;64:50-58. doi:10.1016/j.bbi.2016.12.021

6. Bronson SL, Bale TL. Prenatal stress-induced increases in placental inflammation and offspring hyperactivity are male-specific and ameliorated by maternal antiinflammatory treatment. *Neuroendocrinology*. 2014;155(7):2635-2646. doi:10.1210/en.2014-1040

7. Gumusoglu SB, Fine RS, Murray SJ, Bittle JL, Stevens HE. The role of IL-6 in neurodevelopment after prenatal stress. *Brain Behav Immun*. 2017;65:274-283. doi:10.1016/j.bbi.2017.05.015

8. Weinstock M. Prenatal stressors in rodents: Effects on behavior. *Neurobiol Stress*. 2017;6:3-13. doi:10.1016/j.ynstr.2016.08.004

9. Gur TL, Worly BL, Bailey MT. Stress and the commensal microbiota: Importance in parturition and infant neurodevelopment. *Front Psychiatry*. 2015;6:1-6. doi:10.3389/fpsyt.2015.00005

10. Chen HJ, Gur TL. Intrauterine Microbiota: Missing, or the Missing Link? *Trends Neurosci*. 2019:1-12. doi:10.1016/j.tins.2019.03.008

11. Warner BB. The contribution of the gut microbiome to neurodevelopment and neuropsychiatric disorders. *Pediatr Res*. 2019. doi:10.1038/s41390-018-0191-9

12. Pronovost GN, Hsiao EY. Perinatal Interactions between the Microbiome, Immunity, and Neurodevelopment. *Immunity*. 2019;50(1):18-36. doi:10.1016/j.immuni.2018.11.016

13. Sampson TR, Mazmanian SK. Control of brain development, function, and behavior by the microbiome. *Cell Host Microbe*. 2015;17(5):565-576. doi:10.1016/j.chom.2015.04.011

14. Abrahams VM, Mor G. Toll-like receptors and their role in the trophoblast. *Placenta*. 2005;26(7):540-547. doi:10.1016/j.placenta.2004.08.010

15. Abrahams VM, Bole-Aldo P, Kim YM, et al. Divergent Trophoblast Responses to

Bacterial Products Mediated by TLRs. *J Immunol*. 2004;173(7):4286-4296. doi:10.4049/jimmunol.173.7.4286

16. Boufaied I, Jones RL, Girard S, et al. Differential effect of LPS and IL-1β in term placental explants. *Placenta*. 2018;75:9-15. doi:10.1016/j.placenta.2018.11.006

17. Ahmad R, Al-roub A, Kochumon S, et al. The Synergy between Palmitate and TNF- α for CCL2 Production Is Dependent on the TRIF/IRF3 Pathway: Implications for Metabolic Inflammation. *J Immunol*. 2018;200:3599-3611. doi:10.4049/jimmunol.1701552

18. Khosravi A, Yáñez A, Price JG, et al. Gut microbiota promote hematopoiesis to control bacterial infection. *Cell Host Microbe*. 2014;15(3):374-381. doi:10.1016/j.chom.2014.02.006

19. Castillo-Ruiz A, Mosley M, George AJ, et al. The microbiota influences cell death and microglial colonization in the perinatal mouse brain. *Brain Behav Immun*. 2018;67:218-229. doi:10.1016/j.bbi.2017.08.027

20. Lian S, Guo J, Wang L, et al. Impact of prenatal cold stress on placental physiology, inflammatory response, and apoptosis in rats. *Oncotarget*. 2017;8(70):115304-115314. doi:10.18632/oncotarget.23257

21. Deshmane SL, Kremlev S, Amini S, Sawaya BE. Monocyte Chemoattractant Protein-1 (MCP-1): An Overview. *J Interf Cytokine Res*. 2009;29(6):313-326. doi:10.1089/jir.2008.0027

22. Mckim DB, Weber MD, Niraula A, et al. Microglial recruitment of IL-1β-producing monocytes to brain endothelium causes stress-induced anxiety. *Mol Psychiatry*. 2018;23(6):1421-1431. doi:10.1038/mp.2017.64

23. Miller AH, Raison CL. The role of inflammation in depression: From evolutionary imperative to modern treatment target. *Nat Rev Immunol*. 2016;16(1):22-34. doi:10.1038/nri.2015.5

24. Renaud SJ, Sullivan R, Graham CH. Tumour Necrosis Factor Alpha Stimulates the Production of Monocyte Chemoattractants by Extravillous Trophoblast Cells via Differential Activation of MAPK Pathways. *Placenta*. 2009;30(4):313-319. doi:10.1016/j.placenta.2009.01.001

25. Pavličev M, Wagner GP, Chavan AR, et al. Single-cell transcriptomics of the human placenta: inferring the cell communication network of the maternal-fetal interface. *Genome Res*. 2017;27(3):349-361. doi:10.1101/gr.207597.116

26. Du MR, Wang SC, Li DJ. The integrative roles of chemokines at the maternal-fetal interface in early pregnancy. *Cell Mol Immunol*. 2014;11(5):438-448. doi:10.1038/cmi.2014.68

27. Ginhoux F, Greter M, Leboeuf M, et al. Fate Mapping Analysis Reveals That Microglia Derive from Primitive Macrophages. *Science (80-)*. 2010;701(November):841-845. doi:10.1126/science.1194637

28. Kierdorf K, Erny D, Goldmann T, et al. Microglia emerge from erythromyeloid precursors

via Pu.1-and Irf8-dependent pathways. *Nat Neurosci*. 2013;16(3):273-280. doi:10.1038/nn.3318

29. Bolós M, Perea JR, Terreros-Roncal J, et al. Absence of microglial CX3CR1 impairs the synaptic integration of adult-born hippocampal granule neurons. *Brain Behav Immun*. 2018;68:76-89. doi:10.1016/j.bbi.2017.10.002

30. Lenz KM, Nelson LH. Microglia and beyond: Innate immune cells as regulators of brain development and behavioral function. *Front Immunol*. 2018;9. doi:10.3389/fimmu.2018.00698

31. Ślusarczyk J, Trojan E, Głombik K, et al. Prenatal stress is a vulnerability factor for altered morphology and biological activity of microglia cells. *Front Cell Neurosci*. 2015;9(March):1-14. doi:10.3389/fncel.2015.00082

32. Diz-Chaves Y, Pernía O, Carrero P, Garcia-Segura LM. Prenatal stress causes alterations in the morphology of microglia and the inflammatory response of the hippocampus of adult female mice. *J Neuroinflammation*. 2012;9(71). doi:10.1186/1742-2094-9-71

33. Götz M, Huttner WB. The cell biology of neurogenesis. *Nat Rev Mol Cell Biol*. 2005;6(10):777-788. doi:10.1038/nrm1739

34. Müntener M, Hsu YC. Development of the trophoblast and placenta of the mouse. *Acta Anat*. 1977;98:241-252.

35. Schambra U. *Prenatal Mouse Brain Atlas*. Vol 53. Springer Science+Business Media, LLC; 2008. doi:10.1017/CBO9781107415324.004

36. Hantsoo L, Kornfield S, Anguera MC, Epperson CN. Inflammation: A Proposed Intermediary Between Maternal Stress and Offspring Neuropsychiatric Risk. *Biol Psychiatry*. 2019;85(2):97-106. doi:10.1016/j.biopsych.2018.08.018

37. Miller GE, Borders AE, Crockett AH, et al. Maternal socioeconomic disadvantage is associated with transcriptional indications of greater immune activation and slower tissue maturation in placental biopsies and newborn cord blood. *Brain Behav Immun*. 2017;64:276-284. doi:10.1016/j.bbi.2017.04.014

38. Kéri S, Szabó C, Kelemen O. Expression of Toll-Like Receptors in peripheral blood mononuclear cells and response to cognitive-behavioral therapy in major depressive disorder. *Brain Behav Immun*. 2014;40:235-243. doi:10.1016/j.bbi.2014.03.020

39. Kiecolt-Glaser JK, Wilson SJ, Bailey MT, et al. Marital distress, depression, and a leaky gut: Translocation of bacterial endotoxin as a pathway to inflammation. *Psychoneuroendocrinology*. 2018;98(August):52-60. doi:10.1016/j.psyneuen.2018.08.007

40. Maes M, Kubera M, Leunis J-C. The gut-brain barrier in major depression- translocation of LPS from gram negative enterobacteria plays a role in the inflammatory pathophysiology of depression. *Neuroendocrinol Lett*. 2008;29(1):117-124. doi:NEL290108A12 [pii]

41. Lauder AP, Roche AM, Sherrill-Mix S, et al. Comparison of placenta samples with contamination controls does not provide evidence for a distinct placenta microbiota.

Microbiome. 2016;4:1-11. doi:10.1186/s40168-016-0172-3

42. Aagaard K, Ma J, Antony KM, Ganu R, Petrosino J, Versalovic J. The Placenta Harbors a Unique Microbiome. *Sci Transl Med*. 2015;6(237):229-262. doi:10.1007/978-1-4614-5915-6

43. Leiby JS, McCormick K, Sherrill-Mix S, et al. Lack of detection of a human placenta microbiome in samples from preterm and term deliveries. *Microbiome*. 2018;6(1):196. doi:10.1186/s40168-018-0575-4

44. Desbonnet L, Clarke G, Shanahan F, Dinan TG, Cryan JF. Microbiota is essential for social development in the mouse. *Mol Psychiatry*. 2014;19(2):146-148. doi:10.1038/mp.2013.65

45. Luczynski P, Neufeld KAMV, Oriach CS, Clarke G, Dinan TG, Cryan JF. Growing up in a bubble: Using germ-free animals to assess the influence of the gut microbiota on brain and behavior. *Int J Neuropsychopharmacol*. 2016;19(8):1-17. doi:10.1093/ijnp/pyw020

46. Sudo N, Chida Y, Aiba Y, et al. Postnatal microbial colonization programs the hypothalamic-pituitary-adrenal system for stress response in mice. *J Physiol*. 2004;558(1):263-275. doi:10.1113/jphysiol.2004.063388

47. Iwasaki A, Medzhitov R. Control of adaptive immunity by the innate immune system. *Nat Immunol*. 2015;16(4):343-353. doi:10.1038/ni.3123

48. Rankine EL, Hughes PM, Botham MS, Perry VH, Felton LM. Brain cytokine synthesis induced by an intraparenchymal injection of LPS is reduced in MCP-1-deficient mice prior to leucocyte recruitment. *Eur J Neurosci*. 2006;24:77-86. doi:10.1111/j.1460-9568.2006.04891.x

49. Swiecki M, Miller H, Sesti-costa R, Cella M, Gilfillan S, Colonna M. Microbiota induces tonic CCL2 systemic levels that control pDC trafficking in steady state. *Mucosal Immunol*. 2017;10(4):936-945. doi:10.1016/j.physbeh.2017.03.040

50. Thompson WL, Karpus WJ, Van Eldik LJ. MCP-1-deficient mice show reduced neuroinflammatory responses and increased peripheral inflammatory responses to peripheral endotoxin insult. *J Neuroinflammation*. 2008;5:1-13. doi:10.1186/1742-2094-5-35

51. DePaolo RW, Lathan R, Rollins BJ, Karpus WJ. The chemokine CCL2 is required for control of murine gastric Salmonella enterica infection. *Infect Immun*. 2005;73(10):6514-6522. doi:10.1128/IAI.73.10.6514-6522.2005

52. Round JL, Mazmanian SK. The gut microbiome shapes intestinal immune responses during health and disease. *Nat Rev Immunol*. 2009;9(5):313-323. doi:10.1038/nri2515.The

53. Kuhn KA, Stappenbeck TS. Peripheral education of the immune system by the colonic microbiota. *Semin Immunol*. 2013;25(5). doi:10.1038/jid.2014.371

54. Nukina H, Sudo N, Aiba Y, Oyama N, Koga Y, Kubo C. Restraint stress elevates the plasma interleukin-6 levels in germ-free mice. *J Neuroimmunol*. 2001;115(1-2):46-52. doi:10.1016/S0165-5728(01)00260-0

55. Lu B, Rutledge BJ, Gu L, et al. Abnormalities in Monocyte Recruitment and Cytokine Expression in Monocyte Chemoattractant Protein 1 – deficient Mice. *J Exp Med*. 1998;187(4):601-608.

56. Semple BD, Frugier T, Morganti-Kossmann MC. CCL2 modulates cytokine production in cultured mouse astrocytes. *J Neuroinflammation*. 2010;7:1-11. doi:10.1186/1742-2094-7-67

57. Haage V, Semtner M, Vidal RO, et al. Comprehensive gene expression meta-analysis identifies signature genes that distinguish microglia from peripheral monocytes/macrophages in health and glioma. *Acta Neuropathol Commun*. 2019;7(1):1-18. doi:10.1186/s40478-019-0665-y

58. Zohar I, Shoham S, Weinstock M. Perinatal citalopram does not prevent the effect of prenatal stress on anxiety, depressive-like behaviour and serotonergic transmission in adult rat offspring. *Eur J Neurosci*. 2016;43(4):590-600. doi:10.1111/ejn.13150

59. Salomon S, Bejar C, Schorer-Apelbaum D, Weinstock M. Corticosterone Mediates Some but Not Other Behavioural Changes Induced by Prenatal Stress in Rats. *J Neuroendocrinol*. 2011;23(2):118-128. doi:10.1111/j.1365-2826.2010.02097.x

60. Ramborger ME, Zorrilla Zubilete MA, Beatriz Acosta G. Prenatal Stress and its effects of human cognition, behavior and psychopathology: A review of the literature. *Pediatr Dimens*. 2018;3(1):1-6. doi:10.15761/pd.1000159

61. Beydoun H, Saftlas AF. Physical and mental health outcomes of prenatal maternal stress in human and animal studies: A review of recent evidence. *Paediatr Perinat Epidemiol*. 2008;22(5):438-466. doi:10.1111/j.1365-3016.2008.00951.x

62. Graham AM, Rasmussen JM, Rudolph MD, et al. Maternal Systemic Interleukin-6 During Pregnancy Is Associated With Newborn Amygdala Phenotypes and Subsequent Behavior at 2 Years of Age. *Biol Psychiatry*. 2018;83(2):109-119. doi:10.1016/j.biopsych.2017.05.027

63. Smith SEP, Li J, Garbett K, Mirnics K, Patterson PH. Maternal Immune Activation Alters Fetal Brain Development through Interleukin-6. *Neurobiol Dis*. 2007;27(40):10695-10702. doi:10.1523/JNEUROSCI.2178-07.2007

64. Hsiao EY, Patterson PH. Activation of the maternal immune system induces endocrine changes in the placenta via IL-6. *Brain Behav Immun*. 2011;25(4):604-615. doi:10.1016/j.bbi.2010.12.017

65. Wu W-L, Hsiao EY, Yan Z, Mazmanian SK, Patterson PH. The Placental Interleukin-6 Signaling Controls Fetal Brain Development and Behavior. *Brain Behav Immun*. 2017;62:11-23. doi:10.1097/CCM.0b013e31823da96d.Hydrogen

66. Diaz Heijtz R, Wang S, Anuar F, et al. Normal gut microbiota modulates brain development and behavior. *Proc Natl Acad Sci U S A*. 2011;108(7):3047-3052. doi:10.1073/pnas.1010529108

67. Clarke G, Grenham S, Scully P, et al. The microbiome-gut-brain axis during early life regulates the hippocampal serotonergic system in a sex-dependent manner. *Mol*

Psychiatry. 2013;18(6):666-673. doi:10.1038/mp.2012.77

68. Luo Y, Zeng B, Zeng L, et al. Gut microbiota regulates mouse behaviors through glucocorticoid receptor pathway genes in the hippocampus. *Transl Psychiatry*. 2018;8(1). doi:10.1038/s41398-018-0240-5