The Role of Maternal Stress and Gut Microbiome in Modulating Maternal, Fetal, and Adult Offspring Immune Function and Offspring Behavioral Outcomes.

Undergraduate Honors Research Thesis

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Allison Bischoff

Undergraduate Molecular Genetics Major

School of Arts and Sciences

The Ohio State University

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Project Advisors:

Dr. Tamar Gur, Department of Neuroscience, Department of Obstetrics and Gynecology, Department of Psychiatry and Behavioral Health

Dr. Harold Fisk, Department of Molecular Genetics

TABLE OF CONTENTS

Abstract

Exposure to stress *in utero* is associated with the development of mood and neurodevelopmental disorders in offspring, including generalized anxiety disorder, depression, and autism spectrum disorder (ASD). While the mechanisms underlying how prenatal stress (PNS) modulates offspring neurodevelopment are largely unknown, a significant body of research has investigated the role of maternal immune dysregulation in eliciting stress-related neurodevelopmental effects in offspring. Due to the well-documented cross-talk between the host immune system and gut microbiome, we hypothesized that stress-induced disruptions of the maternal gut microbiome could reshape maternal and fetal immune function—consequently impacting neurodevelopment as reflected by changes in offspring behavior. Using antibiotics to disrupt the maternal gut microbiome in combination with our mouse model of prenatal stress, immune function was evaluated in maternal, fetal, and adult offspring tissues using RT-qPCR to assess relative mRNA abundance for immunomodulatory genes. PNS and antibiotic conditions had broadly immunosuppressive effects in the maternal gastrointestinal (GI) tract. Sex-specific analyses in the placenta and fetal brain revealed differential effects of PNS and antibiotics on cytokine and chemokine mRNA concentrations in the male placenta and female fetal brain, as well as trending, but not significant, alterations in adult offspring microglia. PNS and antibiotics did not elicit changes in adult offspring behavior as measured by limited replicates of marble burying and light-dark preference tests. Overall, these findings further reveal the complex interactions between PNS, maternal gut dysbiosis, and maternal and fetal immune function.

Acknowledgements

I would like to thank my research advisor Dr. Tamar Gur for her support over the past three years. Despite entering her lab without any prior to knowledge in prenatal stress and neuroimmunology, her passion and encouragement has kept me inspired throughout the hardships presented by the COVID-19 pandemic. Importantly, this thesis project would not have been possible without the exceptional mentorship of MSTP student Helen Chen. Beyond being an incredibly kind person, she is a critically-thinking scientist who finds fulfillment through teaching others. Allowing me to join her on this journey to understand the connections between the gut microbiome and prenatal stress, she has taught me wet lab skills, the nuances of scientific communication and data interpretation, and what to look for in future mentor relationships.

Both Dr. Tamar Gur and Helen Chen have been key female role models in my scientific development, helping me to develop the confidence and skills that will allow me to succeed in University of Michigan's PhD program in Biomedical Sciences this fall. I also want to thank Dr. Kathryn Lenz and Dr. Harold Fisk for serving on my defense committee. Lastly, I want to thank all of the previous and current members of the Gur Lab that I have interacted with for joining me on this journey, including Dr. Adrienne Antonson, Dr. Jeff Galley, Therese Rajasekera, Sydney Schiff, Hannah Rashidi, Zach Waite, Jen Smith, and Amber Dalal. Thank you all for the advice, the support, and the good memories!

Chapter 1

Introduction

Maternal stress and subsequent physiological responses have been associated with lasting effects on offspring neurodevelopment and behavior in both animal models and clinical studies[1–4]. Using a model of restraint stress, we have shown that prenatally stressed adult offspring display sex-specific changes in behavior, specifically social deficits in males and anxiety-like behavior in females[5–7]. Although the behavioral effects of prenatal stress (PNS) are case-specific and dependent upon timing of exposure[8], clinical research has also identified sex-specific behavioral effects of PNS[9,10]. Mimicking the behavioral outcomes observed in our model, clinical evidence has associated circulating levels of maternal cortisol with increased anxiety-like behavior and negative emotionality in female, but not male offspring[9,11]. Additionally, as decreased sociability is a canonical symptom of ASD[12], male-specific social deficits in our model correspond with clinical observations of male-biased development of ASD[13,14]. Both of these findings strengthen the clinical relevance of our model.

While the mechanisms underlying how PNS modulates offspring neurodevelopment remain largely unknown, a significant body of research has investigated the role of maternal immune dysregulation in eliciting neurodevelopmental deficits in offspring[6,15–22]. Importantly, pregnancy is characterized by dynamic shifts in immune function that are often generalized as immunosuppressive, with exceptions at blastocyst implantation, placentation, and parturition[23]. The importance of tightly controlled maternal immune function in fetal neurodevelopment has been demonstrated by clinical studies that have associated maternal immune activation with alterations in

offspring behavior and brain structure and connectivity[17,19–21]. Additionally, functional studies in rodents have identified particular pro-inflammatory cytokine signaling pathways, including IL-1β and IL6, that are both sufficient and necessary to alter fetal neurodevelopment in models of maternal immune activation[16,18]. As PNS is associated with maternal and fetal immune dysregulation[5,6,15,22,24], stress-induced changes to immune function during pregnancy may provide a mechanism by which PNS shapes neurodevelopment. Attempting to better understand the immune-mediated mechanisms by which PNS affects fetal development, we have previously demonstrated that our model of restraint stress disrupts immune homeostasis in pregnant dams and within the intrauterine environment[5,6,25].

Notably, it has also been clearly established that psychological stress impacts the composition of the commensal gut microbiome[7,26–28]. In our model of PNS, we have observed altered abundances of bacteria and microbial immunomodulatory genes in colon contents obtained from dams exposed to stress[25]. The influence of psychological stress on gut microbial composition is thought to be mediated by the existence of a gut-brain axis, through which bi-directional communication occurs between the central nervous system (CNS) and gastrointestinal (GI) tract[29,30]. Signals arising from the brain in response to external stimuli can alter intestinal function or vice versa. Through alterations of gut motility, permeability, mucus production, and immune function, autonomic nervous system activation can disrupt homeostasis across the GI tract and, consequently, alter gut microbial composition[29,30]. Intriguingly, modulation of the gut microbiome is also associated with changes in host immune function through

interactions between immune cells and microbial antigens and metabolites at the intestinal mucosal barrier[31–33].

In our model, PNS increases mRNA levels of the pro-inflammatory cytokines *Il6* and *Tnf* in the fetal brain, as well as protein levels of the pro-inflammatory chemokine CCL2 in the placenta and fetal brain[6]. However, these increases are not apparent in germ-free (GF) dams, which lack microbes on all mucosal surfaces. This indicates that in the absence of microbes, restraint stress does not disrupt intrauterine immune homeostasis[6] and implicates the maternal gut microbiome as a key factor in facilitating PNS-induced changes in immune function in the fetal brain. However, results from GF mice have been criticized for their lack of clinical relevance[34] and are confounded by dramatically altered immune development[35], neurodevelopment[34], and baseline behavior[34]. Alternative models of microbial depletion and disruption should thus be employed to confirm the immunomodulatory role of the gut microbiome in response to PNS, including antibiotics administration during gestation.

Due to our previous findings in GF models and the well-recognized role of commensal gut microbes in maintaining immune homeostasis[31,32], we hypothesized that administration of antibiotics would ameliorate the immunomodulatory effects of PNS and associated behavioral phenotypes in adult offspring due to depletion of the maternal gut microbiome. To test this hypothesis, I used a 2x2 factorial design of chronic restraint stress and antibiotics administration during mid-to-late gestation (Figure 1A). Evaluation of immune function was carried out by quantifying mRNA levels of pro-inflammatory cytokines (*Il1b*, *Il6*, and *Tnf*) and chemokines (*Ccl2*). The cytokines explored in this study are largely produced by monocytes and tissue-resident macrophages—in addition

to epithelial and endothelial cells—to propagate inflammatory responses[36,37]. The chemokine CCL2 is involved in the recruitment and migration of monocytes in response to inflammatory stimuli[38], though it has also been implicated in context-specific M1 and M2 macrophage polarization[39]. Macrophages are known for their dynamic plasticity in response to different environmental cues[40], including microbial antigens and metabolites[33,41–43], making them promising mediators of the immunological changes associated with gut dysbiosis and stress.

Quantification of mRNA was first carried out in the maternal GI tract, the primary interface between the host immune system and gut microbes. Analyses were then extended to include the placenta and fetal brain to identify changes in fetal immune function. Additionally, these analyses were conducted on adult offspring microglia—brain-resident macrophages. Due to well-documented sex differences in microglial phenotype and density[44,45], sex-dependent responses of microglia to PNS or maternal gut microbiome depletion could provide a mechanism to explain previously described sexually dimorphic behavioral changes. Finally, repetitive behavior was assessed in the adult offspring using the marble burying test, and anxiety-like behavior was assessed using the light-dark preference test.

Chapter 2:

Methods

2.1. Animals and Experimental Design

C57BL/6 mice were obtained from Jackson Laboratories (Bar Harbor, ME) and housed in the vivarium at The Ohio State University Wexner Medical Center. Mice

were bred over the course of 48 hours and presence of vaginal plugs were used to determine the first gestational day of pregnancy (GD1). Pregnant dams were then randomly assigned different PNS and antibiotic conditions. The PNS dams were subjected to restraint stress from 9:00 am to 11:00 am from GD10-16 using a perforated 50mL conical tube. Non-stressed dams were left undisturbed. A broad-spectrum antibiotic cocktail (0.5 mg/mL vancomycin, 1 mg/mL meropenem, 1mg/mL nystatin, and 1 mg/ mL neomycin) or a vehicle control was administered through drinking water to dams from GD7 until tissue collection or parturition for offspring behavioral experiments. For RT-qPCR, maternal gut, placental, and fetal brain tissues were collected at GD17, frozen on dry ice, and stored at -80°C until processing for RNA. For behavioral experiments, pups were weaned at post-natal day (PND) 28, co-housed with same-sex littermates, and subject to behavioral testing from PND70-76.

2.2. Stool Culturing

Fecal matter was collected from vehicle and antibiotics-assigned dams prior to antibiotics treatment (GD7), after 3 days of treatment prior to the start of restraint stress (GD10), and after 9 days of treatment after the last restraint (GD16) to verify successful depletion of the maternal gut microbiome. Stool was homogenized in 3 mL of PBS. Homogenized stool was serially diluted 1:20 in PBS three times. 50 µL of each of the dilutions and undiluted sample were added to 5 mL of brain heart infusion (BHI) agar and poured into separate petri dishes. Plates were left to set at room temperature for 30 minutes before placing inverted petri dishes in a CO_2-free incubator at 37°C for 24 hours. After 24 hours, colony counting was done manually while blinded to the assigned

conditions. Colony forming units (CFUs) were calculated using the following equation: (# of colonies / 50 µL x 100 µL/mL x dilution factor x 3 mL) / mass of stool.

2.3. Whole Tissue RNA Isolation and Quantitative Real-Time PCR

RNA was isolated from maternal and fetal tissues using Trizol reagent (Invitrogen, Carlsbad, CA) and cDNA synthesis was performed using the High Capacity cDNA Reverse Transcriptase kit (Applied Biosystems, Foster City, CA), per manufacturer's instructions. Using the Taqman Gene Expression Master Mix protocol (Applied Biosystems, Foster City, CA), RT-qPCR reactions were performed. *Gapdh* was used as the housekeeper gene for distal colon, ileum, and fetal brain samples. *Tbp* was used as the housekeeper for placental samples. RT-qPCR data is presented as fold change compared to the vehicle non-stressed group or the female control group for analyses of sex differences, using the $2^{-\Delta\Delta Ct}$ method.

2.4. Microglia and Microglial RNA Isolation

11-12 week old offspring were perfused with ice-cold PBS and brains were collected in PBS and kept on ice until processing. Brains were homogenized using Potter homogenizers, and samples were resuspended 70% stock isotonic percoll (SIP). A discontinuous Percoll gradient was created by layering 50% SIP, 35% SIP, and 1X PBS on top of the 70% SIP Microglia were collected between the 50% and 70% SIP layers, washed with MACS buffer (1% BSA, 1 mM EDTA in 1X PBS), and incubated with CD11b magnetic microbeads (Miltenyi Biotec) for 15 minutes. CD11b+ microglia were isolated using MS columns and a Miltenyi OctoMACS Separator. For RNA isolation, microglia were resuspended in Trizol for cell lysis and kept at -80°C until

further processing. The Zymo Direct-zol Microprep kit was used to isolate RNA, per manufacturer's instructions. Qubit broad range RNA assay was used to quantify and assess quality of isolated RNA. cDNA synthesis, RT-qPCR, and statistics were carried out as previously described (Whole Tissue RNA Isolation and Quantitative Real-Time PCR, p. 20). *Rpl19* was used as the housekeeper for microglial samples.

2.5. Marble Burying Test

All behavioral experiments were completed at PND70-76 and mice were acclimated to the behavior room for one hour prior to testing. For the marble burying test, twenty marbles of assorted colors were equally spaced (4 x 5 grid) in standard cages with fresh bedding. Mice were individually placed in these cages and left undisturbed for 30 minutes. The number of marbles buried was counted by a blind observer to assess repetitive behaviors.

2.6. Light-Dark Preference Test

A 40 x 40 x 25 cm Plexiglass box was divided into two compartments (20 x 40 x 25 cm) by a black Plexiglass separator. A 3 x 10 cm doorway allowed for the mouse to freely transition between light and dark compartments. The dark compartment was covered by black Plexiglass, while the light compartment was illuminated at an intensity of 150 lux. All test mice were placed in the light compartment to start the 5 minute trials. Fusion software (Omnitech Electronics, Inc., Columbus, OH) was used to determine latency to enter the dark compartment, the total time spent in each compartment, and total distance travelled.

2.7. Statistical Analyses

Data was analyzed using GraphPad Prism Software (San Diego, CA). For all analyses, two-way ANOVAs were performed followed by a Tukey's post hoc test to determine possible effects of sex. For tests in which the main effect of sex and the interaction between sex and stress or sex and antibiotics were not significant, data was averaged per litter for further analyses to eliminate the influence of litter-mate effects. To determine the effect of stress and antibiotics, two-way ANOVAs followed by a Tukey's post hoc tests were performed. For comparing stressed and non-stressed offspring (Figure 9A), groups were compared using an unpaired parametric T test. For all tests, the ROUT method (Q = 1%) was use to exclude outliers from analysis and significance was defined as $p < 0.05$.

Chapter 3

Results

3.1. Prenatal stress and antibiotics-mediated gut dysbiosis induce systemic maternal immunosuppression and prenatal stress reduces gestational weight gain.

To deplete the maternal gut microbiome, a cocktail of broad-spectrum antibiotics (neomycin, vancomycin, meropenem, and nystatin) was administered to pregnant dams through drinking water starting from gestational day (GD)7.5, three days prior to the start of restraint stress on GD10.5. Restraint stress was performed for 2 hours per day from GD10.5 to GD16.5, and antibiotic treatment continued until GD17 at tissue collection or until parturition for postnatal studies (Figure 1A). To first validate our model of antibiotics-mediated maternal gut dysbiosis and ensure that antibiotics administered

through drinking water were being consumed, water consumption (mL/day) was measured across conditions. Antibiotics-treated mice did not show reduced consumption of water when compared to vehicle-treated mice (Figure 1B). Fecal samples collected on GD7.5, GD10.5, and GD16.5 were cultured in BHI agar to determine bacterial load, and reduction in colony-forming units/g of stool was observed by GD16.5 (Figure 1C). These results represent a limited sample size (n = 2/group). Thus, future experiments will confirm reduction of gut microbial load through additional stool culturing experiments or 16s RNA-sequencing.

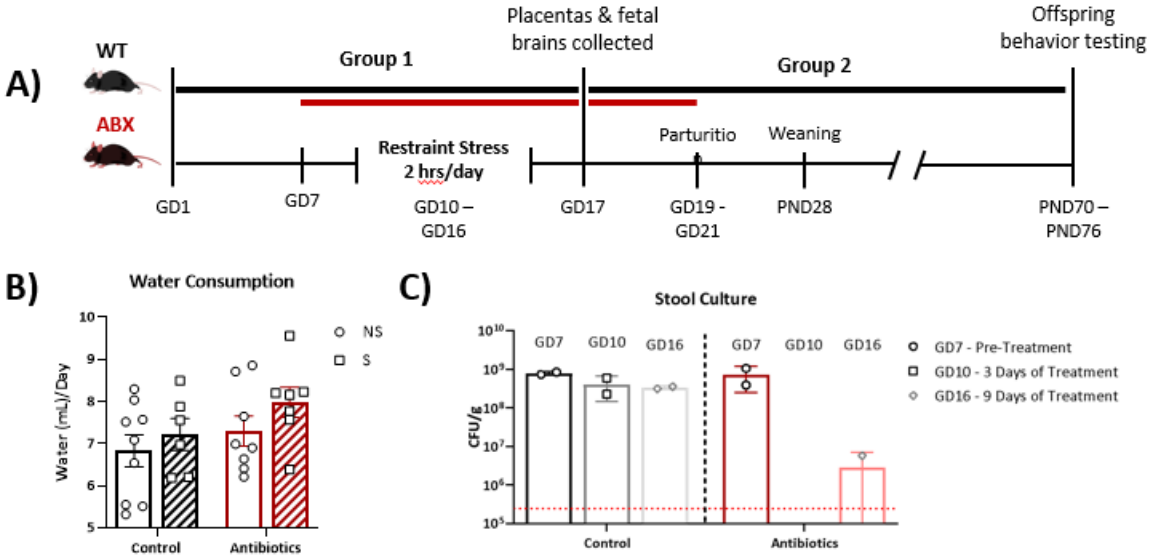

Figure 1. Model of antibiotics reduces maternal gut bacterial load. (A) Study design. (B) Antibiotics administration did not reduce water consumption in pregnant dams (n = 6-9/group). (C) Stool culturing showed a reduction in colony-forming units (CFU) per gram of fecal matter obtained from antibiotics-treated dams. (n = 2/group). GD = gestational day; WT = wild type; ABX = antibiotics.

To assess the impact of PNS and antibiotics administration on pregnancy-related characteristics, I measured maternal spleen mass and litter size on GD17—24 hours following the last round of restraint stress. I also measured gestational body mass

gained from GD10.5-16.5 and average food consumption per day. Both PNS and administration of antibiotics resulted in a reduction in maternal spleen mass (Figure 2A, main effect of stress: f(1,26) = 7.289, p = 0.0120; main effect of antibiotics: f(1,26) = 23.61, p<0.0001), an indicator of systemic maternal immunosuppression.

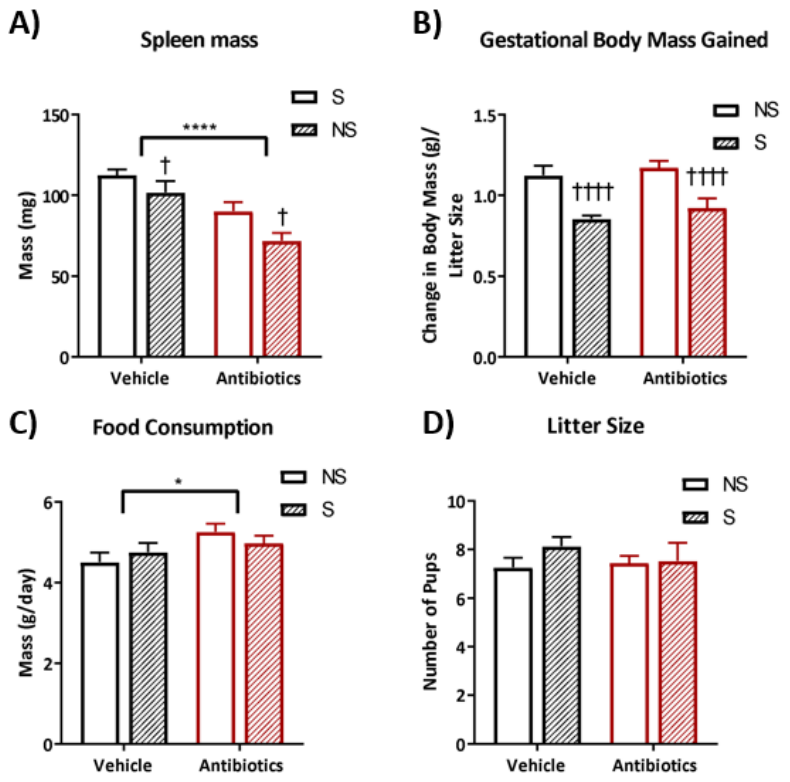

Figure 2. Model of PNS and antibiotics display characteristics of stress and systemic maternal immunosuppression. (A) Spleen mass at GD17 is reduced by PNS and antibiotics (n = 7-8/group). (B) Gestational body mass gained normalized to litter size from GD10.5 to 16.5 is reduced by PNS (n =15-18/group), but PNS does not have a main effect (C) food consumption (n = 9-15/group) or (D) litter size (n = 15-19/group). Although, antibiotics had a main effect of increasing food consumption (C). Two-way ANOVA: asterisks represent main effects of antibiotics (*p < 0.05, ****p<0.0001), daggers represent main effects of stress (†p< 0.05, ††††p<0.0001). Bars represent mean ± SEM.

Additionally, dams exposed to stress gained significantly less body mass from gestational day (GD) 10.5 to GD16.5 when normalized to litter size (Figure 2B, $f(1,61) = 29.38$, $p<0.0001$), which is not due to differences in food consumption (Figure 2C) or litter size across conditions (Figure 2D). Antibiotics administration did not affect gestational body mass gain (Figure 2B), but increased food consumption in dams (Figure 2C, $f(1,43) = 4.461$, $p = 0.0405$). (n = 9-15). Altogether, this suggests that our model of PNS leads to maternal immunosuppression and restricts gestational weight gain, replicating prior findings[25].

3.2. PNS and antibiotics induce immunosuppressive changes in cytokine and chemokine mRNA abundance across the maternal gastrointestinal tract.

I first sought to identify whether PNS induced microbiome-dependent changes in immune function in the maternal GI tract, the primary interface between gut microbes and the host immune system. Analysis of RT-qPCR data in the maternal distal colon and ileum revealed immunosuppressive mRNA profiles associated with both stress and antibiotics administration (Figure 3-4). In the distal colon, stress significantly reduced transcript levels of the pro-inflammatory cytokine TNF (Figure 3A, $f(1,20) = 5.105$, $p = 0.0352$). There was also a trending decrease in *Ccl2* abundance following stress exposure (Figure 3C, $f(1,18) = 3.89$, $p = 0.0641$). Additionally, antibiotics treatment reduced *Tnf* (Figure 3A, $F(1,20) = 6.345$, $p = 0.0204$) and *Il1b* (Figure 3C, $F(1,20) = 10.82$, $p = 0.0037$), though *Il6* abundance was not altered (Figure 3D).

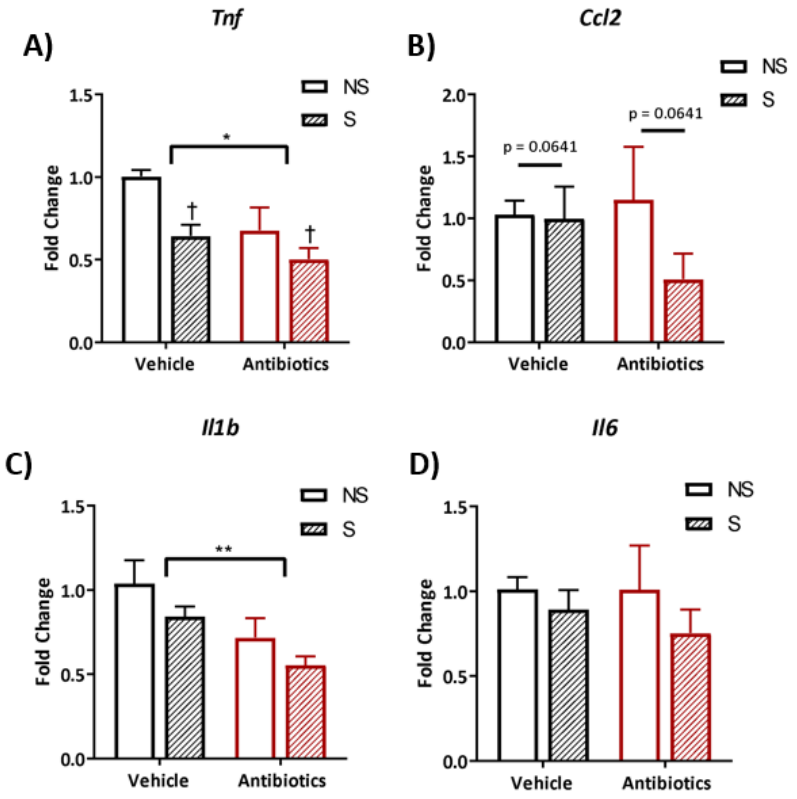

Figure 3. PNS and antibiotics induce immunosuppressive phenotypes as measured by mRNA quantification of cytokines and chemokines in the maternal distal colon. PNS significantly decreased *Tnf* (A) in the maternal distal colon (GD17), and had trending main effect of stress on *Ccl2* (B, f(1,18) = 3.89, p =0.0641). Antibiotics reduced *Tnf* (A) and *Il1b* (C) in the maternal distal colon, while Ccl2 remained unaffected (B). PNS and antibiotics had no effect on *Il6* (D). (n =6-8/group). Two-way ANOVA: asterisks represent main effects of antibiotics (*p < 0.05, **p < 0.01), daggers represent main effects of stress (†p< 0.05). Bars represent mean ± SEM.

In the maternal ileum, antibiotics significantly reduced *Il1b* (Figure 4C, f(1,24) = 34.9, p<0.0001), mimicking the results observed in the distal colon. Similarly, abundance of *Tnf* was also significantly reduced by PNS and antibiotic administration (Figure 4A, main effect of stress: f(1,27) = 6.994, p = 0.0135; main effect of antibiotics: f(1,27) = 6.994, p =0.0135). Moreover, PNS and antibiotics significantly reduced *Ccl2* in the maternal ileum (Figure 4B, main effect of stress: f(1,24) = 5.024, p = 0.0345); main effect of antibiotics: f(1,24) = 7.35, p = 0.0122). Unlike the maternal distal colon, both

stress and antibiotics administration resulted in significant reductions in *Il6* (Figure 4D, main effect of stress: f(1,24) = 5.044, p = 0.0342; main effect of antibiotics: f(1,24) = 18.35, p = 0.0003), a complex pro-inflammatory cytokine and anti-inflammatory myokine.

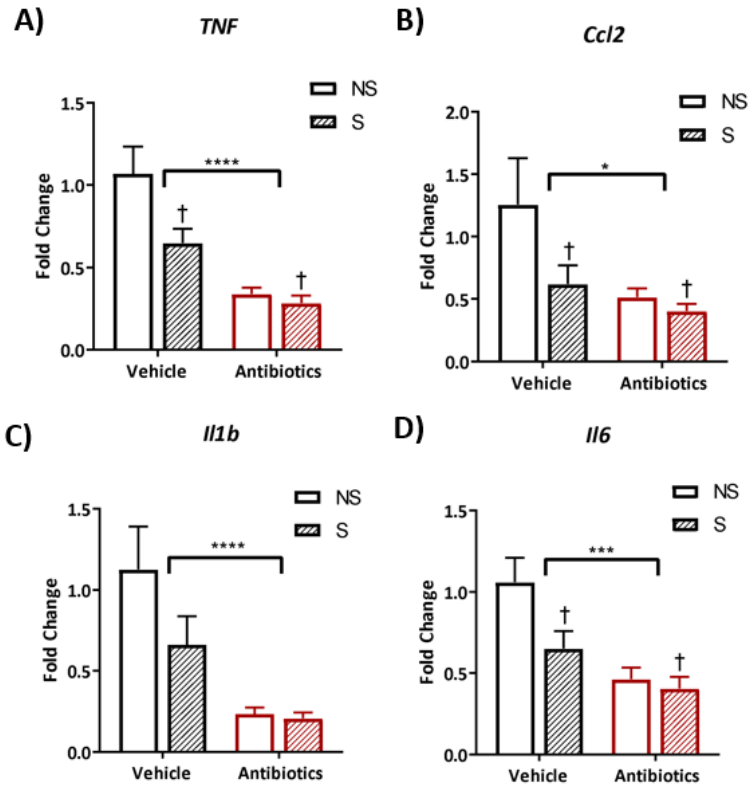

Figure 4. PNS and antibiotics induce immunosuppressive changes in the maternal Ileum as measured by mRNA quantification of cytokines and chemokines. There is a main effect of PNS and a main effect of antibiotics on *Tnf* (A), *Ccl2* (B), and *Il6* (D) in the maternal Ileum at GD17. Antibiotics also reduced *Il1b* (C). (n = 6-8/group). Two-way ANOVA: asterisks represent main effects of antibiotics (*p < 0.05, ***p < 0.001, ****p <0.0001), daggers represent main effects of stress (†p< 0.05). Bars represent mean ± SEM.

3.3. Antibiotics modulate placental abundance of *Ccl2* in a sex-specific and stress-dependent manner.

To identify whether PNS- and antibiotics-mediated changes in immune function extended beyond the maternal GI tract to fetal tissues, I sought to identify changes in placental immune function. One female and one male placenta were collected per dam to analyze sex differences; however, in the absence of sex differences or sex-dependent effects, data were averaged per litter for further analyses.

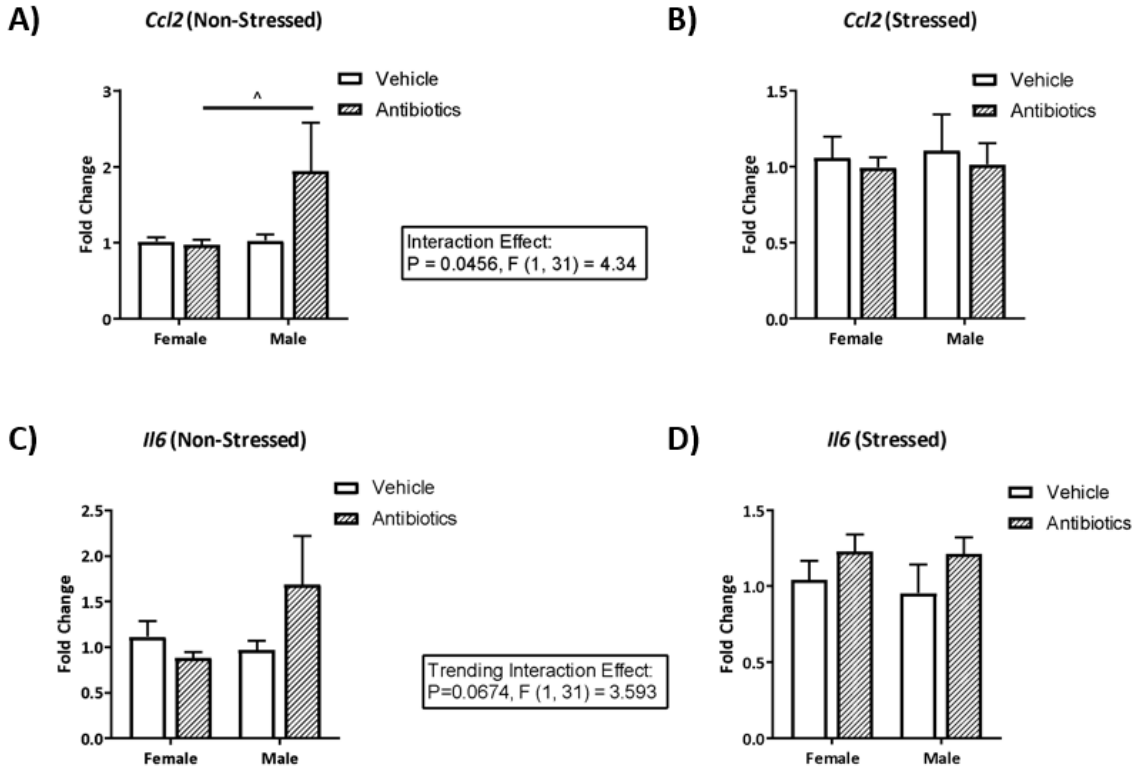

Figure 5. Antibiotic administration in non-stressed dams significantly increases *Ccl2* in male-associated placentas, with similar trends reflected in *Il6*. A sex x antibiotics interaction effect was observed for placental abundance of *Ccl2* in non-stressed dams (A). *Ccl2* was increased in antibiotics-treated male placentas relative to antibiotics-treated female placentas (A). This antibiotics-induced, male-specific increase was not apparent in placentas from PNS conditions (B). There was a similar trending effect observed with placental *Il6* (C, f(1,31) = 3.593, p = 0.0674). Male-specific, antibiotic-induced increases in *Il6* were not apparent in placentas exposed to PNS (D). (n = 5-10/group). Two-way ANOVA: carets represent interaction effects between sex and antibiotics (^p < 0.05). Bars represent mean ± SEM.

Sex x stress and sex x antibiotics two-way ANOVAs revealed sex-specific changes in placental *Ccl2* abundance in response to maternal exposure to antibiotics. Specifically, there was a sex x antibiotics interaction in placental *Ccl2* abundance in non-stressed tissues (Figure 5A, f(1,31) = 4.34, p = 0.0456). Tukey's post-hoc test revealed a significant increase in *Ccl2* in antibiotics-treated male placentas relative to antibiotics-treated female placentas (p = 0.0406). Similarly, there was a trending sex x antibiotics interaction effect on levels of placental *Il6* (Figure 5C, f(1,31) = 3.593, p = 0.0674). Interestingly, antibiotics-induced increases in male placenta *Ccl2* and *Il6* were not observed in the PNS condition (Figure 5B, D).

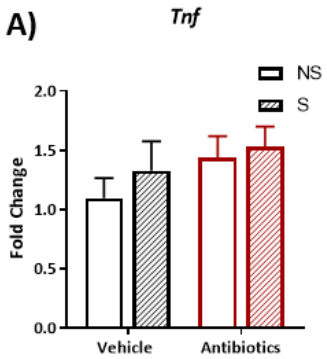

Figure 6. PNS and antibiotics do not induce overt changes in *Tnf* abundance in whole placentas. mRNA quantification of cytokines and chemokines in the placenta reveal no effects of PNS or antibiotics on abundance of *Il6* (A) or *Tnf* (B). (n = 6-10/group). Bars represent mean ± SEM.

No main effects of sex or sex-dependent effects were observed in placental abundance of *Tnf* (data not shown), and further by litter did not reveal any changes in *Tnf* abundance in response to PNS or antibiotics (Figure 6).

3.4. PNS increases abundance of *Tnf* in female fetal brains from vehicle-treated dams.

Next, I quantified cytokine and chemokine mRNA in the fetal brain to identify neuroinflammatory sequelae of PNS and maternal antibiotics treatment. Sex-specific analyses revealed a significant sex x stress interaction effect on *Tnf* abundance in fetal brains (Figure 7A, f(1,26) =5.279, p = 0.0299). In particular, Tukey's post-hoc test showed that PNS elevated *Tnf* in female fetal brains from vehicle-treated, stressed dams relative to female fetal brains from vehicle-treated, non-stressed dams (p = 0.0472). This pro-inflammatory, female-specific effect of PNS was not observed in fetal brains derived from antibiotic-treated dams, suggesting that stress fails to elicit increases in *Tnf* under conditions of maternal microbe depletion (Figure 7B).

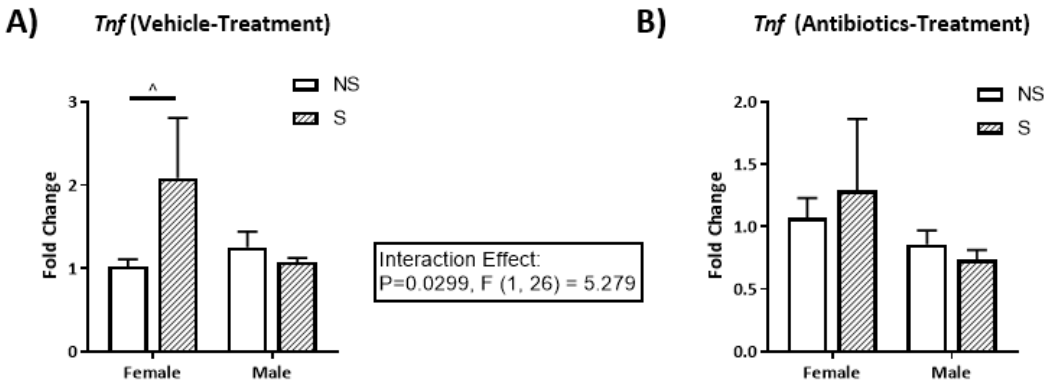

Figure 7. PNS increases *Tnf* in female fetal brains from vehicle-treated dams. A sex x stress interaction effect was observed in fetal brains obtained from vehicle-treated dams at GD17.5 (A). In vehicle-treated dams, PNS increased abundance of *Tnf* in female fetal brains, but not in males. This interaction was not apparent in fetal brains obtained from antibiotics-treated dams (B). (n =5-10/group). Two-way ANOVA: carets represent interaction effects between sex and stress (^p < 0.05). Bars represent mean ± SEM

As sex x stress and sex x antibiotics analyses revealed no sex differences or sex-dependent effects on fetal brain *Il6 or Ccl2* abundance, data was averaged per litter (Figure 8). Further analyses revealed a main effect of PNS and a main effect of

antibiotics in decreasing fetal brain *Il6* (Figure 8A, main effect of stress: f(1,27) = 8.65, p = 0.0066); main effect of antibiotics: f(1,27) = 7.722, p = 0.0098), but no changes in *Ccl2* (Figure 8C).

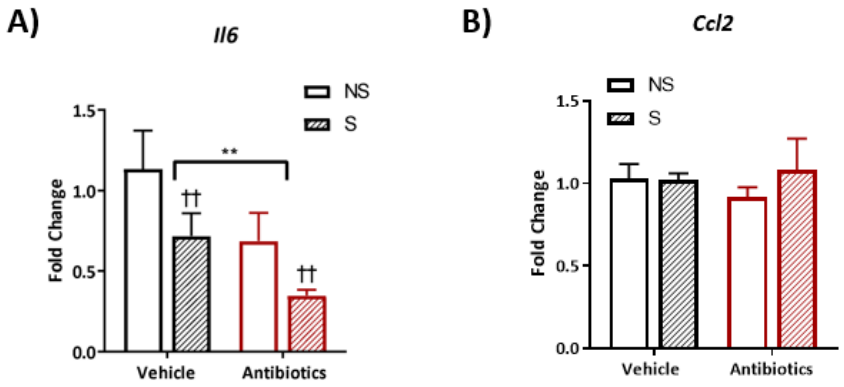

Figure 8. PNS and antibiotics reduce *Il6* in the fetal brain. At GD17, PNS and antibiotics decreased abundance of *Il6* in the fetal brain (A), while *Tnf* and *Ccl2* remained unchanged. (n = 6-10/group). Two-way ANOVA: asterisks represent main effects of antibiotics (**p < 0.01), daggers represent main effects of stress (††p< 0.01). Bars represent mean ± SEM.

3.5. Trending increases in adult offspring microglial *Tnf* in response to prenatal stress appear to be sex-specific.

I then sought out to determine whether changes in cytokine and chemokine transcript abundance in the fetal brain persisted postnatally and into adulthood. As microglia are resident innate immune cells of the brain and produce cytokines both at baseline and in response to infection, inflammation, and injury[45,46], I isolated microglia from adult offspring exposed to PNS *in utero* for RT-qPCR.

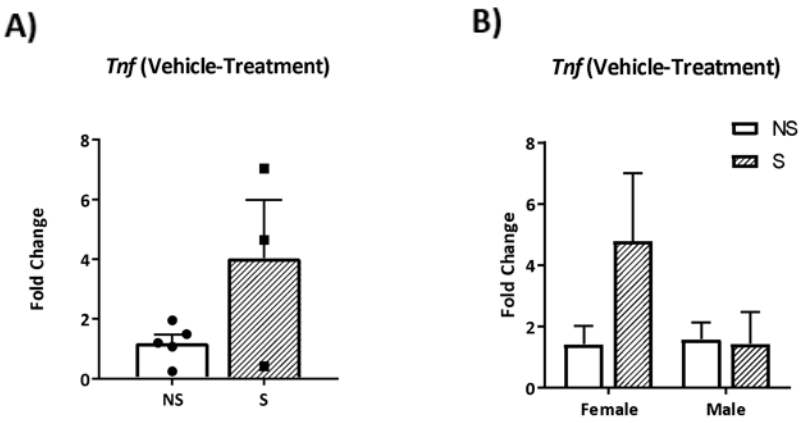

Figure 9. Preliminary data suggests PNS increases *Tnf* in microglia from adult, female offspring. PNS does not induce significant changes in microglial *Tnf* abundance (A, n =3-5/group, p = 0.3098). However, preliminary data suggest that PNS may increase microglial *Tnf* in female offspring, but not males, though this interaction did not reach significance. (B, n = 2-5/group, F (1, 10) = 0.6614, p = 0.4350).

In adult offspring, exposure to PNS appeared to increase microglial *Tnf*, though this trend did not reach statistical significance due to low sample size and high variability (Figure 9A, p = 0.3098). Notably, there was a visually apparent, albeit non-significant, increase in *Tnf* abundance in adult, female offspring microglia from prenatally stressed mice, but not in male offspring (Figure 9B, f(1,10) = 0.6614, p = 0.4350). Further replicates will be required to increase the power of our microglial analyses.

3.6. Prenatal stress and antibiotics do not alter repetitive and anxiety-like behaviors in adult offspring

The marble burying test is used to identify repetitive behaviors in rodents, thought to be characteristic of ASD in humans[47]. As prenatal stress is associated with increased risk of developing ASD in the offspring[13,14], I sought to determine whether our

model of stress induced repetitive behaviors in the offspring and whether this effect could be ameliorated by antibiotics-mediated maternal gut microbiome depletion. No main effects of stress or sex-dependent changes were observed. Data presented was consequently averaged across litters. Stress had no significant effect on the percentage of marbles buried (Figure 10A), indicating that our model of PNS does not induce repetitive behaviors in adult offspring. Antibiotics also had no significant effect on the percentage of marbles buried (Figure 10A). Furthermore, there were no interaction effects between PNS and antibiotics on the percentage of marbles buried.

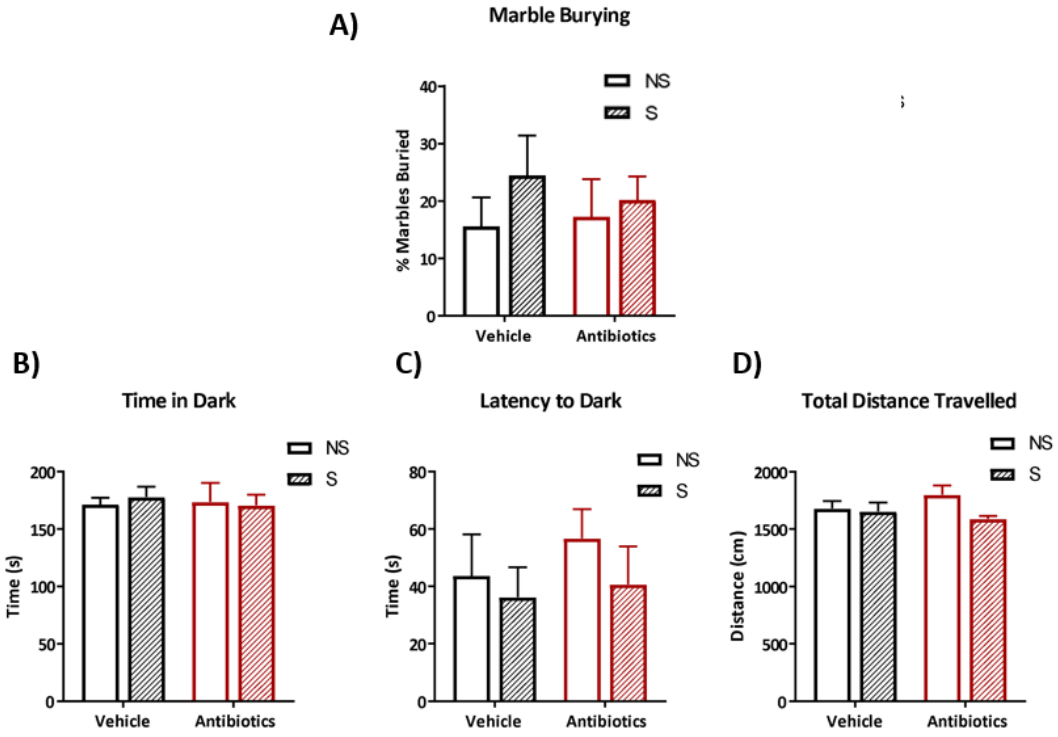

Figure 10. Prenatal stress and antibiotic administration do not alter repetitive and anxiety-like behaviors in adult Both PNS ($p = 0.3256$) and antibiotics ($p = 0.8222$) had no effect on marble burying (A, n = 4-5/group). In light-dark preference tests, PNS and antibiotics did not impact time spent in the dark compartment (B, PNS: $p = 0.8679$; ABX:

p = 0.8136), latency to enter the dark compartment (C, PNS: p = 0.3746; ABX: p = 0.5040), or total distance travelled (D, PNS: p =0.1071; ABX: p = 0.6923) (n =4-5/group). No interaction effects between PNS and antibiotic conditions were observed in results from marble burying and light-dark preference tests.

As PNS has previously been shown to increase anxiety-like behavior[5,6,48], I used the light-dark preference test to assess the ability of our PNS model to induce anxiety-like behavior in offspring. Anxiety-like behaviors are defined by increased time spent in the dark compartment and reduced latency to enter the dark compartment. Once again no main effects of stress or sex-dependent changes were observed and data was averaged across litters. PNS did not alter time spent in the dark compartment (Figure 10B) or latency to enter the dark compartment (Figure 10C), which indicates that anxiety-like behavior, as measured by the light-dark preference test, is not affected by our PNS model. Antibiotics also had no effect on time spent in the dark compartment (Figure 10B) or latency to enter the dark compartment (Figure 10C). Additionally, neither PNS nor antibiotics (Figure 10D) impacted offspring locomotion as measured by distance traveled in the light-dark preference test.

Chapter 4:

Discussion

The gut-brain axis, through which bidirectional communication between the central nervous system and the GI tract occurs[29,30], has emerged as a key mechanism by which stress can impact physiology. In particular, it has been established by our lab and others that psychological stress disrupts the composition of the commensal gut

microbiome[7,26–28]. Due to the effects of the gut microbiome on immune function[31–33] and the unique physiology of the maternal and fetal immune systems[23], I aimed to investigate how PNS and antibiotic-induced alterations to the maternal gut microbiome affect maternal and fetal immune function and offspring neurodevelopment.

Both PNS and antibiotics reduced maternal spleen mass at GD17, an indicator of systemic maternal immunosuppression. This finding is consistent with prior observations of increased circulating corticosterone in dams with our model of PNS[25], as glucocorticoids are well-recognized for their immunosuppressive properties[49]. PNS also reduced maternal weight gain from GD10.5 to GD16.5. This result reinforces the stress-eliciting effects of our model of PNS, replicating previous research demonstrating that stress and glucocorticoid administration during pregnancy results in reduced gestational weight gain and offspring birth weights[50,51]

In the maternal GI tract, PNS and antibiotics independently and differentially impacted colonic and ileal immune function; however, there were no significant stress x antibiotics interactions suggesting that antibiotics-induced maternal gut microbiome does not ameliorate the effects of stress on maternal gut immune function. In fact, both PNS and antibiotics elicited changes in mRNA abundance that reflect broadly immunosuppressive effects in the maternal ileum and distal colon. Previous research has identified immunosuppressive effects of broad-spectrum antibiotics on innate and adaptive immunity, increasing susceptibility to bacterial and viral infections[52–55]. Clinical studies and animal models have also identified immunosuppressive effects of chronic stress on cellular immunity[56]. These findings corroborate the immunosuppressive effects of PNS and antibiotics observed in this research, including reduced stable

mRNA levels of pro-inflammatory cytokines and chemokines in the maternal gastrointestinal tract and reduced maternal splenic weight, and further validates our model. As both PNS and antibiotics induce broadly immunosuppressive effects on the maternal GI tract, these results may suggest that PNS induces gut microbiome disruption and similar consequent host immune responses to that of antibiotics-mediated disruption. Future studies are warranted to identify particular microbes or microbial immunomodulatory genes that are disrupted by PNS and antibiotics, correlating with maternal GI immune function, intrauterine immune function, and neurodevelopment.

In the placenta, there was a significant sex x antibiotics interaction in placental *Ccl2* abundance, with increased *Ccl2* in male placentas from antibiotics-treated dams compared to females from antibiotics-treated dams. Similar effects were observed with placental *Il6*, though the interaction did not reach significance. This result suggests that antibiotics have sex-specific immunological effects in the placenta. While it is unclear whether these immunomodulatory effects are mediated through disruption of the maternal gut microbiome or direct interactions with the placenta, this is the first observation of antibiotics-induced changes in placental immune function as far as I am aware. PNS also elicited increases in pro-inflammatory *Tnf* in female fetal brains, but not males. Sexually dimorphic immune responses to maternal gut dysbiosis, induced by methods such as PNS or antibiotics, may present a potential mechanism by which sex-specific behavioral effects of the maternal microbiome disruption arise. Notably, PNS-induced increases in female fetal brain *Tnf* were not observed in antibiotics-treated mice, suggesting a role of the maternal gut microbiome in eliciting this effect. This is

consistent with previous findings from our lab that unlike specific-pathogen-free (SPF) mice, GF fetal brains are protected from PNS-induced inflammation[6]. In contrast to indicators of systemic and GI tract maternal immunosuppression elicited by PNS and antibiotics (Figure 1B, 3, and 4), elevated stable mRNA from pro-inflammatory genes in male placentas and female fetal brains may reflect a compensatory mechanism to counteract maternal immunosuppression.

Following up on this finding of sex-dependent effects of PNS on fetal brain immune function, long-term changes in adult offspring CNS immune function were evaluated. To do so, microglia, the resident innate immune cell of the CNS, were isolated from adult offspring and mRNA quantification for previously described cytokines and chemokines was completed. Preliminary data suggests that our model of PNS increases pro-inflammatory *Tnf* in adult microglia from female offspring, but not males, though this trend did not reach statistical significance. This suggests that PNS differentially affects CNS immune function in fetal and adult brains in a sex-specific manner. This finding would be consistent with extensive evidence of sexually dimorphic microglial phenotypes and responses to early-life perturbations[44,45]. These differences in immune function could potentially account for previously observed sex differences in adult offspring behavior in our PNS model[5,7]. Additionally, previous research has documented PNS-induced shifts in adult microglial density, morphology, and secretion of cytokines *in vitro*[57,58] that are consistent with pro-inflammatory developmental responses of adult microglia. Altogether, my findings and the current literature suggest that PNS modulates microglia development, eliciting effects that persist into adulthood in a sex-specific manner.

Lastly, I assessed offspring behavior to identify whether PNS and antibiotics-mediated maternal microbiome depletion led to offspring behavioral changes. Many models for induction of PNS exist, including restraint stress, social defeat, and predatory exposure. Depending upon the intensity, duration, and timing of PNS, different behavioral changes in offspring can arise[8]. To gain a comprehensive understanding of how prenatal stress impacts neurodevelopment and behavior, it is crucial that different intensities and durations of stress exposure are evaluated in a sex-specific manner. Previous studies with our PNS model have demonstrated that PNS reduces social interaction in adult male offspring, and that social interaction elicits increased corticosterone release[7]. In contrast, PNS elicited anxiety-like behavior in adult female offspring as evaluated by elevated plus maze[5]. Due to the complexity of behavioral phenotypes and their translation from mouse models to human phenomena, a variety of behavioral tests are required to comprehensively understand how our model of PNS affects offspring behavior. Thus, I assessed repetitive behaviors with the marble burying test—a behavioral assay that has not yet be done on our model. Preliminary data suggest that PNS and antibiotics do not affect repetitive behaviors in the adult offspring.

Furthermore, I used the light-dark preference test to evaluate anxiety-like behavior. Previously, we have observed decreased latency to enter dark compartments in light-dark preference tests in both female and male offspring exposed to PNS[6]. However, in this light-dark preference test, PNS did not significantly affect latency to enter the dark compartment ($p = 0.3746$). Additional replicates of this data will be collected to either confirm or refute previously identified significance in the light-dark preference test. In the future, social behavior of offspring will also be evaluated in this

model, due to previous evidence of reduced social behavior in offspring exposed to PNS[7].

Overall, these data provide further insight into how PNS and antibiotics-mediated gut microbiome disruption impacts maternal and fetal immune function. By identifying and clarifying mechanisms by which PNS impacts offspring immune development, this research brings us closer to identifying immune and gut microbial-targeted therapeutic interventions to prevent PNS-induced psychiatric and behavioral disorders.

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