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Prenatal stress disrupts social behavior, cortical neurobiology and commensal microbes in adult male offspring

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Abstract

In utero and early neonatal exposure to maternal stress is linked with psychiatric disorders, and the underlying mechanisms are currently being elucidated. We used a prenatal stressor in pregnant mice to examine novel relationships between prenatal stress exposure, changes in the gut microbiome, and social behavior. Here, we show that males exposed to prenatal stress had a significant reduction in social behavior in adulthood, with increased corticosterone release following social interaction. Male offspring exposed to prenatal stress also had neuroinflammation, decreased oxytocin receptor, and decreased serotonin metabolism in their cortex in adulthood, which are linked to decreased social behavior. Finally, we found a significant difference in commensal microbes, including decreases in *Bacteroides* and *Parabacteroides*, in adult male offspring exposed to prenatal stress when compared to non-stressed controls. Our findings indicate that gestation is a critical window where maternal stress contributes to the development of aberrant social behaviors and alterations in cortical neurobiology, and that prenatal stress is sufficient to disrupt the male gut-brain axis into adulthood.

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Conflict of interest

The authors declare no competing financial interests.

Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:<https://doi.org/10.1016/j.bbr.2018.06.025>.

Keywords

Prenatal stress; Microbiome; Social behavior; Neuroinflammation

1. Introduction

Prenatal stress (PNS) is recognized as a major contributor to neurodevelopmental disorders [1]. The mechanisms underlying this contribution include alterations in epigenetic regulation, the hypothalamic pituitary adrenal (HPA) axis, and inflammation [2]. There is increased focus on changes in the microbiome following PNS as a contributor to increased risk of neuropsychiatric disorders [3,4]. For instance, stress during gestation altered the maternal vaginal microbiome [5] and gut microbiome [6], and the composition of the gut microbiome in adult offspring [6,7]. An individual's microbiome is recognized as an important contributor to health, including mental health. During birth and the early postnatal period, microbes are passed from the mother to her offspring. Receiving altered microbiota from the mother may contribute to aberrant neurodevelopment in the offspring, including alterations in social behaviors. Previously, we demonstrated that gestational stress is associated with alterations in the maternal microbiome. For example we found a tendency towards alteration in placental microbes and a significant alteration in gut microbiota [6]. Moreover, female offspring exposed to PNS had concomitant increased cytokines and decreased brain derived neurotrophic factor (BDNF) in placenta, fetal brain, and amygdala in adulthood. Finally, exposure to PNS was associated with continued alterations in microbiome in females and concomitant increased anxiety-like behavior in adulthood. This increased anxiety in females is reflective of what is found in the human population, where anxiety is more common in women [8]. Autism Spectrum disorder (ASD), on the other hand, is more prevalent in males [9], and alterations in the microbiome are hypothesized to contribute to some aspects of this disorder [10,11]. Altered social behavior is a key aspect of ASD, thus we tested if maternal stress during gestation impacts social behavior in male offspring and leads to long term microbiome changes.

2. Methods

2.1. Mice and prenatal stress paradigm

Nulliparous female C57/Bl6 mice were obtained from Jackson Laboratories and bred at the Ohio State University Wexner Medical Center. Pregnant females were randomly assigned to either the stressed experimental group or non-stressed control group. The stressor used was restraint stress, which involved placement of the female pregnant mouse in a 50 mL conical tube with perforations to allow for ventilation. At the end of the two-hour period, mice were removed from the device and left undisturbed until the following day when stress was repeated. The stressed group underwent restraint stress daily between embryonic days (E) 10-E16 for a period of two hours between the hours of 09:00–11:00. Restraint stress was chosen because it has been clearly demonstrated to alter the microbiome [12]. Other stress models like chronic mild stress involve activities, such as cage crowding, that could confound interpretation of changes in microbiota, due to coprophagia. Animals were weaned at 21 days and separated into female and male cages at that time. Males were group housed

with siblings and left undisturbed with food and water *ad libitum*. For all experiments described in this manuscript, no more than 3 pups per dam were used. For behavioral testing, different cohorts of offspring were used in each behavioral paradigm. Behavioral testing was undertaken between P60–P70. Only male offspring were included in the present report; findings in female offspring are previously reported [6]. All experiments were conducted in accordance with the principles and procedures outlined in 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.

2.2. Behavioral testing

2.2.1. Social behavior testing—Testing of social behavior (SB) was used to evaluate sociability in rodents, as previously described [13]. Briefly, testing was done in a three chamber box in which the two end chambers contained metal cells in the middle of each chamber. The testing chamber (total of three compartments) is 20.5" × 10.5" and 9.25" tall. The outer compartments are 7.5" × 10.5" while the middle compartment is 4.5" × 10.5". The compartment dividers are 3" long; two dividers on opposite sides of the compartment allow for ~4.5" opening between compartments. The metal cells are 3.75" × 3.75" × 5.75" rectangular prisms with a plastic base/lid. Its sides (5.75" tall) are lined with thin metal rods (~0.2 cm in diameter) such that contact between animals is possible, but neither animal can cross in/out of the cell. Directly prior to the 10 min testing phase with exposure to the social stimulus mouse, test subjects were subjected to a 10 min habituation phase during which they roamed the social behavior apparatus which had empty metal cells in the far-right and far-left compartments. After 10 min, a novel object (orange Pyrex bottle cap (Corning, MA)) and a social stimulus mouse of the same sex and similar age (DBA2J strain) were placed in the metal cells in the nonsocial and social chambers, respectively, initiating the test phase. The social stimulus animal was unfamiliar to the testing subject and had not been subject to any prenatal stress. Chambers were randomized for each mouse. During the testing phase, the test mouse explored the three chambers for 10 min. The Noldus EthoVision video tracking system recorded time spent in each of the three chambers, time spent actively investigating each cell, and the number of transitions between chambers during acclimatization and testing. Social approach behavior was assessed using time spent actively investigating the social stimulus mouse in relation to time spent sniffing the novel object. The testing apparatus, metal cells, and novel object were cleaned with 70% ethanol between trials. A sample size of 7 non-stressed and 9 stressed mice per group with a maximum of 3 pups per dam was used in this experiment.

2.2.2. Elevated plus maze—Anxiety-like behaviors were tested in the elevated plus maze (EPM). The maze used was exposed to fluorescent lighting and elevated 75 cm above the floor. A mouse was placed onto the central area of the maze facing an open arm, at the beginning of each trial. Mice were tested for a period of 5 min. Their movements on the maze were tracked and subsequently analyzed by the Noldus EthoVision video tracking system. The following parameters were assessed: time spent in closed and open arms and distance travelled in closed and open arms. A sample size of 9 non-stressed and 10 stressed mice per group with a maximum of 3 pups per dam was used in this experiment.

2.2.3. Novel object recognition—The NOR test consisted of two phases. In the familiarization phase, two identical objects (two orange Pyrex bottle caps(2", Corning, MA)) were placed at opposite ends of an otherwise empty cage (11.5" × 7" × 4.5") containing clean bedding. Mice were allowed to explore the cage and the two identical objects for 10 min. Mice were then returned to their home cage. Three hours later during the test phase, a novel object (Blue 15 mL centrifuge cap, 7/8") was placed in the cage. The mouse was then placed in the center of the cage, and allowed to explore for 5 min. Novel object preference was assessed using the time spent investigating original object and the time spent investigating novel object, in relation to the total time spent investigating both objects. NOR was hand-scored by trained personnel blinded to subject condition, using a stopwatch and video recordings of the NOR testing trials. Cages and bedding were replaced after each trial. A sample size of 6 non-stressed and 5 stressed mice per group with a maximum of 3 pups per dam was used in this experiment.

2.2.4. Tail suspension test—Mice were individually suspended by the tail to a horizontal ring-stand bar at a distance of 35 cm from the floor using adhesive tape. A 5 min test session was videotaped and scored by a trained observer who was blind to the experimental conditions. The behavioral measure scored was the duration of "immobility," which was defined as the time when mice were judged to cease escape-motivated behaviors. A sample size of 11 non-stressed and 10 stressed mice per group with a maximum of 3 pups per dam was used in this experiment.

2.3. Tissue collection

Tissue samples were collected sterily. Brains collected from a subset of adult male mice were sliced into 1 mm sections using a brain matrix. Specific regions were identified and macrodissected using their approximate mouse stereotaxic coordinates (cortex- Bregma 0.26 corresponding to anterior cingulate cortex) [14]. Brain tissue was stored at -80 °C for RNA isolation and cDNA synthesis.

2.4. HPLC for 5HT and 5HIAA

High Performance Liquid Chromatography (HPLC) was used to quantify 5-HT and 5-HIAA levels in male offspring cortex. HPLC was carried out in the AB Sciex QTRAP 5500-Agilent 1290 LC (LC/MS/MS system) using a C18 Kinetex biphenyl (100 × 2.1 mm, 2.6 µm) column and a security guard column (10 × 2.1mm) from Phenomenex. Metabolites of interest were extracted from cortex samples via a series of hot water baths and homogenization before adding 0.2% FA in ACN solution. Supernatants were transferred to new microcentrifuge tubes for evaporation before adding 0.1% aqueous formic acid and transferring to 3 kDa Amicon filtering devices. Finally, 5 µl of sample was injected into the column and calibration curves were constructed by plotting the peak area against the nominal concentration of each compound. External standards (5 HIAA, serotonin, and 13C glycine) and internal standards (13C glycine) were used throughout.

2.5. Corticosterone measurement

Directly following the social interaction testing, blood samples were obtained by submandibular bleed. Corticosterone was measured in blood plasma, according to the

manufacturer's instructions, by ELISA (Correlate-EIA kit; Assay Designs). All samples, standards, and replicates were assayed in duplicate.

2.6. Immunohistochemistry

Immunohistochemistry was performed and quantified as previously described [15]. Briefly, following perfusion, brains were post-fixed in paraformaldehyde followed by incubation in 30% sucrose at 4 °C. Fixed brains were frozen in isopentane (−78 °C) and dry ice, and stored at −80 °C. Frozen brains were sectioned for prefrontal cortex (Bregma=2.46 mm) at 25 µm. Sections were labeled with rabbit anti-Iba1 (1:1000; Wako, Richmond, VA; Catalog# 019–19741) followed by donkey anti-rabbit Alexa Fluor 488 antibody (1:500; abcam, Cambridge, MA; Catalog# ab150073). Images were taken using a Zeiss 510 Meta confocal microscope and analyzed using ImageJ software. IBA-1 labeling was analyzed to determine microglial activation in the brain. Following exposure to stress, previously homeostatic microglia take on an activated phenotype that is marked by an amoeboid soma and thicker processes [16]. These morphological changes are reflected by an increase in the IBA-1-positive area [17]. Therefore, increased IBA-1 expression was used as a proxy for microglial activation in the brain. For the digital imaging analysis of IBA-1 images, a threshold for positive labeling (full view of the microglial morphology with the background excluded) was determined for each image. Data were processed by ImageJ using the densitometric scanning of the threshold targets, and results expressed as the average percent area with positive labeling.

2.7. Quantitative PCR

TRIzol reagent (Invitrogen) was used for total RNA extraction from tissue and RNA quantity was assessed using spectrophotometry. cDNA was synthesized from RNA (1 µg/15 µL) using qScript cDNA SuperMix system, per the manufacturer's instructions (Quanta Biosciences). Wells contained 5 µL of Taqman Gene Expression Master Mix (Applied Biosystems), 2 µL of RNase-free water, and 0.5 µL of appropriate probes giving a total well volume of 10 µL. The ABI Prism 7000 PCR machine (Applied Biosystems) was used to perform qPCR. Cycling parameters were: 1) 15 s at 95 °C, 2) 1 min at 60 °C repeated for 40 cycles. qPCR data is presented as the relative change in expression (−CT) calculated using the 2^{−CT} method. GAPDH was used as a housekeeping gene.

2.8. Microbial community analysis

Fecal samples for microbial analysis were collected at the time of behavioral testing. Following behavioral testing mice were placed in a clean cage without bedding until they defecated, and sample was collected and placed in a microcentrifuge tube for further processing. Qiagen (Hilden, Germany) DNA Mini Isolation Kit was used to isolate genomic DNA with modifications as previously described [18]. The Illumina MiSeq platform was used for targeted 16S ribosomal RNA gene sequencing to determine microbial diversity. 2 × 300 bp paired-end sequences were produced, targeting the V1–V3 regions of the bacterial 16S rRNA using the 27 F and 519R primers. Raw sequencing data was provided in fastq format for downstream analysis. Unifrac distances and community similarity and diversity were computed from the sequence data using QIIME (Quantitative Insights into Microbial Ecology) version 1.9.1. Taxonomic data was generated at each rank level (e.g. Phylum,

Class, Order, Family, Genus) to develop a composite microbial profile. The proportions of each taxonomic classification in each sample were computed as a percent of the total microbial community.

2.9. Experimental design and statistical analysis

Prenatal stress was carried out in several separate cohorts of mice, with matched group of control non-stressed dams. Each behavioral paradigm was carried out in a separate cohort of PNS-exposed offspring and control. Unpaired T-tests were performed to establish statistical significance for RT-PCR, and behavioral experiments. The analysis was implemented in Stat View Version 5.0.1. Error bars represent the standard error of the mean. Principal Coordinates Analysis (PCoA) of weighted and unweighted UniFrac distance matrices was used to determine clustering between the two groups (Control vs. Stress). Differences in bacterial taxa were determined using t-tests. P-values were corrected for multiple tests using the Benjamini-Hochberg method [19], with a q-value of 10%. The Adonis statistic, a beta-diversity statistic that is available through the vegan package of R and accessible with QIIME, was used with 999 permutations to calculate statistical significance between the distance matrices of sample groups. For all multivariate statistics, significance was set to $p = 0.05$.

3. Results

3.1. Prenatal stress leads to decreased social interaction in male offspring

To determine the behavioral sequelae of prenatal stress, sociability, anxiety-like behavior, cognitive function, and depressive-like behavior were measured. Previously this model of prenatal stress was found to increase anxiety-like behavior in the dams, confirming the deleterious nature of this paradigm [6]. Offspring were tested during adulthood, between P60-P70. Different cohorts of mice were used for each behavioral test. Male offspring exposed to prenatal stress had a significant decrease in social-approach behavior when compared to non-stressed males as reflected by decreased amount of time spent socializing with a male stimulus mouse (Fig. 1A; $t(16)=2.080$, $p = 0.05$). They did not differ in total distance travelled (Fig. 1B; $t(16)=0.913$, $p=0.38$) or preference for one side of the apparatus prior to placement of stimulus mouse or object (Fig. 1C; $t(16)=1.042$, $p=0.32$). Time spent in the open arm of the EPM did not differ in males exposed to prenatal stress when compared to non-stressed controls (Fig. 1D; $t(19)=0.070$, $p=0.95$). Behavior in the male offspring was also examined in the NOR paradigm, a cognitive task where preference for a novel object is measured. Prenatally stressed male mice did not show an alteration in preference for a novel object (Fig. 1E; $t(9)=0.475$, $p=0.65$). Male offspring also did not demonstrate an alteration in the TST, which reflects depressive-like behavior (Fig. 1F; $t(8) = 0.453$, $p=0.66$). Thus, alterations in behavior were evident only in the domain of social behavior.

3.2. Prenatal stress decreases serotonergic metabolism in adult males

HPLC was performed in the cortex and plasma of adult male mice and offspring exposed to prenatal stress were compared with control male offspring. In adult male offspring cortex we found a trend towards an increase in 5HT (Fig. 2A; $t(20)=-1.358$, $p=0.1$), a significant decrease in 5HIAA (Fig. 2B; $t(20)=2.910$, $p=0.008$) and overall decrease serotonin turnover

[5-HIAA/5-HT] (Fig. 3C; $t(20)=3.172$, $p=0.005$), reflective of an overall increase of 5HT. We found a significant reduction of MAO-A in adult male cortex (Fig. 2B; $t(22)=2.696$, $p=0.01$), suggesting that reduced turnover was due to decreased degradation of 5HT. In the periphery, we found a significant increase in plasma 5HT concentration (Fig. 2C; $t(15)=3.095$, $p=0.01$) in males exposed to PNS when compared to control. 5HIAA levels in plasma were low to undetectable (data not shown) therefore turnover was not calculated.

3.3. Prenatal stress increases corticosterone response to social interaction and reduces oxytocin receptor levels in adult males

We examined the corticosterone response in adult male offspring directly following social interaction, and found a significant increase in plasma corticosterone following social interaction in males exposed to prenatal stress (Fig. 3A; $t(17)=2.588$, $p=0.02$). In addition, we found increased CRH in the cortex of adult males exposed to prenatal stress when compared to controls (Fig. 3B; $t(22)=2.696$, $p=0.01$). Together, this is suggestive of heightened reactivity of the HPA axis following PNS. We then determined whether increased cortical CRH was associated with a downregulation in oxytocin receptor (OXTR) levels in the cortex. We found a significant reduction (Fig. 3C; $t(22)=2.782$, $p=0.01$) in cortex from adult male offspring exposed to PNS when compared to unexposed controls.

3.4. Prenatal stress leads to elevated markers of neuroinflammation in adult males

We examined the cortex of male offspring exposed to prenatal stress for evidence of increased cytokines, reflective of neuroinflammation. We found a significant increase in IL-6 (Fig. 4A; $t(21)=2.487$, $p=0.02$) and IL-1 β (Fig. 4B; $t(22)=2.770$, $p=0.01$) in the cortex of PNS exposed males when compared to non-PNS controls. We then measured IBA-1 expression in the brain since microglial activation is associated with morphological restructuring of microglia that leads to increased IBA-1-positive area [16]. Compared to non-stressed controls, males exposed to prenatal stress had a significant increase in the IBA-1-positive area in the prefrontal cortex (Fig. 4C; $t(14)=3.463$, $p=0.01$). Together, these data indicate longstanding neuroinflammatory abnormalities that contribute to reduced social behaviors following prenatal stress.

3.5. Prenatal stress causes microbial changes in male offspring

The male adult offspring stool microbiome significantly differed based upon whether or not the offspring were exposed to the stressor during gestation. Weighted UniFrac distance matrices were used to assess differences between the microbial communities of offspring of stressor-exposed and non-stressed control dams. When UniFrac distances were plotted using principle coordinate analysis, samples from male offspring exposed to the stressor plotted separately from samples from non-stressed control offspring (Fig. 5A). Testing with the Adonis statistic indicated that this different clustering was statistically significant ($p < 0.05$). When unweighted UniFrac distances were plotted on a PCoA, there was no separation of samples from offspring of stressor-exposed and non-stressed control dams (Supplemental Fig. 1A). Likewise, there were no significant differences in measures of alpha diversity (Fig. 5B, and Supplemental Fig. 1B). These analyses indicate that samples from male offspring of dams exposed to the stressor have significantly different microbial community composition than samples from offspring of non-stressed control dams that is primarily due to differences

in the relative abundances of bacterial taxa, and not due to the emergence or loss of bacterial taxa. When individual bacterial taxa were assessed, it was evident that there was substantial intergroup variability in the relative abundances of bacterial genera (Fig. 5C). Despite the variability, PNS and control offspring had significant differences in bacterial relative abundances when they reached adulthood. Adult male offspring exposed to PNS had significantly lower relative abundances of bacteria in the genera *Bacteroides* $t(20)=3.26$, $p=0.003$ and *Parabacteroides* $t(20)=3.75$, $p=0.001$ (Fig. 5D). Differences in other taxa did not reach statistical significance after correcting for multiple comparisons.

4. Discussion

Stress, and its psychiatric sequelae of anxiety and depression, are widespread during pregnancy [20] and associated with behavioral outcomes in the offspring [21,22]. Here, we demonstrate that in rodents, prenatal stress leads to alterations in microbiota in adult males, as well as decrease in social behavior, and alterations in cortical neurobiology including reduction in serotonin turnover, elevated markers of neuroinflammation, alterations in CRH axis, and concomitant reduction in OXTR. Social behavior is mediated by multiple neural circuits and brain regions [23]; cortical dysfunction has previously been tied with social behavior in both humans and rodents [24,25]. To our knowledge this is the first time in males that the gut microbiota has been considered in the link between PNS, social behavior, and concomitant alterations in cortical neurobiology.

Recent studies show that exposure to PNS reduced social interaction in adult male rodent offspring [26–28]. Here, we confirm and extend this observation to show that in male offspring there is a specific deficit in social interaction, without disturbance in anxiety- and depressive-like behavior, or cognition. This is in contrast to our previous study in female offspring showing that females do have increased anxiety-like behavior and cognitive deficit following prenatal stress [6], but they did not show deficits in social interactions (data not shown). Sex-differences are a characteristic of psychopathology, and the finding that behavioral outcomes in this PNS model mirror those found in psychiatric disorders, namely increased affective disorders in females and social deficits in males strengthens the interpretation of these findings. Of note, social deficits are a hallmark of ASD, albeit not the only feature [29]. Future work will focus on determining whether there are alterations in other ASD-associated behaviors, such as repetitive behaviors.

The cortex, and prefrontal cortex are implicated in social behaviors in both mouse and man [24]. Therefore, we examined the impact of prenatal stress on several parameters within the cortex including oxytocin, serotonin (5HT), and neuroinflammation. Commensal microbes in early life modulate levels of 5-HT in the brain [30]. Of note, male mice in that study were specifically found to have aberrant 5HT, with increases in both 5HT and 5HIAA. We measured serotonin turnover (5HIAA/5HT) and found a significant reduction in cortex from males exposed to PNS when compared with non-stressed controls. In addition, we found a reduction in MAO-A, an enzyme responsible for the breakdown of amines including 5HT, in the cortex of male mice exposed to PNS when compared to non-stressed controls, mirroring what was found in post-mortem tissue of children with ASD who have abnormal social behavior [31]. Together, these data reflect an overall increase in 5HT in the cortex of the

male mice exposed to PNS. We also examined peripheral 5HT, as commensal microbes produce metabolites that stimulate the enterochromaffin cells of the colonic epithelium to increase 5HT synthesis [32]. We found a significant increase in plasma 5HT concentration, suggesting that PNS influences peripheral levels of 5HT in addition to brain levels of 5HT. Additional studies examining luminal contents in different portions of the digestive tract are required to establish a conclusive role for enterochromaffin cells. Overall, our findings indicate that there is extended impairment in 5HT turnover in males following PNS.

Confirming the hypothesis that prenatal stress may increase stress-induced activity of the HPA-axis, which has been associated with reduced social interactions [33,34], we found a significant increase in corticosterone following exposure to the social behavior paradigm in males exposed to PNS. We found a concomitant reduction in CRH in the cortex as well, pointing to sustained HPA axis dysregulation in adulthood in males exposed to PNS. Cortical CRH has been implicated in aberrant neuronal communication following stressful situations [35]. Cortical CRH has been implicated in a circuit regulating social behavior through regulation of oxytocin [36]. Oxytocin is a neuropeptide that modulates social behavior in rodents [37] and humans [38]; several rodent models of ASD demonstrate deficits in the oxytocin system [39]. Centrally acting oxytocin inhibits the stress-induced activity of the HPA axis [40], in addition to the well-known peripheral role in parturition and lactation. Oxytocin is widely distributed throughout the CNS by smaller parvocellular neurons, and has been found to exert behavioral and physiologic stress-attenuating effects and to promote positive social interaction [41]. Previous studies have demonstrated a reduction of OXTR in the paraventricular nucleus and increased oxytocin receptor binding in the central amygdala of rats following an unpredictable mild stressor during the final week of pregnancy [27] with a concomitant reduction in social behaviors. Social deficit was reversed with central administration of oxytocin. We found a significant reduction in oxytocin receptor (OXTR) mRNA in cortex of male offspring exposed to prenatal stress. Interestingly, post-mortem findings from individuals with ASD also show decreases in OXTR [42,43] suggesting that these findings have translational relevance to individuals with ASD.

Neuroinflammation can also lead to social withdrawal in rodents [44] and decreased sociability in humans [45,46]. We therefore assessed markers of neuroinflammation in our offspring exposed to PNS. Previous studies of prenatal stress demonstrated an increase in cytokine production and Iba-1 labeling in the hippocampus of males exposed to prenatal stress [47]. Indeed here we demonstrate a significant increase in IL-1p and IL-6 mRNA in the cortex of males exposed to PNS compared to controls. Consistent with the proinflammatory cytokine expression, there was an increase in the Iba-1-positive area, indicative of microglial activation, in males exposed to PNS. It is important to note that besides microglia, other cells, such as macrophages, express IBA-1, and here we did not further assess the different cell types in the brain. Nevertheless, microglia are the most prevalent mononuclear phagocytes in the brain, and microglial activity has been demonstrated to influence social behavior [48]. We surmise that microglia activation following prenatal stress contributes to impairment in social behavior.

The microbiome is linked with alterations in social behavior in studies assessing the offspring of dams whose immune systems were activated during pregnancy [10]. In addition, treating animals with antibiotics to disrupt the microbiome led to changes in social behavior [49]. Stress during gestation alters the composition of the maternal microbiome and these changes are transmitted to the offspring [3,5,6]. Our study shows that offspring microbial changes persist into adulthood with males exposed to PNS demonstrating a significant difference in the gut microbiome. In addition, a significant reduction in *Bacteroides* and *Parabacteroides* was found in males exposed to PNS. We previously have demonstrated a significant reduction in *Bacteroides* in males exposed to restraint stress and a social defeat stressor, and significantly increased circulating levels of IL-6 and CCL2 [18] [Bailey et al., unpublished observation]. Of note, a recent study has linked *Bacteroides* and *Parabacteroides*, along with a number of other microbes, to contributing to sex-specific changes in behavior in a mouse model of autism [50]. In addition, treatment with *Bacteroides fragilis* was able to ameliorate the behavioral and inflammatory abnormalities in the maternal immune activation (MIA) model of autism [10]. Together, these findings suggest that the significant reductions in *Bacteroides* and *Parabacteroides* in the male offspring exposed to PNS are contributing to the subsequent behavioral and inflammatory sequelae.

Prenatal stress is now widely known to impact the developing CNS in deleterious ways, and the mechanism underlying its influence on social behaviors is of vital importance. Here, we demonstrate that PNS alters the microbiome of adult male offspring, with concomitant reduction in social interaction. In addition, we found that this early life exposure affects cortical 5HT metabolism, elevated markers of neuroinflammation, and oxytocin, as well as corticosterone levels in response to social interaction. This constellation of changes reflects neurobiological changes seen in individuals with ASD [9,31,42,43,50–52]. Our findings suggest that the roots of the psychopathology of this disorder may lie *in utero*, and be connected to aberrant establishment of the Gut-brain-axis. Further studies are required to prove a causative relationship between these findings. However, this pattern demonstrates that PNS is sufficient to disturb commensal microbes, social behavior and cortical neurobiology into adulthood. Our results contribute to a growing number of studies that have linked gestational and early life microbiota to behavioral disorders [30,4] and outlines a specific and contributory role of maternal stress.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

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Fig. 1.

Prenatal stress leads to decreased social interaction in male offspring.

Pregnant Female C57BL6 mice were exposed to restraint stress for 2h a day from E10–E16 or left as non-stressed control mice. Separate cohorts of male offspring at P60–70 were tested in a battery of behavioral tests. A) Social interaction index was determined in male offspring of PNS exposed mice. Social approach was reduced in males exposed to PNS when compared with control ($t(16)=2.080$, $p=0.05$) (B) distance travelled ($t(16)=0.913$, $p=0.38$) and C) side preference ($t(16)=1.042$, $p=0.32$) in the social approach paradigm did not differ. D) Time spent in the open arm of the elevated plus maze did not change with PNS ($t(19)=0.070$, $p=0.95$). E) Cognition, as assessed by preference for a novel object was not influenced by PNS ($t(9)=0.475$, $p=0.65$). F) Depressive-like behavior was examined in the tail suspension test and did not change following PNS ($t(8)=0.453$, $p=0.66$). Bars represent the mean $\pm$ SEM. Means with (*) are significantly different from controls.

Fig. 2.

Prenatal stress decreases serotonergic metabolism in adult males.

Cortex and plasma were collected from adult males exposed to prenatal stress (PNS) and control C57BL/6 mice and HPLC analysis of 5HT and its metabolite 5HIAA were performed. A. HPLC analysis shows a trend towards an increase in 5HT (Fig. 2A; $t(20) = -1.358$, $p=0.1$) in the cortex of males exposed to PNS when compared to non-exposed male controls. B. HPLC analysis demonstrates a significant decrease in 5HIAA (Fig. 2B; $t(20)=2.910$, $p=0.008$) in the cortex of males exposed to PNS when compared to non-exposed male controls. C. Significant decrease in the ratio of 5HIAA/5HT ($t(20)=3.172$, $p=0.005$) in the cortex of males exposed to PNS when compared to non-exposed controls indicates an overall increase in serotonin. D. qPCR analysis shows significant decrease ($t(22)=2.696$, $p=0.01$) in MAO-A in the cortex of adult males exposed to PNS when compared to non-exposed males. ($n=10-12$ /group) E. In a separate cohort, HPLC analysis of 5HT shows significant increase in the plasma of males exposed to PNS when compared to non-exposed controls ($t(15)=3.095$, $p=0.01$). Bars represent the mean $\pm$ SEM. Means with (*) are significantly different from controls.

Fig. 3.

Prenatal stress increases corticosterone response to social interaction and reduces oxytocin receptor levels in adult males.

A cohort of male C57/BL6 offspring exposed to prenatal stress (PNS) and control offspring were tested in the social behavior paradigm. Blood was collected immediately after completion of social behavior. A. Corticosterone (CORT) significantly increased in males immediately after social interaction ($t(17)=2.588$, $p=0.02$) B. qPCR analysis shows a significant increase in CRH in the cortex of PNS exposed males when compared to non-exposed controls ($t(22)=2.696$, $p=0.01$) C. qPCR analysis shows a significant decrease in OXTR in the cortex of adult males exposed to PNS when compared to non-exposed males. ($t(22)=2.782$, $p=0.01$) Bars represent the mean $\pm$ SEM. Means with (*) are significantly different from controls.

Fig. 4.

Prenatal stress leads to cortical neuroinflammation in adult males.

Prenatal Stress (PNS) increased cytokine production and microglial activation in brain tissue collected from adult males. A, B. qPCR analysis shows a significant increase in IL-6 ($t(21)=2.487$, $p=0.02$) and IL-1 β ($t(22)=2.770$, $p=0.01$) in the cortex of PNS exposed males when compared to non-exposed controls. C. Representative images of IBA-1 labeled prefrontal cortex from PNS exposed adult males, and non-exposed controls. D. Exposure to PNS caused microglia activation with increased proportional area of Iba-1 immunofluorescence in the prefrontal cortex ($t(14)=3.463$, $p=0.01$). Bars represent the mean $\pm$ SEM. Means with (*) are significantly different from controls.

Fig. 5.

Prenatal stress causes microbial changes in male offspring.

Sequences from stool samples from stressor-exposed male offspring in adulthood plotted separately from samples from non-stressed control male offspring on a Principal Coordinate Analysis (PCoA) using weighted UniFrac distances. This difference was statistically significant using the Adonis statistic ($p < 0.05$). B. PD Whole Tree measure of alpha diversity was not significantly different in males exposed to PNS and unexposed males. C. Relative abundances of sequences classified at the family or genus taxonomic level in male offspring exposed to PNS compared to non-exposed males. D. The relative abundances of *Bacteroides* and *Parabacteroides* were significantly different in males exposed to PNS compared to non-exposed males. A total of 14 non-stress male offspring and 15 stressed male offspring from 6 non-stress and 9 stressed dams were examined for microbiome samples. * $p < .001$ after multiple test correction.