SSRI treatment modifies the effects of maternal inflammation on in utero physiology and offspring neurobiology

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ABSTRACT

Perturbations to the in utero environment can dramatically change the trajectory of offspring neurodevelopment. Insults commonly encountered in modern human life such as infection, toxins, high-fat diet, prescription medications, and others are increasingly linked to behavioral alterations in prenatally-exposed offspring. While appreciation is expanding for the potential consequence that these triggers can have on embryo development, there is a paucity of information concerning how the crucial maternal-fetal interface (MFI) responds to these various insults and how it may relate to changes in offspring neurodevelopment. Here, we found that the MFI responds both to an inflammatory state and altered serotonergic tone in pregnant mice. Maternal immune activation (MIA) triggered an acute inflammatory response in the MFI dominated by interferon signaling that came at the expense of ordinary development-related transcriptional programs. The major MFI compartments, the decidua and the placenta, each responded in distinct manners to MIA. MFIs exposed to MIA were also found to have disrupted sex-specific gene expression and heightened serotonin levels. We found that offspring exposed to MIA had sex-biased behavioral changes and that microglia were not transcriptionally impacted. Moreover, the combination of maternal inflammation in the presence of pharmacologic inhibition of serotonin reuptake further transformed MFI physiology and offspring neurobiology, impacting immune and serotonin signaling pathways alike. In all, these findings highlight the complexities of evaluating diverse environmental impacts on placental physiology and neurodevelopment.

1. Introduction

Not long ago, in the early 1900s, the fetus was viewed as a “perfect parasite” that was “afforded protection against nutritional damage that might be inflicted on the mother” (Almond and Currie, 2011). This line of thinking emphasized a lack of concern for environmental insults on fetal development. In the present day, it is generally well-accepted that perturbations to a pregnant mother’s healthy physiology, such as with smoking or alcohol consumption, can negatively impact offspring development. Such environmental alterations may occur in states of malnutrition, obesity, infection, autoimmune conditions, mental health struggles, psychiatric conditions, and more (Almond and Currie, 2011; Bordeleau et al., 2021; Cortés-Albornoz et al., 2021; Estes and McAllister, 2016; Fitzgerald et al., 2021; Knuesel et al., 2014; Susser and Lin, 1992). Insults that induce an altered immune state during pregnancy (e.g. infection, autoimmune condition, pollutants) have been linked to altered offspring neurodevelopmental trajectories (Estes and McAllister, 2016; Knuesel et al., 2014; Zengeler and Lukens, 2021).

Abbreviations: ADD, anti-depressant drug; BBB, blood–brain barrier; DEG, differentially expressed gene; dNK, decidual natural killer cell; E, embryonic day; GO, gene ontology; GSEA, gene set enrichment analysis; hpi, hours post-injection; IFN, interferon; IGF, insulin growth factor; lip., intraperitoneal; MACS, magnetic-activated cell sorting; MAO, monoamine oxidase; MFI, maternal-fetal interface; MIA, maternal immune activation; P, postnatal day; PCA, principal component analysis; polyEC, polyclininosinic-polycytidylic acid; RNA-seq, RNA-sequencing; SLC, solute carrier; scRNA-seq, single-cell RNA-sequencing; SSRI, selective serotonin reuptake inhibitor; TLR, Toll-like receptor; USV, ultrasonic vocalization.

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Stress and depression during pregnancy have also been shown to increase the risk of similar cases or other psychiatric conditions in children (Kinsella and Monk, 2009; Talge et al., 2007). The common thread between these varied cases is an environmental trigger that causes a shift in maternal physiology which may propagate ill-affecting signaling cascades to the developing embryo.

Accumulating epidemiologic studies have linked maternal immune activation (MIA) to an increased penetrance of neurodevelopmental disorders, psychiatric conditions, and other neurologic disorders in offspring (Brown and Derkits, 2010; Han et al., 2021; Knuesel et al., 2014; Patterson, 2009). Diverse immune triggers including viral or bacterial infection, autoimmune conditions, and exposure to environmental pollutants have all been associated with elevated rates of mental conditions including autism, schizophrenia, depression, and more (Bilbo et al., 2018; Brown and Derkits, 2010; Ellul et al., 2022; Estes and McAllister, 2016; Knuesel et al., 2014; Patterson, 2009). Further subtleties in this model exist, as not all cases of MIA may outright trigger neurologic alternations in offspring but could instead act as a primer for future insults which collectively spur symptom onset (Giovannelli et al., 2013; Knuesel et al., 2014). Immune activation may heighten levels of inflammatory mediators that can impact the placental environment and the developing embryo (Deverman and Patterson, 2009; Estes and McAllister, 2016; Knuesel et al., 2014; Patterson, 2009). For example, heightened maternal serum levels of cytokines such as IL-6 and IL-17a can lead to offspring behavioral alterations in MIA animal models (Choi et al., 2016; Lammert et al., 2018; Smith et al., 2007; Wu et al., 2017).

Another pertinent environmental trigger that merits further exploration is the impact of the use of antidepressant drugs (ADDs) during pregnancy. As the incidence of depression in the population has risen in past decades, so has the use of ADDs (Millard et al., 2017). ADDs constitute one of the most commonly prescribed group of drugs as they are currently prescribed to approximately 10 % of the worldwide population (Millard et al., 2017). While adverse maternal mental health has been consistently linked to an increased rate of neurodevelopmental and psychiatric disorders in offspring (Fitzgerald et al., 2021; Millard et al., 2017), it is unclear how pharmacological treatment for these conditions in the mother may impact the developing embryo.

About 80% of pregnant women prescribed an ADD are given a selective serotonin reuptake inhibitor (SSRI), as this class of ADDs are known to be relatively safe to take during pregnancy (Millard et al., 2017). SSRIs work by blocking serotonin reuptake by serotonin transporters, thereby increasing extracellular serotonin concentrations and prolonging serotonin signaling (Homberg et al., 2010; Marchocki et al., 2013). Though SSRIs are often used during pregnancy, there is some evidence to suggest that SSRIs can be teratogenic and that intake during pregnancy may increase the risk of premature delivery, low birth weight, neonatal cardiovascular abnormalities, and offspring metabolic and neurologic disorders (Fitzgerald et al., 2021; Homberg et al., 2010; Marchocki et al., 2013). Given that the placenta is the sole source of serotonin for the developing embryo early in pregnancy, paired with the importance of serotonin for brain development, prenatal disruption of maternal serotonin levels could presumably alter offspring neurodevelopment (Ansorge et al., 2008, 2004; Araujo et al., 2020; Bonnin et al., 2011; Bonnin and Levitt, 2011; Homberg et al., 2010; Malm et al., 2016; Oberlander et al., 2009; Sujan et al., 2019).

A critical component of studying the impact of the maternal environment on the developing embryo is the maternal-fetal interface (MFI). The maternally-derived decidua and embryo-derived placenta constitute a temporary barrier organ that represents the first site of interaction between the mother and the embryo (Maltepe and Fisher, 2015). This interface allows for maternal support of the developing embryo with nutrients, gases, and hormones and also protection from harmful stimuli (Maltepe and Fisher, 2015). Therefore, the MFI must balance tolerogenic immune responses to a non-self-embryo, but also protect the developing embryo from deleterious consequences of infection or other triggers of maternal inflammation (Ander et al., 2019; Maltepe and Fisher, 2015; Sun et al., 2021).

Understanding how the MFI responds to a shift in a healthy baseline milieu in the maternal environment may be key in elucidating any impacts on fetal development. Studies into how perturbations to this important interface influence neurodevelopment are lacking. We therefore sought to investigate the murine MFI response to either MIA or SSRI exposure during pregnancy, as well as how these stressors impact offspring neurodevelopment. Given the evidence that combined stressors during pregnancy in a “two-hit” model have a greater impact on offspring development (Bilbo et al., 2018; Knuesel et al., 2014), we also aimed to examine whether a combinatorial effect of SSRI exposure and MIA exists.

To explore how environmental stressors impact in utero physiology and neurodevelopment, we exposed pregnant mice to a mimicked viral infection and/or treated them with SSRIs. We found that the MFI undergoes a rapid and robust immune response following MIA that was largely interferon-driven. Each MFI compartment (decidua and placenta) responded in distinct fashions to MIA. Moreover, we uncovered basic transcriptional sex differences in the MFI that were sub-acute eliminated following MIA. Offspring of these MIA pregnancies displayed altered sex-specific behaviors that were not accompanied by an appreciable transcriptional shift in microglia, the resident immune cells of the brain. Like exposure to MIA alone, we found that SSRI treatment on its own during pregnancy altered the MFI immune signaling landscape. Intriguingly, dual exposure to both MIA and SSRIs elicited combinatorial effects. Specifically, the MFI immune response to MIA was reshaped in the presence of SSRIs and the embryonic brain transcriptional response to either MIA or SSRIs alone was exacerbated when the treatments were combined. This study demonstrates that the maternal-fetal interface is sensitive to environmental insults such as MIA and SSRI exposure and that these triggers can impact offspring neurobiology in a complex fashion.

2. Methods

2.1. Mice

All mouse experiments were performed in accordance with the relevant guidelines and regulations of the University of Virginia and approved by the University of Virginia Animal Care and Use Committee. 8-week-old C57BL/6J wild-type mice were obtained from Taconic Biosciences and housed in University of Virginia facilities for at least 1 week before use. Mice were housed in specific pathogen-free conditions under standard 12-hour light/dark cycle conditions in rooms equipped with control for temperature (21 ± 1.5 °C) and humidity (50 ± 10 %).

2.2. SSRI treatment and maternal immune activation

Virgin female wild type mice were placed on fluoxetine water (160 mg/L) or standard water as a control at least 2 weeks prior to mating. Duos of females were set up with male mice for mating and checked every morning for vaginal plugs. The presence of a vaginal plug was marked as embryonic day (E)0.5 and males were subsequently removed from the cage. Maternal immune activation was initiated by intraperitoneal (i.p.) injection of 20 mg/kg polyinosinic-polycytidylic acid (polyIC:E) at E11 and E12 or saline was injected as a control (Lammert and Lukens, 2019). Fluoxetine and control groups were kept their respective water treatments throughout the entirety of pregnancies.

2.3. Tissue collection

Mice were euthanized by i.p. Euthasol injection (440 mg/kg; Anada, 200-071). Maternal blood was immediately collected by cardiac puncture, incubated at room temperature for 10 min, and then spun down for 15 min at 1500 rpm. The top layer of serum was collected for...
downstream analyses. For sample isolation at embryonic timepoints, whole maternal-fetal interface tissue was dissected away from the embryo, flash frozen, and stored at −80 °C. Unless otherwise noted, E12 or E14 maternal-fetal interface tissue was kept all together, including the decidua, placenta, and surrounding supportive uterine muscle tissue. For qPCR experiments comparing the decidua to the placenta, the surrounding supportive uterine muscle tissue was removed and the decidua was dissected away from the placenta. For all experiments fetal bodies were removed from the placenta, the top of embryo heads were isolated, and brains were scooped out. Embryonic brains were either placed in Buffer A (DMEM/F12 with 10 % FBS, 0.1 % Glutamax, and 0.1 % antibiotic antimycotic) on ice for downstream magnetic-activated cell sorting (MACS) or were flash frozen and stored at −80 °C. The remaining embryonic body was flash frozen and stored at −80 °C for downstream sex genotyping. For postnatal brain collection (postnatal day (P)5 or P90) euthanized mice were perfused with 5 U/ml heparin in 1x PBS, brains were harvested, and meninges carefully removed. Brains were placed in Buffer A on ice for downstream MACS preparation.

2.4. Sex genotyping

Flash frozen fetal body tissue was incubated in 200 µl DirectPCR Lysis Reagent (Viagen Biotech, 102-T) with 4 µl Proteinase K (Thermo Fisher 3115879001) overnight at 55 °C. Samples were heat shocked at 85 °C for 45 min and then chilled at 4 °C for 5 min and spun down at 16,000 rpm for 10 min. DNA supernatants were collected and stored at −20 °C until use for PCR. PCR reactions were set up using MyTaq Red Mix (Meridian Bioscience BIO-25043) and Sx primer pair (sequences below) with extracted DNA, and then run on an agarose gel.

Rev: 5′ CTT ATG TTT ATA GGC ATG CAC CAT GTA 3′
Fwd: 5′ GAT TAT TGG AGT GGA AAT GTG AGG TA 3′

2.5. RNA extraction

Frozen tissues (whole MFs, placentas, deciduae, or whole brains) were thawed on ice and then mechanically homogenized in Tissue Protein Extraction Reagent (7-PER; Thermo Fisher, 78510) containing phosphatase inhibitor cocktail PhosSTOP (Roche, 04906845001) and protease inhibitor cocktail Complete (Roche, 11873580001). Whole maternal-fetal interface tissue, placentas, and deciduae were homogenized in 500 µl and whole E12 or E14 brains in 250 µl of this TPER cocktail. Slurries (50 µl of placenta decidua slurry or the entire embryonic brain slurry) were then added to 500 µl TRIzol reagent (Life Technologies, 15596018) and stored at −80 °C until further use. For RNA extraction, TRIzol-supended samples were thawed on ice, vortexed, and 100 µl of chloroform (Fisher Scientific, BP1145-1) was added. Samples were incubated at room temperature for 5 min and then spun down at 14,000 rpm at 4 °C for 15 min. The clear aqueous top layer was collected and an equal volume of isopropanol (Sigma, I9516) was added then vortexed vigorously. Samples were then incubated at room temperature for 10 min and spun down at 12,000 rpm at 4 °C for 5 min. The resulting RNA pellet was then washed with 1 ml of 70 % ethanol in RNAse−/ DNAse-free water and spun down at 14,000 rpm at 4 °C for 5 min. This wash step was then repeated and finally the RNA pellet was air dried at room temperature and resuspended in 30 µl of DNAse−/RNAse-free water. RNA quality and quantity were evaluated using NanoDrop 2000 Spectrophotometer (Thermo Scientific).

2.6. Magnetic-activated cell sorting

Fresh brains held in Buffer A (DMEM/F12 with 10 % FBS, 0.1 % Glutamax, and 0.1 % antibiotic antimycotic) on ice were transferred to Buffer B (HBSS + Ca + Mg with 4 U/ml papain, and 50 U/ml DNase I) at room temperature. For embryonic timepoints (E12 and E14) brains were added to 1.5 ml Buffer B. For postnatal timepoints (P5 and P90) brains were added to 5 ml Buffer B. Samples were triturated using a 5 ml serologic pipette and incubated for 15 min at 37 °C. Samples were then triturated using a 1 ml pipette and incubated again for 15 min at 37 °C. This step was repeated twice (giving a total of 3 trituration steps and 45 min of incubation at 37 °C) to yield a smooth single cell suspension. Samples were triturated once more with a 1 ml pipette and then filtered through a 70 µm cell strainer into a clean tube. 20 ml Buffer A was then added and cells were spun down at 300 g at 4 °C for 10 min (fast acceleration, slow brake). Supernatants were aspirated and the remaining cell pellets were resuspended in 180 µl 1x MACS buffer (Miltenyi Biotec, 130-0910376) in PBS. 90 µl of CD11b magnetic microbeads (Miltenyi Biotec, 130-093-634) were added and samples were incubated for 15 min at 4 °C. Samples were then washed with 1 ml MACS buffer and spun down at 300 g at 4 °C for 10 min (fast acceleration, fast brake). Supernatants were aspirated and the remaining cell pellets were resuspended in 500 µl MACS buffer. LS columns (Miltenyi Biotec, 130-042-401) and a QuadroMACS magnet (Miltenyi Biotec, 130-091-051) were used to isolate CD11b+ cells according to the manufacturer’s instructions. Column-bound microglia (CD11b+ cells) were then spun down at 300 g at 4 °C for 10 min. Cell pellets were either added to 200 µl TRIzol reagent for RNA extraction or resuspended in 1x PBS for flow cytometry.

2.7. Flow cytometry

Flow cytometry was employed to validate MACS purification efficacy. CD11b-positive and -negative fractions in 1x PBS were transferred to a 96-well round bottom plate and spun down at 1500 rpm for 5 min at 4 °C. Cells were then stained with fixable viability dye (eBioscience, 65-0866-14) at 1:1000 for 30 min at 4 °C. Cells were washed with FACS buffer (1x PBS, 1 mM EDTA, and 1 % BSA) and spun down at 1500 rpm at 4 °C. Cells were then stained 1:200 with CD11b (APC), CD45 (PE-Cy7), and TCR β chain (Brilliant Violet 510) flow antibodies (all from eBioscience) in FACS buffer for 15 min at 4 °C. Cells were washed once with FACS buffer, spun down at 1500 rpm at 4 °C, then resuspended in 100 µl of FACS buffer. Microglia were identified as CD45hi and CD11b+ after gating for single cells. Data were acquired using a Gallios flow cytometer (10 colors, 3 lasers, B5-R1-V2 Configuration with Kaluza Acquisition; Beckman Coulter) and analyzed using FlowJo software (Becton, Dickinson, & Company).

2.8. RNA-sequencing procedure and data analysis

Isolated maternal-fetal interface RNA and sorted microglia resuspended in TRIzol were sent to GENEWIZ Next Generation Sequencing for sample preparation and bulk RNA-sequencing. The raw sequencing reads (FASTQ files) were aligned to the UCSC mm39 mouse genome build using the splice-aware read aligner HISAT2. Samtools was used for quality control filtering. Reads were sorted into feature counts with HTSeq, DESeq2 (v1.20.0) was used to normalize the raw counts based on quality control filtering. Reads were sorted into feature counts with HTSeq, DESeq2 (v1.20.0) was used to normalize the raw counts based on quality control filtering. Analyses were performed and plots were generated in RStudio using the following packages: lattice, DESeq2, pheatmap, GSA, ggplot2, ggrepel, dplyr, tidyverse, ggprofiler2, pals, EnhancedVolcano, stringr, data.table, and VennDiagram.

2.9. cDNA synthesis and qPCR

Isolated RNA was converted to cDNA using a Sensifast cDNA Synthesis Kit (Bioline, BIO-65054). Gene expression levels were determined using Taqman Gene Expression Assay primer/probe mix (Thermo
tissues (with Fisher), SensifastProbe No-ROX kit (Bioline, BIO-86005), and a CFX384 Real-Time PCR System (BioRad, 1855484). All kits were used according to manufacturer’s instructions. The following primers (Thermo Fisher Scientific) were used: ifit1 (Mm00515153_m1), Mx1 (Mm00487976_m1), Ccl3 (Mm00441259_g1), Trf3 (Mm01207404_m1), Il1b (Mm00434228_m1), Il6 (Mm00446190_m1), Ii17a (Mm00439619_m1), Ilora (Mm01211445_m1), Il17ra (Mm00434214_m1), Cx3cr1 (Mm02620111_s1), Syk (Mm01333935_m1), Chd1 (Mm00514308_m1), Ej/2s1 (Mm00782766_s1), Rp2 (Mm01971861_g1), Rack1 (Hs00272002_m1), Slc6a4 (Mm00439391_m1), Slc22a3 (Mm00488294_m1), Ido1 (Mm00492590_m1), Tph1 (Mm01202614_m1), Maoa (Mm00558004_m1), Maob (Mm00555412_m1), Cdh1 (Mm01247357_m1), Cd56 (Mm01149710_m1), Uly (Mm00447710_m1), Xist (Mm01232884_m1), and Gapdh (Mm99999915_g1). Relative expression levels were calculated based upon Gapdh expression.

2.10. Serotonin ELISA

Serotonin concentrations in maternal sera or MFI homogenates were determined using a serotonin ELISA kit (Enzo Life Sciences, ADI-900-175). Frozen MFI tissues were thawed on ice and then mechanically homogenized in 500 ul TPER cocktail (Tissue Protein Extraction Reagent T-PER (Thermo Fisher, 78510)) containing phosphatase inhibitor cocktail PhosSTOP (Roche, 04906845001) and protease inhibitor cocktail cOmplete (Roche, 11873580001). Slurries were then spun down at 16,000 rpm for 10 min and the soluble supernatants were collected. Serotonin ELISA was conducted on undiluted maternal sera or 1:10 diluted MFI tissue extracts according to the manufacturer’s instructions. The plate was read on an Epoch microplate spectrophotometer (Agilent) at 405 nm.

2.11. Multiplex cytokine assay

Cytokine concentrations in maternal sera and homogenates from MFI, placenta, and decidua were determined using a Bio-Plex multiplex cytokine assay. Frozen tissues (bulk MFI or dissected placenta/decidua tissues) were thawed on ice and then mechanically homogenized in 500 ul TPER cocktail (Tissue Protein Extraction Reagent T-PER (Thermo Fisher, 78510) containing phosphatase inhibitor cocktail PhosSTOP (Roche, 04906845001) and protease inhibitor cocktail cOmplete (Roche, 11873580001). Slurries were then spun down at 16,000 rpm for 10 min and the soluble supernatants were collected. A multiplex cytokine assay was conducted on undiluted samples. All reagents were used according to the manufacturer’s instructions: Bio-Plex Pro Reagent Kit III (Bio-Rad, 171304090M), Bio-Plex Pro Coupled Magnetic Beads (from Mouse Cytokine 23-plex Assay, Bio-Rad, M60009RDPD), Group I Cytokine standards (Bio-Rad, 171150001). The plate was read on a Bio-Plex 200 System with HTS (Bio-Plex, 171000205).

2.12. Behavior

All behavior experiments were performed between 8 am and 5 pm in a blinded fashion. Mice were transported from their home vivarium room to the behavior core and allowed 30 min to habituate before beginning each test. All behavior experiments were conducted as previously described (Lammert et al., 2016).

2.12.1. Ultrasound vocalization recording

Communication following maternal separation was conducted on P10 male and female mice. Pups were separated from their mother with their littermates and allowed to habituate in the testing room for 10 min. Mice were then placed one-by-one in a clean 1 L plastic cup with a microphone suspended overhead. Ultrasonic vocalizations (USVs) were recorded using UltraSoundGate GM16/CMPA microphone (Avisoft Bioacoustics) and analyzed with SASLab Pro software (Avisoft Bioacoustics). USVs were measured between 25 and 125 kHz and background recordings shorter than 0.02 ms were excluded.

2.12.2. Marble burying assay

Repetitive/stereotyped behaviors were assessed on adult male and female mice (8–10 weeks of age) using the marble burying assay. Mice were acclimated to wood chip bedding in their home cages overnight prior to testing. Experimental mice were placed into a clean cage (12 × 7 × 5 in) filled with 3 in tightly packed wood chip bedding with 20 glass marbles arrayed in rows of 4 and columns of 5 equally spaced throughout the cage. Mice were allowed to explore the cage for 15 min and then an index score of marbles buried was calculated. A score of 0–1 was given for each marble (0 = < 50 % buried, 0.5 = ~50 % buried, 1 = >50 % buried) to yield a maximum score of 20 for this assay.

2.12.3. Social preference test

Sociability was assessed on adult male and female mice (8–10 weeks of age) using the social preference test. Two chamber habituation sessions were conducted in which mice were allowed to explore the entire 3-chamber arena with empty wire cages added for 5-min each session. Mice were then housed in solo overnight before testing. The social preference test began with placing the mice in the center of the 3-chamber arena with the portals blocked off in which mice were allowed to explore only the center chamber for 5 min. Then, the barriers were removed and the mice had free access to the entire arena, with the top chamber containing an empty wire cage holding a novel mouse (age and sex-matched) and the bottom chamber containing another empty wire cage holding a novel object (aquarium suction balls). Mice were allowed to explore for 10 min and activity was tracked using EthoVision XT (Noldus).

2.13. Statistics

Sample sizes were chosen on the basis of standard power calculations (with α = 0.05 and power of 0.8). Statistical tests for RNA-seq analyses were conducted using R (4.0.4 GUI 1.74 Catalina build (7936)) in RStudio (1.4.1106). For all other analyses, Prism software (GraphPad, 9.4.0) was used to calculate mean and s.e.m. values and to conduct unpaired Student’s t test, one-way ANOVA, and two-way ANOVA. P values less than 0.05 were considered significant.

3. Results

3.1. MIA triggers a robust immune response acutely at the maternal-fetal interface

There is now an abundance of evidence that inflammation during pregnancy is a significant risk factor for offspring neurodevelopmental disorders yet the response of the maternal-fetal interface to systemic inflammation is less well understood. We sought to better understand how inflammation during pregnancy impacts offspring neurodevelopment through characterizing the MFI transcriptional response to MIA in an unbiased manner. To this end, we employed a model of MIA in which pregnant dams were injected with the viral mimetic polycytidylic acid (polyI:C) on embryonic day (E)11 and E12 (Supplemental Table 1; Lammert et al., 2018; Lammert and Lukens, 2019). This immune response is relatively mild, as polyI:C exposure did not significantly affect dam weight gain throughout pregnancy (Fig. S1A), litter size, or offspring sex distribution (Fig. S1B).

To illuminate the impact of MIA on the maternal-fetal interface in an unbiased manner, we employed bulk RNA-sequencing (RNA-seq) on MFI tissue (comprising both the decidua and placenta) from MIA and control pregnancies. We chose to evaluate the MFI at E12, 3 h-post-injection.
(hpi), in which maternal serum IL-6 levels are highest in this MIA model, as well as E14, 48 hpi, in which IL-17a levels peak (Choi et al., 2016; Fig. 1A). MFI samples were collected from time- and embryo-matched saline and polyIC pregnancies. RNA extracted from MFI samples collected at E12 and E14 was pooled equally by sex within each litter, yielding four samples collected from independent saline and polyIC pregnancies. Principal component analysis (PCA) of all E12 and E14 samples revealed significant clustering of samples by both timepoint and treatment (Fig. S2A), indicating a substantial effect of both developmental stage and MIA on the placental transcriptome.

We first wanted to define the acute response to MIA by comparing polyIC to saline MFI samples at E12 (3 hpi) collapsed across sex. PCA at this timepoint revealed that saline and polyIC MFI samples clustered in distinct groups along PC1, accounting for 86 % of the variance, indicating that MIA causes a predominant shift in the MFI transcriptome (Fig. 1B). The transcriptional response at the MFI was robust and quick at the initiation of MIA. We found 1,877 upregulated genes and 1,674 downregulated genes when comparing polyIC to saline MFIs at E12 (Fig. 1C). When looking at the top 20 polyIC upregulated genes, we noticed a strong pro-inflammatory signature dominated by type I interferon (IFN) response genes including Mx1, Cxc10, and Ift1, among others (Fig. 1C,D). Indeed, we found dramatically increased expression of many interferon-stimulated genes that included IFIT and OAS pattern recognition receptors, STAT transcription factors, and more (Fig. 1E).

We next sought to glean an overall view of the immune response orchestrated at the MFI in response to polyIC exposure. A cytoscape network analysis of polyIC upregulated genes revealed an enrichment of immune terms related to cytokine signaling, lymphocyte and neutrophil migration, autophagy, and leukocyte activation (Fig. 1F). Gene set enrichment analysis (GSEA) of polyIC upregulated genes using KEGG pathways uncovered specific signaling cascades engaged which included PI3K-AKT, NOD-like receptors, Toll-like receptors (TLRs), RIG-1, and NF-kB signaling, among others (Fig. 1G). We also noted pathways related to T cell activation (Fig. 1G) and were intrigued to see indication of a Th17 response, given that maternal Th17-mediated IL17A production is required for the onset of polyIC offspring behavioral phenotypes (Choi et al., 2016; Kim et al., 2017; Reed et al., 2020). Indeed, we found a broad upregulation of many genes related to Th17 differentiation acutely following polyIC exposure in the MFI (Fig. 1H). We also noted a shift toward anti-angiogenic signaling in which polyIC induced the downregulation of many pro-angiogenic factors (i.e. Fg2, Pdgfa, Pdgfc) concomitant with upregulation of anti-angiogenic factors (i.e. Angpt1, Angr2, Cxc9, Cxc10, Cxc11, Thbs1, Thbs4; Fig. S3).

While the largest fold changes in gene expression were found in upregulated genes, we were still curious to explore the pathways disengaged in MIA-exposed MFIs. GSEA of polyIC downregulated genes using the gene ontology (GO) database revealed an interesting dampening of pathways related to offspring development (Fig. 1I). Such downregulated genes when comparing polyIC to saline MFIs at E12 largely driven by type I IFN signaling at the expense of ordinary offspring developmental support. Sex differences in the neurodevelopment of MIA offspring have been reported using multiple different models of infection (Carlezon et al., 2019; Gogos et al., 2020; Kalish et al., 2021; Keever et al., 2020; Smith et al., 2020). Thus, we were interested to see whether there were sex differences in the immune response at the placenta that could begin to explain some of the differences in offspring neurodevelopment. We therefore conducted differential gene expression analysis on E12 MFIs split by sex. We found 1,513 differentially expressed genes (DEGs) in males and 2,272 DEGs in females (Fig. S2B), with the largest fold changes occurring in upregulated genes in both sexes. We found many of these upregulated genes to be similar between the sexes (Fig. S2B,C) with the top 15 upregulated DEGs largely overlapping in male and female MFIs (Fig. S2D). Taken together, this transcriptional analysis of the acute MFI response to MIA revealed an engaged immune response that is largely conserved between the sexes.

3.2. The pro-inflammatory response to polyIC is distinct at the placenta and the decidua

To glean insight into whether this immune response is generated in dam-derived cells (decidua) or embryo-derived cells (placenta), we dissected apart decidua and placenta tissue. Successful dissociation of the decidua from the placenta was confirmed by relative expression of the placenta-enriched marker Cd1b (E-cadherin; Fig. 2A). We additionally confirmed that decidua from either male or female embryos were composed primarily of female (i.e. maternal) cells via expression of the X chromosome gene Xist (Fig. 2B). Placentas from female embryos also expressed Xist but completely lacked expression of the Y chromosome gene Uty (Fig. 2B-C). Accordingly, placentas from male embryos completely lacked Xist expression but highly expressed Uty (Fig. 2B-C).

We next profiled the expression of pro-inflammatory genes by qPCR to understand the response to polyIC in each MFI compartment. We previously found Ifit1, Mx1, and Il6 to have increased expression in bulk maternal-fetal interface tissue (Fig. 1). Strikingly, the placenta, but not the decidua, significantly upregulated expression of Ifit1, Mx1, and Il6, in addition to Ccl3 and Il6ra (Fig. 2D). Meanwhile, a well-characterized marker of decidual NK (dNK) cells, Cd56, was exclusively upregulated in the decidua but not the placenta 3 hpi (Fig. 2D).

These findings heightened our curiosity as to whether protein levels of pro-inflammatory cytokines were differentially enriched in either MFI compartment. We conducted a multiplex cytokine assay on dissected decidua and placenta tissue homogenates from pregnancies 3 h post-saline or -polyIC treatment. In comparing cytokine concentrations within each tissue, we found significantly elevated levels of IL-6, IL-17a, and G-CSF within the placenta but not the decidua (Fig. 2E). No cytokines were found in significantly altered levels within the decidua (Fig. 2E). Further, when comparing polyIC decidua to polyIC placentas we found significantly elevated levels of IL-1β, IL-4, IL-17a, G-CSF, GM-CSF, and TNF-α (Fig. 2E). Other MIA studies have similarly reported increased levels of IL-6 and IL-17a following polyIC treatment (Choi et al., 2016; Lammert et al., 2018; Smith et al., 2007) and we were intrigued to find this response exclusively in the placental compartment. Altogether, we find that both dam- and embryo-derived cells respond to polyIC yet in distinct fashions.

3.3. The MFI immune response to MIA is largely absent by E14

We were next curious to see how long this inflammatory response at the MFI persisted after polyIC exposure. To this end, we compared the transcriptome of polyIC and saline MFI samples at E14 (48 hpi). PCA clustering revealed that saline and polyIC samples still clustered in relatively distinct groups at this time point, though not to the same extent as at 3 hpi (Figs. 1B, 3A). While there was a significant number of DEGs in response to MIA at 3 hpi, we found many fewer DEGs at 48 hpi (Fig. 3B), indicating a potential resolution of the inflammatory response. Interestingly, we noticed that the male control MFIs clustered more closely with the polyIC MFIs by PCA compared to the females (Fig. 3A).

To further explore this pattern, we conducted differential expression analysis with the samples split by sex. Indeed, only 106 genes were significantly differentially expressed in the male MFI exposed to polyIC compared to controls (Fig. 3B). We meanwhile uncovered 541 DEGs in female MFIs, 56 of which were upregulated and 483 of which were downregulated (Fig. 3B). In both sexes, there was a striking lack of the immune response genes that were so strongly upregulated at 3 hpi (Fig. 1C, 3B). We verified this effect by qPCR looking at a selection of relevant DEGs and indeed saw the same strong upregulation of immune-
Fig. 1. The maternal-fetal interface undergoes a robust immune response acutely after polyI:C exposure. Pregnant dams were injected intraperitoneally (i.p.) with 20 mg/kg polyI:C on embryonic day (E)11 and E12 to elicit maternal immune activation (MIA) or with saline as a control. Bulk RNA-sequencing was conducted on maternal-fetal interface (MFI) tissue at E12 (3 hrs post-injection, hpi) and E14 (48 hpi). (A) Experimental design. 3- and 48-hpi placental tissue and fetal bodies were collected. Fetal bodies were genotyped by Sx PCR to demarcate placental extracts by sex. Sex-stratified samples were pooled within litters for E12 and E14 time-points, n = 4 litters/group. (B-G) RNA was isolated from MFI tissue then bulk RNA-sequencing was conducted on the 4 experimental groups with 4 samples per group. (B) Principal component analysis (PCA) showing clustering of groups from E12 placental samples. (C) Volcano plot showing the number of differentially expressed genes in E12 MFI tissue comparing polyI:C to saline (FDR < 0.1) and combined by sex. Dark purple dots indicate genes that passed the combined p-value < 1x10^-5 and log2FC > 2 cutoff of significance, light purple dots indicate genes with p-value < 1x10^-5, and dark grey dots indicate genes with log2FC > 2. (D) Heatmap representation of the top 20 genes upregulated in the polyI:C group, combined by sex (FDR < 0.1). (E) Interferon pathway gene expression (rlog normalized counts) compared between polyI:C and saline groups, combined by sex. (F) Cytoscape network map illustrating select shared gene ontology (GO) terms. (G) Gene set enrichment analysis (GSEA) using KEGG pathways from genes upregulated (FDR < 0.1) in the polyI:C group. Dot plot of select enriched KEGG terms. (H) Genes related to Th17 differentiation compared between polyI:C and saline groups, combined by sex and expression levels visualized by heatmap. (I) GO Terms of PolyC Downregulated.
related genes at E12 which were no longer differentially expressed by sex and/or MIA. This finding spurred our curiosity to define any baseline transcriptional sex differences and whether MIA influences the expression of these genes.

3.4. Baseline sex differences in the MFI transcriptome are dampened subacutely by MIA

We noted in our PCA plots that samples clustered distinctly based on sex when comparing our saline controls at E12 (Fig. 1B) and at E14 (Fig. 3A), indicating significant baseline sex differences in the MFI transcriptome. We were fascinated to see that nearly all of these sex-dependent genes were no longer differentially expressed by sex when comparing polyI:C male to polyI:C female MFIs both at E12 and E14 (Fig. 4A,B). Specifically, there were only 15 genes at E12 and 75 genes at E14 that were differentially expressed when comparing between the sexes in polyI:C MFIs (Fig. 4A,B), and many of these DEGs were sex chromosome genes (Fig. 4A). In other words, the response to polyI:C is largely overlapping in male and female MFIs, consistent with our DEG findings (Fig. S2).

To begin to understand these MFI transcriptional sex differences, we first wanted to better define the biological processes chiefly employed at each developmental stage assessed, as this may inform any perturbations that occur as a result of sex and/or MIA. Thus, we conducted PCA and differential expression analysis comparing subsequent developmental timepoints (E14 v. E12) in saline control samples. PCA revealed that time had the greatest influence on the transcriptional landscape (Fig. S4A); PCI accounted for 69% of the variance in which samples
cluster along this principal component predominately by timepoint. Sex accounted for the second-most variance in transcriptome (14 %), as samples clustered next by female and male along PC2 (Fig. S4A).

We identified 3,727 genes enriched at E12 and 3,858 enriched at E14 in the homeostatic MFI (Fig. S4B). The top DEGs enriched at E12 included hemoglobin subunits Hbb.b1 and Hba.x, the cell cycle regulator Phlda2, and oxidoreductases Dio3 and Hsd3b6 (Fig. S4B). GSEA of all E12 DEGs revealed that networks related to the cell cycle, mitochondrial translation, and rRNA processing dominated the transcriptomic landscape at this time compared to E14 (Fig. S4C). At E14, we identified prevailing enrichment for the prolactin genes Prl3a1, PrlB6, and PrlBA6, pregnancy-specific glycoproteins Prl8a6 and Prl8a8, which are known to be involved in immunoregulation and thromboregulation, as well as the catehepsin-ending gene Cts6. Indeed, GSEA of all E14-enriched genes uncovered pathways related to lipid metabolism (likely related to lactation) and plasma/platelet-related processes, in addition to insulin growth factor (IGF)- and solute carrier (SLC)-mediated transport (Fig. S4C). We also identified significant enrichment for Acc2, the angiotensin-converting enzyme that can also serve as a primary entry point into cells subverted by SARS-CoV-2 when ACE2 is present in a membrane-bound form (Yan et al., 2020; Fig. S4B). This is consistent with studies showing that SARS-CoV-2 can infect placental syncytiotrophoblasts and initiate an inflammatory response at the placenta (Argueta et al., 2021; Bordt et al., 2021).

Males and females conceivably require different support in terms of nutrients, growth factors, and protection (Gabory et al., 2013; Meakin et al., 2021; Rosenfeld, 2015). In addition, male embryos are immunologically viewed as significantly more foreign to the mother’s immune system due to the presence of Y chromosome genes, perhaps necessitating an even more tolerogenic state (Gabory et al., 2013; Rosenfeld, 2015). Moreover, differences in sex chromosome gene dosage (XX vs XY) could influence MFI function both at baseline and in response to a change in the maternal milieu, such as those triggered by environmental insults like MIA. We therefore first sought to more completely understand the gene modules that made up apparent sex-dependent DEGs. At E12, GSEA revealed an abundance of differentially regulated pathways in female MFIs, many of which were related to immune signaling pathways (Fig. 4C). In particular, we noted enrichment of signaling modules involving Fc receptors, TLRs, chemokines, and activation of various immune cell subsets (Fig. 4C). The male MFI at baseline were enriched for protein export and spliceosome pathway KEGG terms (Fig. 4C).

We wondered whether these sex-dependent gene modules persisted at E14 and/or whether new sex-dependent gene modules appeared as pregnancy progressed. A comparison of genes differentially expressed in males at E12 and E14 revealed an overlap of 141 genes, with 210 genes uniquely expressed at E12 and 105 genes uniquely expressed at E14 (Fig. 4D). The largest fold changes in male-enriched genes occurred in Uty, Ef2ksy, and Ddx3y, which were present at both E12 and E14 (Fig. 4E). GSEA using the Reactome dataset found that male MFIs were enriched for mRNA processing-related pathways at E12 while at E14 male MFIs were enriched for fibrin signaling and insulin-like growth factor transport (Fig. 4D). Male MFIs were enriched for pathways related to translation and the calreticulin cycle at both E12 and E14 (Fig. 4D).

We then repeated this analysis on female MFIs and found 338 overlapping genes at E12 and E14, with 179 genes unique to E12 and 117 genes unique to E14 (Fig. 4D). The top 10 DEGs enriched in females were present at E12 and E14, including Stab1, Mrc1, and F13a1 (Fig. 4E). Consistent with our KEGG terms (Fig. 4C), E12 female MFIs were enriched for immune signaling (Fig. 4D). Some of these immune pathways were also apparent at E14, including Dectin- and neutrophil-related signaling (Fig. 4D) and genes such as Cc3, Cxc3r1, and Fcris (Fig. 4E). Female MFIs were also enriched for non-immune-related pathways at both E12 and E14 which included GTPases and modules related to the nervous system (Fig. 4D). At E14 alone, female MFIs were enriched for cell cycle-related signaling (Fig. 4D).

All of these gene modules that are typically employed in males and females separately during development are no longer apparent in poly:
C pregnancies (Fig. 4A,B). Strikingly, though the immune response to polyI:C is largely complete by E14, these dampened sex differences continue to persist at this timepoint, which indicates of a prolonged imprint of polyI:C exposure (Fig. 4A,B). While the female and male MFI immune response to MIA predominantly overlaps, it is possible that the lack of typical employment of sex-specific gene modules contributes to altered offspring development in a MIA environment.

3.5. MIA leads to sex-biased behavioral changes without an accompanying transcriptional shift in microglia

Given the quintessence of the maternal-fetal interface in supporting embryo development paired with the significantly shifted transcriptome triggered by MIA, we next sought to investigate the impact that maternal inflammation may have on offspring neurodevelopment. We wondered whether sex-biased differences in offspring behavior existed in our MIA model, given our findings that the MIA female MFI was found to downregulate many immune-related genes subacutely (Fig. 3B,D) and that baseline transcriptional sex differences in the MFI are dampened by MIA (Fig. 4).

Indeed, our MIA offspring displayed sex-biased behavioral alterations. More specifically, we found that male polyI:C offspring emitted fewer ultrasonic vocalizations (USVs) when briefly separated from their mother and littermates at postnatal day (P)10 (Fig. 5A), indicating impaired communication ability compared to female polyI:C littermates and saline control offspring. Sex-biased behavioral alterations were also present in adulthood, as male polyI:C offspring buried more marbles (Fig. 5B) and interacted equally with an object versus a novel mouse.
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**Fig. 5.** Maternal immune activation elicits sex-biased behavioral alterations in offspring but does not impact microglia on the transcriptional level. Pregnant dams were injected i.p. with 20 mg/kg polyI:C on E11 and E12 to elicit MIA or with saline as a control. (A–C) Male and female offspring of MIA and control pregnancies were assessed for behavioral alterations including communication ability (A), stereotyped/repetitive actions (B), and sociability changes (C). (A) Number of ultrasonic vocalizations elicited during 3-min of separation from mother and littermates at postnatal day (P)10. (B) Number of marbles buried during a 10-min marble burying assay conducted at 8–10 weeks of age. (C) Percent time spent interacting with a novel mouse compared to a novel object during a 10-min three-chamber social preference test conducted at 8–10 weeks of age. (D) Microglia RNA-sequencing experimental design. 3- and 48-hpi embryos were collected and decapitated. Brains were also harvested from P5 and P90 offspring. Single cell suspensions of fresh brain tissue were subject to CD11b + magnetic bead purification by magnetic-activated cell sorting (MACS) to isolate microglia. Sex-stratified samples were pooled within litters for E12 and E14 timepoints (n = 4 litters per group). Individual mice were used for P5 and P90 timepoints (n = 4 mice per group). RNA was isolated from purified microglia and then bulk RNA-seq was conducted on the 4 experimental groups with 4 samples per group at each timepoint. (E) PCA showing clustering of treatment groups from all timepoints. (F,G) Reanalysis of single cell RNA-sequencing data collected from MIA offspring and PBS control brains (Kalish et al. 2020) at E14 (48 h-post-polyI:C) and E18 (96 h-post-polyI:C). tSNE plots show microglia forming two clusters at E14 (F, left) and five clusters at E18 (G, left); however, MIA and PBS microglia do not fall into any distinct cluster at either timepoint or form any distinct clusters (F,G; right). Each point represents an individual mouse (A–C). Statistical significance calculated by one-way ANOVA with Tukey’s post-hoc comparison (A–B) or multiple Student’s t-tests (C). Error bars indicate mean ± s.e.m. ns = not significant, *P < 0.05, **P < 0.001, ***P < 0.0001.

(Fig. 5C) compared to female polyI:C offspring and saline controls; indicative of repetitive/stereotyped behaviors and sociability deficits, respectively. These sex-biased behavioral changes are consistent with published neurodevelopmental alterations using other MIA models (Carlezon et al., 2019; Gogos et al., 2020; Kalish et al., 2021; Smith et al., 2020).

The innate immune cells of the brain, microglia, constitute the first line of defense against brain pathogens and are also critically involved in homeostatic neurodevelopmental processes including circuit refinement, angiogenesis, and cell maturation/differentiation (Cowan and Petri, 2018; Lukens and Eyo, 2022; Zengeler and Lukens, 2021). All of these processes could conceivably be impacted by sex and/or inflammation during neurodevelopment. We therefore wondered whether microglia would be particularly sensitive to any immunologic and/or sex-specific stimuli that could be propagated from the placenta to the developing embryonic brain during MIA.

To investigate in an unbiased manner whether microglial gene expression is impacted by MIA, we conducted bulk RNA-seq on purified offspring microglia at select timepoints post-MIA (Fig. 5D). Given our intriguing findings from the MFI RNA-seq study, we chose to evaluate microglia at E12 (3 hpi) and E14 (48 hpi) in an effort to correlate any MFI findings to a potential impact on microglia. We additionally collected sorted microglia at P5, a timepoint in development in which microglial phagocytosis is distinctly relevant (Nelson et al., 2017; Schafer et al., 2012; Zengeler and Lukens, 2021), as well as at P90 when neurodevelopment is largely complete. Microglia were isolated by
generating single-cell suspensions of brain tissue and then selecting for CD11b+ cells by magnetic-activated cell sorting (MACS). This strategy was effective in purifying CD11b+ cells at all evaluated timepoints (E12, E14, P5, and P90; Fig. S5A,B) and the purity and efficacy of this isolation technique was not impacted by either sex or treatment condition (Fig. S5C).

Using the same strategy as with MFI samples, RNA extracted from microglia samples collected at E12 and E14 was pooled equally by sex within each litter from a total of four litters per group (Fig. 5D). Given the larger brain size, samples isolated at P5 and P90 were collected from a full brain of sorted microglia RNA from four mice per group (Fig. 5D). PCA of all microglia across these developmental points revealed that time had the greatest influence on the transcriptional landscape of microglia: groupings were spread out by timepoints across PC1 which accounted for 91 % of the variance (Fig. 5E, S6A). In looking at each time point individually, we found that microglia were largely unaffected by MIA whereby polyIC and saline samples clustered together at E12, E14, P5, and P90 (Fig. 5E). We noted very few differentially expressed genes when comparing saline to polyIC microglia at each of these timepoints, with none passing the significance threshold (Fig. S7). Moreover, we did not find any major sex differences in microglia at baseline (Fig. S8) or when exposed to polyIC (Fig. S9) on the transcriptional level at any of the assessed timepoints.

To verify the quality and robustness of our microglia RNA-seq dataset, we evaluated the baseline transcriptional landscape and developmental trajectory in control microglia collapsed across sex. We conducted differential expression analysis at subsequent developmental timepoints (E14 v. E12, P5 v. E14, and P90 v. P5) in saline control samples and identified a vast number of DEGs in each of these comparisons as we had found in the placenta (Fig. S6B). We found microglia to be increasingly transcriptionally different at each comparison through our developmental time course (Figs. S6, S10-A-C) in which there was a coordinated loss of immature microglia genes and gain of mature markers (Fig. S10-D-G). Pathway analyses revealed that microglia progress through highly distinct neurodevelopmental phases: these cells first undergo robust replication, then progress to a role of sculpting the extracellular matrix and neuronal development, then become highly active in terms of metabolism, cell growth, and signaling cascades, and finally reach a mature maintenance state (Fig. S10H). This transcriptional assessment of microglial maturation is consistent with published studies (Hanamsagar et al., 2017; Matovcich-Natan et al., 2016).

To independently corroborate these findings, we re-analyzed the transcriptional profile of MIA microglia from a published single-cell RNA-sequencing (scRNA-seq) dataset using a similar polyI:C model (Kalish et al., 2021). Following clustering of all cells at E14 (48 h post-polyIC) and E18 (96 h post-polyIC), we selected for microglia based on enriched expression of Cx3cr1 and Tmem119 and low expression of other cell-type-specific markers (Fig. S11). tSNE re-clustering of microglia yielded two sub-clusters at E14 (Fig. S5F) and 5 sub-clusters at E18 (Fig. S5G). MIA and control PBS microglia did not fall into any distinct cluster at either E14 (Fig. S5F) or E18 (Fig. S5G). In addition, we found very few significantly differentially expressed genes between MIA and PBS microglia. There were no significant DEGs at E14 and only 4 DEGs at E18: mt-Co2, Mrc1, Lyve1, and Fl3a1. Taken together, these findings suggest that this model of MIA does not significantly affect the transcriptome of microglia during development or persisting into adulthood. Whether this MIA model alters microglia at the functional levels and/or primes microglia to respond differently to insults later in life will be important future areas of investigation.

3.6. SSRI exposure reshapes the MFI response to MIA

A recent study found that inflammation during pregnancy due to maternal high-fat diet gave rise to sex-dependent behavioral alterations that were accompanied by reduced placental serotonin levels (Ceasirne et al., 2021). We therefore wondered whether maternal inflammation in our polyIC model also altered the serotonergic tone of the MFI. In addition, we were interested to see whether and how the MFI responds to a different type of environmental insult, and furthermore whether a combination of insults would differentially impact in utero physiology and offspring neurobiology. For this second insult, we used fluoxetine (a common SSRI, brand name Prozac), a medication used to treat depression in addition to other mental conditions, including obsessive compulsive disorder, panic attacks, some eating disorders, and premenstrual syndrome (Sohel et al., 2022). A query study found that fluoxetine is among one of the top 20 most commonly prescribed medications during pregnancy (Mitchell et al., 2011).

For our study, we began treating female mice with fluoxetine at least two weeks prior to mating (Fig. 6A) to investigate the impact on MFI health and offspring neurodevelopment. Control and fluoxetine-treated pregnant dams were then exposed to MIA or a saline control injection as before and then maternal sera, MFIs, and embryonic brains were harvested 3 hpi to compare differential responses to polyIC and fluoxetine as well as interrogate any combinational effects (Fig. 6A). Fluoxetine exposure, like polyIC, did not appreciably affect maternal weight gain during pregnancy (Fig. S12A) nor the number of embryos when collected at E12 (Fig. S12B). We did note a trend toward diminished dam weight gain and offspring numbers in double-hit fluoxetine polyIC pregnancies (Fig. S12A,B).

Serotonin in the developing embryo is supplied by the mother via placental production until the embryo is able to synthesize its own source of serotonin beginning around E15 (Bonnin et al., 2011). MIA exposure in the non-fluoxetine group caused MFI serotonin levels to increase twofold (Fig. 6B). We were fascinated to see the opposite effect of polyIC on fluoxetine pregnancies, whereby MFI serotonin levels were nearly undetectable (Fig. 6B).

Though serotonin is perhaps most well-known for its roles in the brain where it regulates cognition, mood, sleep, and appetite, serotonin also plays a significant part in peripheral immune responses (Herr et al., 2017). Nearly all immune cells express serotonin effector machinery and serotonin can influence cytokine secretion, leukocyte activation and migration, and more (Herr et al., 2017). We therefore questioned whether SSRI exposure, which is known to elevate peripheral serotonin levels, would reshape the MFI immune response to MIA. Indeed, qPCR of key inflammatory mediators identified in our RNA-seq dataset (Fig. 1C-E) revealed that fluoxetine combined with polyIC exposure significantly potentiated the expression levels of these pro-inflammatory genes at the placenta including Ifi1, Cdc5, and Il6 (Fig. 6C). Meanwhile, some of these pro-inflammatory genes remained unchanged by fluoxetine exposure alone (Fig. 6C). Interestingly though, fluoxetine-only exposure diminished MFI expression of the immune signaling components Ili7ra, Syk, and Cc3cr1, some of which were restored to control levels in the case of dual treatment with both polyIC and fluoxetine (Fig. 6D).

These results led us to question whether cytokine levels were changed systemically in the mother or in the MFI in our treatment groups. To this end, we conducted a multiplex cytokine assay on maternal sera collected at 3 hpi. We found significantly elevated levels of IL-4 and IL-6 following polyIC treatment alone and elevated IL-6 levels with polyIC + fluoxetine, but no other significant differences were found in any of the other cytokines analyzed in this assay (Fig. S13A, Supplemental Table 2). Instead, we found that the levels of IL-1α, IL-6, IL-17α, and G-CSF in the MFI were considerably impacted by our treatments (Fig. 6E). Consistent with other studies (Chai et al., 2016), polyIC exposure led to significantly higher levels of IL-6 in MFI tissue, yet these were not impacted by fluoxetine (Fig. 6E). PolyIC treatment also led to higher MFI levels of IL-17α and G-CSF and these were dampened to baseline levels in the presence of fluoxetine (Fig. 6E). We also noted a decrease in IL-1α levels with polyIC or fluoxetine exposure alone that were returned to baseline levels in two-hit fluoxetine + polyIC MFIs (Fig. 6E). In contrast to these effects on IL-1α, IL-6, IL-17α, and G-CSF, we did not observe an appreciable impact of polyIC and/or fluoxetine treatment on MFI levels of IL-1β, IL-4, IL-10,
GM-CSF, IFN-γ, or TNF-α at 3 hpi (Figure S13B, Supplemental Table 3). These collective findings illustrate that polyIC and fluoxetine can impact the MFI cytokine milieu, and that the combination of these treatments can have multimodal effects.

We next questioned how fluoxetine treatment alone or in combination with polyIC-induced MIA modulates MFI serotonin signaling. Tph1, coding for the major serotonin synthesis enzyme tryptophan hydroxylase 1, was significantly upregulated in fluoxetine polyIC MFIs (Figure S13C), perhaps to compensate for the extremely low MFI serotonin levels in this double-hit group (Fig. 6B). Next, we turned to the serotonin transporters 5-HTT (also known as SERT; Slc6a4) and OCT3 (Slc22a3). While there is little serotonin present in the two-hit placentas (Fig. 6B), we were surprised to find diminished expression of Slc6a4 and elevated expression of Slc22a3 in fluoxetine polyIC placentas (Figure S13C). PolyIC treatment alone elevated serotonin levels (Fig. 6B) and simultaneously decreased Slc22a3 expression (Figure S13C).

Serotonin is synthesized from tryptophan and IDO1 is an essential tryptophan degrading enzyme producing kynurenine to shunt tryptophan resources away from serotonin production (Campbell et al., 2014). We found no significant changes in MFI Ido1 expression in any of our treatment groups (Figure S13C). Monoamine oxidase (MAO) enzymes can catalyze the oxidative deamination of serotonin which inhibits serotonin active signaling at 5-HT receptors. We did not find differential expression of either Maoa or Maob in MFI tissue 3 hpi in any of our treatment groups (Figure S13C), suggesting serotonin deactivation likely does not account for differential MFI serotonin levels at this timepoint (Fig. S6B). Altogether, these data suggest that fluoxetine may reshape MFI serotonin signaling when combined with maternal inflammation.

3.7. SSRI exposure potentiates the offspring brain response to MIA

Given the significance of the placenta as a source of serotonin to the developing embryo (Bonnin et al., 2011; Bonnin and Levitt, 2011), as well as the importance of serotonergic signaling during neurodevelopment (Bonnin and Levitt, 2011; Marchicki et al., 2013; Velasquez et al., 2013), we next wanted to investigate the impact of MIA and SSRI exposure on the developing embryonic brain. MIA has been previously reported to decrease the expression of protein synthesis-related
genes due to activation of the integrated stress response (Kalish et al., 2021). We noticed a trending decrease in the expression of the translation initiation factor Elf2s1 in polyI:C brains 3 hpi (Fig. 7A). Conversely, the expression of Elf2s1 as well as Chd1, a chromatin remodeler linked to the stress response, and Rack1, a core ribosomal subunit, were all strongly increased in polyI:C brains that were also exposed to fluoxetine (Fig. 7A).

SSRIs, including fluoxetine, are known to cross the maternal-fetal interface barrier and blood–brain-barrier to enter the developing embryo (Homberg et al., 2010; Rampone et al., 2004). Moreover, maternal inflammatory mediators such as IL-6 and IL-17a can also influence offspring developmental neurobiology (Choi et al., 2016; Lammert et al., 2018; Smith et al., 2007). Given this, we sought to investigate the impact of MIA and SSRI treatment on serotonin-related gene expression. Serotonin-related genes Slc6a4, Slc22a3, Ido1, and Tph1 were all non-significantly diminished in expression with either fluoxetine or polyI:C exposure alone but dramatically increased when fluoxetine and polyI:C were combined (Fig. 7B). These trends match that which we found for protein synthesis machinery and highlight the combinatorial impact of MIA and SSRI treatment on the developing brain.

The impact that we saw of MIA and SSRI treatment on MFI inflammatory signaling (Fig. S6C-E) spurred us to see if a shift in cytokine signaling was occurring simultaneously in the embryonic brain. We focused on IL-6 and IL-17a signaling given the dependence of MIA-induced behavioral alterations on these molecules (Choi et al., 2016; Lammert et al., 2018; Smith et al., 2007). We found significantly elevated expression of Il6, Il6ra, and Il17ra in embryonic brains exposed to combined fluoxetine polyI:C treatment, but not those exposed to either fluoxetine or polyI:C treatment alone (Fig. 7C). Altogether, these results illustrate that pro-inflammatory agents combined with SSRI treatment may impact offspring neurobiology by shifting the transcription of protein synthesis, serotonin, and cytokine machinery.

The severe impact of polyI:C treatment on fluoxetine pregnancies was further noted when pregnancies were allowed to carry to term. While most control, polyI:C, and fluoxetine pregnancies successfully produced live pups, we found that a large fraction of fluoxetine pregnancies were lost following the second hit polyI:C treatment (Fig. S12C). Moreover, only a few fluoxetine polyI:C pups made it to weaning age (P21; Fig. S12D) while most pups from the other treatment groups were viable. In totality, our study reported herein finds that different manipulations of the maternal environment led to distinct responses at the maternal-fetal interface and in offspring neurobiology (Fig. 7D).

4. Discussion

The placenta is a remarkable organ whose temporary existence is essential for eutherian mammal life. Maternal-fetal interface cells mediate indispensable nutrient and waste exchange between the mother and developing embryo (Maltepe and Fisher, 2015). Disturbed placental well-being can thus severely compromise embryo development. Knowledge of how the MFI supports offspring neurodevelopment during homeostasis and the impact of common environmental insults on in utero physiology and subsequent neurodevelopmental consequences are lacking. Expanding knowledge in these areas is important to achieve better understanding of both healthy neurodevelopment as well as the etiology of environmentally-triggered neurodevelopmental

Fig. 7. Fluoxetine exposure potentiates the embryonic brain response to MIA. Female mice were given fluoxetine in the drinking water (160 mg/L) for at least 2 weeks prior to addition of a male to the cage for mating. The presence of a vaginal plug was marked as E0.5. Pregnant dams were injected i.p. with 20 mg/kg polyI:C on E11 and E12 to elicit MIA or with saline as a control. Fetal brains were collected 3 hpi on E12. (A-C) qPCR of bulk brain tissue assessing expression of genes related to protein synthesis (A), the serotonin signaling (B), and cytokine signaling (C). (D) Graphical abstract summarizing the findings from the present study. Samples were collected from at least two independent pregnancies per group. n = 6–8 mice per group (A-C). Statistical significance calculated by one-way ANOVA with Tukey’s post-hoc comparison (A-C). Error bars indicate mean +/- s.e.m. *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001.
disturbances.

In the study reported herein, we investigated the impact of maternal inflammation and disruption in serotonic tone via SSRI exposure on in utero physiology and offspring neurodevelopment. Our bulk RNA-seq dataset revealed that the MFI undergoes a notable pro-inflammatory response to MIA within 3 h that has lasting impacts on the placental transcriptome out to at least 48 h. A recent study found that maternal SARS-CoV-2 infection perturbed placental expression of IFN-stimulated genes and Fc receptors which was accompanied by impaired placental antibody transfer to the fetus (Bordt et al., 2021). Even brief disruption of the delicate maternal-fetal interface milieu has the potential to alter nutrient, waste, and gas exchange with the developing embryo which may have severe consequences.

We noted a strongly upregulated type I IFN signature in polyI:C MFIs. This is consistent with previous findings that type I IFNs are a large source of antiviral immunity in the placenta (Bordt et al., 2021; Hoo et al., 2020; Yockey and Iwasaki, 2018). We noted significantly elevated expression of Ifitm1 and Ifitm3 in MIA-exposed MFIs (Supplemental Gene Lists). IFITM can block viral entry into cells, yet it also can permit the fusion of placental cytotrophoblasts into syncytiotrophoblasts, a key step in placental barrier formation (Buchrieser et al., 2019). While limiting viral spread into the developing embryo is essential, the generation of highly pro-inflammatory IFNs may negatively impact both placental physiology and offspring neurodevelopment.

Our MIA model recapitulated at the maternal-fetal interface the well-known IL-6 and IL-17a signatures that are necessary for the onset of MIA behavioral changes (Choi et al., 2016; Lammert et al., 2018; Smith et al., 2007). While it is unclear whether cytokines can cross either the placental barrier and/or the blood–brain barrier (BBB), cytokine signaling at the placenta itself can contribute to ill-affecting neurodevelopmental consequences (Hsiao and Patterson, 2012, 2011). For instance, impairing IL-6Ra function (thereby obstructing IL-6 signaling) in placental trophoblasts was shown to protect against MIA-induced offspring cerebellar pathologies and behavioral changes (Wu et al., 2017). In all, the MFI inflammatory response characterized with this dataset may help to illuminate some of the developmental effects of MIA.

One may be surprised to learn that the homeostatic MFI is jam packed with maternal immune cells; leukocytes constitute nearly 40 % of all cells (Anders et al., 2019; Faas and De Vos, 2018). The majority of these immune cells are dNK cells with the remainder largely being decidual macrophages and T cells (Anders et al., 2019). This large immune population serves essential tolerogenic roles as well as homeostatic uterine and vascular remodeling functions (Anders et al., 2019; Faas and De Vos, 2018; Hoo et al., 2020). One could postulate that alterations in the immune landscape that occur in response to MIA, SSRIs, or other insults may ultimately disrupt the delicate tolerogenic state and potentially cause immune-driven harm to the embryo. We speculate that in MIA pregnancies, immune cells generally dedicated to homeostatic functions in the maternal-fetal interface are instead concerned with engaging an inflammatory response. Perhaps a loss of homeostatic immune cell function contributes to altered MIA offspring outcomes.

Our current study is limited in that it lacks cell specificity. Future studies will be necessary to shed light on the specific cell types in which the signaling pathways identified herein are altered. For now, we may only speculate on potential responders based on known cell-type expression. dNK cells produce a variety of growth factors, angiogenic factors, and cytokines in response to stimuli including VEGF, IL-8, IFN-γ, and CXCL10 at the maternal-fetal interface (Anders et al., 2019). We found dramatically elevated Cxcl10 expression in polyI:C-exposed MFIs. Interferons can induce Cxcl10 expression and CXCL10 can contribute to immune cell chemotaxis, T cell activation, and angiogenesis (Vaziri-injejad et al., 2014). Correspondingly, we found an increase in the dNK cell marker Cd56 specifically in the decidua following polyI:C treatment. Whether dNK cells are the source of CXCL10 and how CXCL10 contributes to the MFI immune response and neurodevelopment are important future areas of investigation.

Circulating cells present in the vasculature are another potential reservoir responsible for driving some of the DEGs in our maternal-fetal interface RNA-seq study. It is possible that the amount of peripheral blood (originating from either the mother or the embryo) in our MFI samples is changed upon initiation of the maternal immune response. Indeed, studies are beginning to reveal that maternal infection can lead to abnormal placental villous architecture and vascular remodeling (Weckman et al., 2019). Many immune molecules like cytokines and chemokines, including some of those which we found to have elevated expression at the MIA maternal-fetal interface, are known to impact placental vascular development and function. Mechanistically, these inflammatory mediators can cross-talk with angiogenic factors, such as sFlt-1 and VEGF (Weckman et al., 2019), which may cause improper placental vascular development and contribute to adverse offspring outcomes. dNK cells are a critical population involved in vascular remodeling (Faas and De Vos, 2018). It is possible that polyI:C exposure affects this homeostatic dNK function. Moreover, we found altered expression of many factors known to impact angiogenesis acutely after polyI:C exposure. Though beyond the scope of the current study, it would be interesting to more mechanistically evaluate how these blood transport processes are impacted by polyI:C, as this could explain some of the alterations to fetal development reported in this MIA model.

The transcriptional landscape is altogether transformed in the MIA environment, having the potential to alter typical transfer of oxygen, nutrients, antibodies, and more to the embryo. It is conceivable that male and female embryos are differentially sensitive to theoretic disruptions in typical placental support. One of the findings we were most surprised by in this study was that baseline MFI transcriptional sex differences are nearly eliminated by MIA. Even more striking to us was that this dampening of sex-specific signaling persisted beyond the cessation of the pro-inflammatory response to polyI:C. A few studies have begun to define sex differences in placental structure and function (Gabory et al., 2013; Meakin et al., 2021; Rosendal, 2015) but there is yet much insight to be gleaned in this realm. We found that female control MFIs were enriched for immune- and neurodevelopment-related modules in addition to pathways related to the cell cycle, semaphorin interactions, and platelet activation. Male embryos are thought to be more metabolically demanding, requiring greater levels of immune tolerance and of nutrient transport to support larger growth requirements (Braun et al., 2021; Meakin et al., 2021). Our dataset found that the male MFI was enriched for pathways related to protein export, the spliceosome, the fibrin clotting cascade, and translation. It is imaginable that a loss of sex-specific MFI support on its own disturbs embryo development beyond the known pro-inflammatory environment effects. Moreover, the sex of the embryo may render different responses to such conditions of stress, potentially precipitating some of the neurodevelopmental consequences commonly reported in MIA offspring.

We were initially surprised to find no major consequence of MIA on the transcriptome of offspring microglia. Other studies using different MIA models have indeed reported that MIA can impact microglia number, motility, and phagocytic capacity (Ben-Yehuda et al., 2020; Hui et al., 2020; Ozaki et al., 2020; Smolders et al., 2015). It is possible that our relatively mild polyI:C-driven model is below a certain threshold for impacting microglia on the transcriptional level; an argument supported by the lack of transcriptional differences seen in the Kalish et al. 2021 dataset collected from a similar model. Microglia exposed to our MIA paradigm may have no detectable transcriptional differences yet still harbor differences in protein levels, post-translational modification, or other aspects that contribute to alterations in form and function. Alternatively, microglia exposed to this type of MIA could exist in a “primed” state in which differences in function or response is revealed in the presence of a stimulus or second hit. Indeed, such was found to be the case in one study in which scRNA-seq analysis of control versus MIA-exposed microglia yielded only 7 DEGs at baseline, yet 401 DEGs following adult immune challenge (Hayes et al., 2022). While MIA microglia may
be similar to control microglia in terms of transcriptional state, density, morphology, and activation at baseline (Garay et al., 2013; Giovannoni et al., 2016, 2015; Hayes et al., 2022; Smolders et al., 2018, 2015), new work suggests that epigenetic priming of MIA microglia leads to dysfunctional responses when confronted with stimuli later in life (Hayes et al., 2022). The variability in the type and strength of the immune response generated with different MIA models, dam microbiome, developmental stage targeted, and other experimental considerations could all additionally account for different outcomes on microglia.

Stress, depression, and anxiety may also contribute to inflammation during pregnancy and have the potential to alter the maternal environment in other facets. Major depressive disorder has a devastating prevalence of 13% amongst pregnant women (Velasquez et al., 2013). SSRIs are the most commonly prescribed treatment for depression (Millard et al., 2017). 25% of women taking SSRIs continue use during pregnancy and another 0.5% of women begin taking an SSRI at some point during pregnancy (Homberg et al., 2010). Maternal SSRI intake can potentially lead to systemic disruption of serotonergic signaling. Circulating and tissue resident immune cells, placental syncytiotrophoblasts and cytotrophoblasts, and developing neural cells all utilize serotonergic transporters and signaling (Heerr et al., 2017; Homberg et al., 2010; Kliman et al., 2018). SSRI action on any or all of these populations could therefore potentially impact neurodevelopment. Our findings reveal that SSRI exposure alone can modulate the expression of genes involved in immune and serotonin signaling at the maternal-fetal interface.

Immune cell function can be directly impacted by SSRI exposure in the mother and embryo, both of which could potentially affect neurodevelopment given that maternal and embryonic immune cells contribute to both placental support and neurodevelopment (Ander et al., 2019; Faas and De Vos, 2018; Filiano et al., 2015; Tanabe and Yamashita, 2018; Zengeler and Lukens, 2021). Serotonin can modulate immune cell activation including cytokine release, migration, adhesion, antigen presentation, phagocytosis, and more, in both pro- and anti-inflammatory ways (Herr et al., 2017). The serotonin transporter SERT, whose function is blocked by SSRIs, can be found on monocytes, macrophages, T and B cells, and mast cells, while serotonin receptors can be found on nearly all types of immune cells (Herr et al., 2017). Thus, SSRIs may broadly affect the immune system and have been investigated as immunomodulatory drugs (Gobin et al., 2014; Szalach et al., 2019). SSRIs have been shown to act in immunosuppressive or immunostimulatory manners depending on the underlying immunologic state (Gobin et al., 2014; Szalach et al., 2019).

Given that SSRIs can directly modulate immune cell function, it logically follows that our study finds a differential impact of poly(I:C) alone compared to combined fluoxetine and poly(I:C) treatment. We found that prenatal fluoxetine exposure influenced the MFI immune response to poly(I:C) by exacerbating the levels of some immunomodulatory factors while dampening others. These complex effects of combined fluoxetine and poly(I:C) exposures could partially be explained by the fact that SSRIs can have both pro- and anti-inflammatory effects (Gobin et al., 2014; Szalach et al., 2019); thereby, SSRI exposure may heighten some inflammatory response pathways while dampening others. For instance, we found heightened expression of Ifi119, Cx36, and Tbx3 in fluoxetine poly(I:C) MFIIs when compared with poly(I:C) treatment alone. On the other hand, levels of IL-1α, IL-17a, and G-CSF were returned to control levels in fluoxetine poly(I:C) MFIIs compared to poly(I:C) alone. Meanwhile other pathways may be unaffected by fluoxetine exposure. For instance, we found no change in the MFI expression levels or protein levels of IL-6 comparing double-hit fluoxetine poly(I:C) to single-hit poly(I:C) alone. Similar effects can be seen when comparing fluoxetine-only exposure to combined fluoxetine poly(I:C) treatment. For example, fluoxetine alone dramatically dampened MFI expression of the cytokine receptor II17ra, the critical pro-inflammatory mediator Syk, and the chemokine receptor Cx3cr1. In the presence of fluoxetine, poly(I:C) treatment restored the expression of II17ra and Syk to control levels.

We found that poly(I:C) elicited heightened MFI levels of serotonin relative to controls, while the combination of poly(I:C) and fluoxetine exposure instead lowered MFI serotonin to nearly undetectable levels. A recent study also found heightened placental serotonin levels in a prenatal stress model that was accompanied by maternal inflammation (Chen et al., 2020). The embryo is not capable of synthesizing its own source of serotonin until after E15 (Bonnin et al., 2011; Bonnin and Levitt, 2011) yet serotonin is used for a variety of developmental processes prior to E15 (Homberg et al., 2010). Serotonin transfer from the mother via the placenta therefore acts as the sole source of serotonin until sufficient embryo-sourced serotonin synthesis is achieved (Bonnin et al., 2011; Bonnin and Levitt, 2011). Thus, disrupted serotonin tone in the maternal-fetal interface prior to E15, as we have detected at E12 following out environmental manipulations, have the potential to severely limit embryo serotonin levels. How disrupted placental serotonin signaling impacts offspring neurodevelopment is certainly underexplored and warrants future investigation.

While poly(I:C) has not been shown to be able to cross the placenta or BBB, many SSRIs are able to cross both of these barriers and may therefore directly influence serotonin signaling in the developing embryo (Homberg et al., 2010; Rampono et al., 2004). Serotonin is involved in a wide array of neurodevelopmental events including cell proliferation, migration, death, neurite guidance, dendrite maturation, and synaptogenesis (Homberg et al., 2010). Conceivably, any of these events may therefore be disrupted if embryonic brain serotonin levels are elevated by SSRIs. While serotonin transporters are only expressed by serotonergic neurons in the adult brain, a wide variety of neurons in developing brain express serotonin transporters and could potentially be affected by SSRIs (Homberg et al., 2010).

Longitudinal human studies tracking the outcomes of children from SSRI pregnancies are currently limited, but murine studies investigating the impact of prenatal SSRI exposure on offspring neural circuits and behavior have begun to uncover some notable consequences (Bonnin and Levitt, 2011; Homberg et al., 2010; Velasquez et al., 2013). While the impact of SSRIs on neural circuit formation and glial development are minimal, studies from Slc6a4+/− mice have revealed that disruption of serotonin transporter function during neurodevelopment disrupts neuroanatomy in the somatosensory cortex and corticollimbic system (Homberg et al., 2010; Lee, 2009). Altered function in these regions maps to the behavioral impacts of prenatal SSRI exposure in which treated offspring display delayed motor development, anxiety- and depressive-like behaviors, reduced impulsivity, improved spatial learning, and increased susceptibility to addictive-like behaviors (Bairy et al., 2007; Forcelli and Heinrichs, 2008; Lee, 2009; Lisboa et al., 2007).

Our dual-environmental-hit studies revealed a complex brain transcriptional response following combined fluoxetine and poly(I:C) treatment compared to either fluoxetine or poly(I:C) exposure alone. In one case, MFI serotonin levels are significantly elevated following poly(I:C) treatment yet are almost undetectable following poly(I:C) treatment in fluoxetine pregnancies when compared to baseline control levels. Another stark example of this combinational effect is observed when looking at the embryonic brain transcriptome: while protein synthesis, serotonin-related, and cytokine-related transcription showed a trending reduction with either fluoxetine or poly(I:C) exposure alone, the combined exposure of fluoxetine and poly(I:C) significantly elevated expression above control levels across these modules. These findings highlight the critical importance of taking the entire maternal environment into account when assessing the impact of triggers on placental physiology and neurodevelopment.

Among eutherian mammals, the placenta has so far been found in at least 20 different variations, making it the most mutable organ (Maltepe and Fisher, 2015). Differences in cell type composition, maternal-fetal interface structure, and cellular function are known to exist between mice and humans (Ander et al., 2019; Hoo et al., 2020; Maltepe and Fisher, 2015). It is therefore imperative that reported findings from murine studies such as ours be investigated secondarily in a human
setting, as any environmental impacts on the human placenta and downstream neurodevelopmental consequences could differ.

Our study suggests that SSRI intake during pregnancy could potentially have lasting consequences on offspring neurobiology. Yet, SSRI use provides necessary reprieve from detrimental mental health states. Untreated maternal stress, depression, and anxiety can all be on their own a perturb offspring neurodevelopment, contributing to adverse behavioral and cognitive outcomes (Bleker et al., 2019). It will therefore be of utmost importance to consider both the relative benefits and potential consequences of SSRIs as a therapeutic option during pregnancy.

5. Conclusions

Our findings illustrate that commonly encountered environmental insults have the potential to cause notable alterations to maternal-fetal interface physiology which may then impact offspring neurodevelopment. In particular, we found that prenatal inflammation and SSRI exposure reshape the signaling milieu of the maternal-fetal interface and offspring brain (Fig. 5D). The placenta remains a highly understudied organ despite its absolute necessity for human life. Uncovering details concerning placental physiology during homeostasis and in response to environmental stressors will undoubtedly illuminate novel developmental biology, and in particular, that which concerns deviation from baseline.

Data availability

All data are available from the authors upon request. Raw gene counts from bulk RNA-seq of maternal-fetal interface tissue and isolated microglia are publicly available on Mendelely [https://doi.org/10.176](https://doi.org/10.176) and GitHub [https://github.com/LukensLab/MIA-RNAseq.git]. All code used to process RNA-seq data is also publicly available on GitHub [https://github.com/LukensLab/MIA-RNAseq.git].

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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K.E.Z. and J.R.L. designed the study; K.E.Z., C.R.L., and H.E. performed experiments; K.E.Z., D.A.S., and K.R.B performed bioinformatics analyses; K.E.Z. analyzed data; K.E.Z. and J.R.L. wrote the manuscript; J.R.L. oversaw the project.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bjibio.2022.10.024.

References

Almond, D., Currie, J., 2011. Killing me softly: the fetal origins hypothesis. J. Econ. Perspect. 25, 153–172. https://doi.org/10.1257/jep.25.3.153.

Ander, S.E., Diamond, M.S., Coyne, C.B., 2019. Immune responses at the maternal-fetal interface. Sci. Immunol. 4, eaat114. https://doi.org/10.1126/sciimmunol.aat114.

Anson, M.S., Zhou, M., Lira, A., Hen, R., Gingrich, J.A., 2004. Early-life blockade of the 5-HT transporter alters emotional behavior in adult mice. Science 306, 879–881. https://doi.org/10.1126.science.1101678.

Anson, M.S., Morelli, E., Gingrich, J.A., 2008. Inhibition of serotonin but not norepinephrine transport during development produces delayed, persistent perturbations of emotional behaviors in mice. J. Neurosci. 28, 199–207. https://doi.org/10.1523/JNEUROSCI.9793-07.2008.

Argueta, L.B., Lacko, L.A., Bram, Y., Taiba, T., Carraro, L., Zhang, T., Uhl, S., Lubor, B.C., Chandar, V., Gil, C., Zhang, W., Dodson, B., Bastianas, J., Prabhuj, M., Salvatore, C. M., Yang, Y.J., Baergen, R.N., tenOver, B.R., Landau, N.R., Chen, S., Schwartz, R.E., Stuhldreher, H., 2021. SARS-CoV-2 infects syncytiotrophoblast and activates inflammatory responses in the placenta. bioRxiv 2021.06.01.446676. https://doi.org/10.1101/2021.06.01.446676.

Bailry, K.L., Madhyastha, S., Ashok, K.P., Bailry, I., Malini, S., 2007. Developmental and behavioral consequences of prenatal fluoxetine. Pharmacology 79, 1–11. https://doi.org/10.1515/00096645.

Ben-Yehuda, H., Macovitch-Natan, O., Kertser, A., Spinrad, A., Prinz, M., Amit, I., Schwartz, M., 2020. Maternal Type-1 interferon signaling adversely affects the microglia and the behavior of the offspring accompanied by increased sensitivity to stress. Mol. Psychiatry 25, 1050–1067. https://doi.org/10.1038/s41380-019-0604-0.

Bilbo, S.D., Block, C.L., Bolton, J.L., Hanamsagar, R., Tran, P.K., 2018. Beyond infection - Maternal immune activation by environmental factors, microglial development, and relevance for autism spectrum disorders. Exp. Neurol. 299, 241–251. https://doi.org/10.1016/j.expneurol.2017.07.002.

Bleker, L.S., De Rooij, S.R., Roseboom, T.J., 2019. Programming effects of prenatal stress on neurodevelopment—The pitfall of introducing a self-fulfilling prophecy. Int. J. Environ. Res. Public Health 16, 2501. https://doi.org/10.3390/ijerph16092301.

Bonnin, A., Levisit, P., 2011. Fetal, maternal, and placental sources of serotonin and new implications for developmental programming of the brain. Neuroscience 197, 1–7. https://doi.org/10.1016/j.neuroscience.2011.10.005.

Bonnin, A., Goedner, N., Chen, K., Wilson, M.L., King, J., Shi, J.C., Blakely, R.D., Deneris, E.S., Levisit, P., 2011. A transient placental source of serotonin for the fetal forebrain. Nature 472, 347–350. https://doi.org/10.1038/nature09972.

Bordeleau, M., Fernández de Cosío, L., Chakravarty, M.M., Tremblay, M.-É., 2021. From maternal diet to neurodevelopmental disorders: A story of neuroinflammation. Frontiers in Cellular Neuroscience 14.

Bordi, E.A., Shook, L.L., Atayev, C., Pullen, K.M., De Guzman, R.M., Meinsohn, M.-C., Chaunin, M., Fischinger, S., Vockey, L.J., James, K., Lima, R., Yonger, L.M., Fasano, A., Brigida, S., Behell, I.M., Roberts, D.J., Pépin, D., Huh, J.R., Bilbo, S.D., Li, J.Z., Kaimal, A., Schust, D.J., Gray, K.J., Lauffenburger, D., Alter, G., Edlow, A.G., 2021. Maternal SARS-CoV-2 infection elicits sexually dimorphic placental immune responses. Sci. Transl. Med. 13, eabi7428. https://doi.org/10.1126/scitranslmed.abi7428.

Braun, A.E., Muench, K.L., Robinson, B.G., Wang, A., Palmer, T.D., Winn, V.D., 2021. Examining sex differences in the human placental transcriptome during the first androgen peak. Reprod. Sci. 28, 801–818. https://doi.org/10.1177/19337191201032020-00355.5.

Brown, A.S., Derkits, E.J., 2010. Prenatal infection and schizophrenia: a review of epidemiological and translational studies. Am. J. Psychiatry 167, 261–280. https://doi.org/10.1176/appi.ajp.2009.09030381.

Buchrieser, J., Degrelle, S.A., Couderc, T., Nevers, Q., Dixon, O., Manet, C., Donahue, D. A., Porrott, F., Hillion, K.-H., Perithame, E., Arroyo, M.V., Souqueire, S., Ruigrok, K., Dupressoir, A., Heidmann, T., Montagutelli, X., Fournier, T., Lecuit, M., Schwartz, O., 2019. IFITM proteins inhibit placental syncytiotrophoblast formation and promote fetal demise. Science 365, 176–180. https://doi.org/10.1126/science.365.6471.176.

Campbell, B.M., Charych, E., Lee, A.W., Möller, T., 2014. Kynurenines in CNS disease: regulation by inflammatory cytokines. Front. Neurosci. 8, 27. https://doi.org/10.3389/fnins.2014.00012.

Carlestone, M.A., Khaw, M., Misig, G., Fidge, B.C., Landino, S.M., Alexander, A.J., Möller, E.L., Robbins, J.O., Li, Y., Bollhasko, V.Y., McDoogle, C.J., Kim, K.-S., 2019. Maternal and early postnatal immune activation produce sex-specific effects on autism-like behaviors and neuroimmune function in mice. Sci. Rep. 9, 1067. https://doi.org/10.1038/s41598-019-53294-9.

Ceasrine, A.M., Devlin, B.A., Bolton, J.L., Ho, Y.C., Huynh, C., Patrick, B., Washington, K., Joo, F., Campos-Salazar, A.B., Lockshin, E.R., Murphy, S.R., Simmons, L.A., Bilbo, S. D., 2021. Maternal diet disrupts the placenta-brain axis in a sex-specific manner. J. Neurosci. 2021.11.02.464808.

Chen, H.J., Antonson, A.M., Rajasekera, T.A., Patterson, J.M., Bailey, M.T., Gur, T.L., 2020. Prenatal stress causes intracellular inflammation and serotoninergic dysfunctions, and long-term behavioral deficits through microbe- and CCL2-dependent mechanisms. Translational Psychiatry 10 (1).
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Brain Behavior and Immunity 108 (2023) 80–97

Choi, G.B., Yim, Y.S., Wong, H., Kim, S., Kim, H., Kim, S.V., Huh, J.R., 2016. The maternal interleukin-17a pathway in mice promotes autism-like phenotypes in offspring. Science 351, 953–959. https://doi.org/10.1126/science.aad3114.

Cortés-Albornoz, M.C., García-Guápeta, D.P., Velez-van-Meerbeke, A., Talero-Gutiérrez, C., 2021. Maternal nutrition and neurodevelopment: A scoping review. Movements 13, 153-163. 2020. Lasting and second-generation impact of maternal immune activation on molecular pathways of the amygdala. Front. Neurosci. 14.

Kim, S., Kim, H., Yim, Y.S., Ha, S., Atarashi, K., Tan, T.G., Longman, R.S., Honda, K., Littman, D.R., Choi, G.B., Huh, J.R., 2017. Maternal gut bacteria promote neurodevelopmental abnormalities in mice offspring. Nature 549, 528–532. https://doi.org/10.1038/nature23910.

Kinsella, M.T., Monk, C., 2009. Impact of maternal stress, depression & anxiety on fetal neurodevelopmental behavior. Clin. Obset. Gynecol. 52, 425–440. https://doi.org/10.1097/GRF.0b013e3181b5021a.

Kliman, H.J., Quartarella, S.B., Setaro, A.C., Siegmam, E.C., Subba, Z.T., Tal, R., Milano, M.K., Steele, T.L., 2018. Pathway of maternal serotonin to the human embryo and fetus. Endocrinology 159, 1659–1669. https://doi.org/10.1210/endo-2017-03252.

Kunesel, I., Chieca, I., Britschgi, M., Schobel, S.A., Bodem, M., Hellings, J.A., Toovey, S., Prinsen, E.P., 2014. Maternal immune activation and abnormal brain development across CNS disorders. Nat. Rev. Neurol. 10, 643–660. https://doi.org/10.1038/nrneurol.2014.147.

Lammert, C.R., Favor, E.L., Bolte, A.C., Payour, M.J., Shaw, M.E., Bellinger, C.C., Weigel, T.K., Zeder, E.R., Lukens, J.R., 2018. Cutting Edge: critical roles for microbiota-mediated regulation of the immune system in a prenatal immune activation model of autism. J. Immunol. 199, (2), 513–518. 2016. Modeling autism-related disorders in mice with maternal immune activation (MIA). Methods Mol. Biol. 1960, 227–236. https://doi.org/10.1007/978-1-5344-0802-1_17.

Lee, I., 2009. Neonatal Brain Infection exposure affects the neural structure in the somatosensory cortex and somatosensory-related behaviors in adolescent rats. Neurotox. Res. 15, 212–223. https://doi.org/10.1007/s11065-009-9224-x.

Llabia, S.F.S., Oliveira, P.E., Costa, L.C., Vencesáio, E.J., Moreira, E.E., 2007. Behavioral evaluation of male and female pup survival exposed to maternal microRNA during pregnancy and lactation. Pharmacol. 80, 49–56. https://doi.org/10.1109/10015097.

Lukens, J.R., Eyo, U.B., 2022. Microglia and neurodevelopmental disorders. Annu. Rev. Neurosci. 45 (1), 425–445. https://doi.org/10.1146/annurev-neuro-070121-023246.

Malm, H., Brown, A.S., Gisler, M., Gyllenborg, D., Hininki-Y-Salomaki, S., Måckeg, J.W., Weissman, M., Wickramatne, P., Artama, M., Gimpich, J., Sauer, A., 2016. Gestational exposure to selective serotonin reuptake inhibitors and offspring psychiatric disorders: A National Register-Based study. J. Am. Acad. Child Adolesc. Psychiatry 55, 359–366. https://doi.org/10.1097/CHI.0000000000000212.

Maltepe, E., Fisher, S.J., 2015. Placenta: the forgotten organ. Annu. Rev. Cell Dev. Biol. 31, 523–555. https://doi.org/10.1146/annurev-cellbio-011714-120732.

Marchozchi, X., Russell, N.E., Domoghov, K.O., 2013. Selective serotonin reuptake inhibitors and pregnancy: A review of maternal, fetal and neonatal risks and benefits. Obstet Med. 6, 155–158. https://doi.org/10.1177/1753489913491594.

Matcovitch-Natan, O., Winter, D.R., Giladi, A., Aguilar, S.V., Spinard, A., Sarrazin, S., Ben-Yehuda, H., David, E., Gonzalez, F.Z., Perrin, P., Keren-Shaul, H., Gury, M., Lara-Astasio, D., Thais, C.A., Cohen, M., Halberp, K.B., Baruch, J., Decozaekk, A., Lorenzo-Vivas, E., Ikizovski, S., Elievski, E., Sieweke, M.H., Schwartz, M., Amit, I., 2016. Microglia development follows a stepwise program to regulate brain development. Science 355, 552–558. https://doi.org/10.1126/science.aaf7255.

Meakin, A.S., Cuffe, J.M., Darby, J.R.T., Morrison, J.L., Clifton, V.L., 2021. Let-7e in the placenta and preeclampsia. Placenta 69, 125–132. https://doi.org/10.1016/j.placenta.2021.04.001.

Nguyen, D.C., Ho, H., Bao, N., 2020. Maternal immune activation in the neonate. Int. J. Neuropsychopharmacol. 7, 329–336. https://doi.org/10.1038/s41398-019-01843-y.

Ozaki, K., Date, K., Ichikawa, A., Hashimoto, A., Sugio, S., Guo, Z., Shibushita, M., Tatematsu, T., Haruwaka, K., Moorhouse, A.J., Yamada, H., Wase, K., 2022. Maternal immune activation in the neonate. Int. J. Neuropsychopharmacol. 25, 604–610. https://doi.org/10.1016/j.bbi.2010.12.017.

Patterson, P.H., 2009. Immune involvement in schizophrenia and autism: Etiology, pathology and animal models. Behav Brain Res. Special Issue on Modeling Autism 3 (2), 1326–1330. 2018. Integrating maternal nutrition and neurodevelopment: A review of maternal nutritional influences on brain development. Science 353. https://doi.org/10.1126/science.aad8670.

Patterson, P.H., 2009. Cytokines and CNS development. Neuron 64, 1–8. https://doi.org/10.1016/j.neuron.2009.09.002.

Patterson, P.H., 2009. Immune involvement in schizophrenia and autism: Etiology, pathology and animal models. Behav Brain Res. Special Issue on Modeling Autism 3 (2), 1326–1330. 2018. Integrating maternal nutrition and neurodevelopment: A review of maternal nutritional influences on brain development. Science 353. https://doi.org/10.1126/science.aad8670.

Patterson, P.H., 2009. Immune involvement in schizophrenia and autism: Etiology, pathology and animal models. Behav Brain Res. Special Issue on Modeling Autism 3 (2), 1326–1330. 2018. Integrating maternal nutrition and neurodevelopment: A review of maternal nutritional influences on brain development. Science 353. https://doi.org/10.1126/science.aad8670.

Patterson, P.H., 2009. Immune involvement in schizophrenia and autism: Etiology, pathology and animal models. Behav Brain Res. Special Issue on Modeling Autism 3 (2), 1326–1330. 2018. Integrating maternal nutrition and neurodevelopment: A review of maternal nutritional influences on brain development. Science 353. https://doi.org/10.1126/science.aad8670.

Patterson, P.H., 2009. Immune involvement in schizophrenia and autism: Etiology, pathology and animal models. Behav Brain Res. Special Issue on Modeling Autism 3 (2), 1326–1330. 2018. Integrating maternal nutrition and neurodevelopment: A review of maternal nutritional influences on brain development. Science 353. https://doi.org/10.1126/science.aad8670.

Patterson, P.H., 2009. Immune involvement in schizophrenia and autism: Etiology, pathology and animal models. Behav Brain Res. Special Issue on Modeling Autism 3 (2), 1326–1330. 2018. Integrating maternal nutrition and neurodevelopment: A review of maternal nutritional influences on brain development. Science 353. https://doi.org/10.1126/science.aad8670.

Patterson, P.H., 2009. Immune involvement in schizophrenia and autism: Etiology, pathology and animal models. Behav Brain Res. Special Issue on Modeling Autism 3 (2), 1326–1330. 2018. Integrating maternal nutrition and neurodevelopment: A review of maternal nutritional influences on brain development. Science 353. https://doi.org/10.1126/science.aad8670.
Rosenfeld, C.S., 2015. Sex-specific placental responses in fetal development. Endocrinology 156, 3422–3434. https://doi.org/10.1210/en.2015-1227.
Schafer, D.P., Lehman, E.K., Kautzman, A.G., Koyama, R., Martinly, A.R., Yamasaki, R., Ransohoff, R.M., Greenberg, M.E., Barres, B.A., Stevens, B., 2012. Microglia sculpt postnatal neural circuits in an activity and complement-dependent manner. Neuron 74, 691–705. https://doi.org/10.1016/j.neuron.2012.03.025.
Smith, C.J., Kingsbury, M.A., Dziabalski, J.E., Hanamsagar, R., Malcon, K.E., Tran, J.N., Norris, H.A., Gulino, M., Bordi, E.A., Bilbo, S.D., 2020. Neonatal immune challenge induces female-specific changes in social behavior and somatostatin cell number. Brain Behav. Immun. 90, 332–345. https://doi.org/10.1016/j.bbi.2020.08.015.
Smith, S.E.P., Li, J., Garbett, K., Mirnics, K., Patterson, P.H., 2007. Maternal immune activation alters fetal brain development through Interleukin-6. J. Neurosci. 27, 10695–10702. https://doi.org/10.1523/JNEUROSCI.2178-07.2007.
Smolders, S., Notter, T., Smolders, S.M.T., Rigo, J.-M., Brüne, B., 2018. Controversies and prospects about microglia in maternal immune activation models for neurodevelopmental disorders. Brain Behav. Immun. 73, 51–65. https://doi.org/10.1016/j.bbi.2018.06.001.
Sun, J.-Y., Wu, R., Xu, J., Xue, H.-Y., Lu, X.-J., Ji, J., 2021. Placental immune tolerance and organ transplantation: underlying interconnections and clinical implications. Front. Immunol. 12.
Susser, E.S., Lin, S.P., 1992. Schizophrenia after prenatal exposure to the Dutch Hunger Winter of 1944–1945. Arch. Gen. Psychiatry 49, 983–988. https://doi.org/10.1001/archpsyc.1992.01820120071010.
Szlach, L.P., Lisowska, K.A., Cubala, W.J., 2019. The influence of antidepressants on the immune system. Arch. Immunol. Ther. Exp. (Warsz) 67, 143–151. https://doi.org/10.1007/s00005-019-00543-8.
Tanabe, S., Yamashita, T., 2018. The role of immune cells in brain development and neurodevelopmental diseases. Int. Immunol. 30, 437–444. https://doi.org/10.1093/intimm/dxy041.
Vazirinejad, R., Ahmad, Z., Arababadi, M.K., Hassanshahi, G., Kennedy, D., 2014. The biological functions, structure and sources of CXCL10 and its outstanding part in the pathophysiology of multiple sclerosis. NIM 21, 322–330. https://doi.org/10.1159/000357780.
Velasquez, J., Goeden, N., Bonnin, A., 2013. Placental serotonin: implications for the developmental effects of SSRIs and maternal depression. Front. Cell. Neurosci. 7.
Wu, W.-L., Hsiao, E.Y., Yan, Z., Mazmanian, S.K., Patterson, P.H., 2017. The placental interleukin-6 signaling controls fetal brain development and behavior. Brain Behav. Immun. 62, 11–23. https://doi.org/10.1016/j.bbi.2016.11.007.
Yan, R., Zhang, Y., Li, Y., Xia, L., Guo, Y., Zhou, Q., 2020. Structural basis for the recognition of SARS-CoV-2 by full-length human ACE2. Science 367, 1444–1448. https://doi.org/10.1126/science.abc2762.
Yockey, L.J., Iwasaki, A., 2018. Interferons and proinflammatory cytokines in pregnancy and fetal development. Immunity 49, 397–412. https://doi.org/10.1016/j.immuni.2018.07.017.
Zengeler, K.E., Luken, J.R., 2021. Innate immunity at the crossroads of healthy brain maturation and neurodevelopmental disorders. Nat. Rev. Immunol. 21 (7), 454–468.