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## ► To cite this version:

Jodi L. Pawluski, Pauline Murail, Florine Grudet, Lena Bys, Anna V Golubeva, et al.. Gestational stress and perinatal SSRIs differentially impact the maternal and neonatal microbiome-gut-brain axis. *Journal of Neuroendocrinology*, 2023, pp.e13261. 10.1111/jne.13261 . hal-04099966

**HAL Id: hal-04099966**

**<https://hal.science/hal-04099966>**

Submitted on 31 May 2023



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# Gestational stress and perinatal SSRIs differentially impact the maternal and neonatal microbiome-gut-brain axis

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## Funding information

Institute des Neurosciences Cliniques de Rennes; Science Foundation Ireland (SFI), Grant/Award Number: SFI/12/RC/2273

## Abstract

Selective serotonin reuptake inhibitors (SSRIs) are the most popular antidepressant medications used to manage perinatal mood disturbances, yet our understanding of how they affect the microbiome-gut-brain axis of the mother and offspring is limited. The purpose of this study was to determine how peripartum SSRI treatment may prevent the effects of gestational stress on plasticity in the maternal hippocampus, plasticity in the neonatal brain and related changes in gut microbiota. To do this Sprague-Dawley female rats were left untreated or subjected to unpredictable stress during pregnancy. Half of the females were supplemented daily with fluoxetine. On postpartum day 2 brains were collected for measurement of plasticity (neurogenesis and microglia content) in the maternal hippocampus and in the neonatal brain. Glucocorticoid receptor density was also investigated in the maternal hippocampus. Microbiota composition was analyzed in fecal samples of dams during and after pregnancy, and colon tissue samples from offspring on postnatal day 2. Main findings show there are significant changes to the maternal microbiome-gut-brain axis that may be fundamental to mediating plasticity in the maternal hippocampus. In addition, there is significant impact of gestational stress on neonatal gut microbiota and brain microglia density, while the effects of SSRIs are limited. This is the first study to explore the impact of gestational stress and SSRIs on the microbiome-gut-brain axis in the mother and neonate. Findings from this study will help inform pathways to intervention strategies including stress reduction techniques and/or microbiota targeted nutritional approaches directed towards improving maternal gut health and outcomes for mother and neonate.

## KEYWORDS

development, hippocampus, microbiome, motherhood, neuroplasticity

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## 1 | INTRODUCTION

Selective serotonin reuptake inhibitors (SSRIs) are prescribed to 3%–10% of pregnant women globally<sup>1–3</sup> to treat perinatal mood and anxiety disorders.<sup>4–9</sup> SSRIs and their metabolites can cross the placenta<sup>10–12</sup> and are detected in breast milk<sup>13</sup> raising questions about the effect of using these serotonergic medications on fetal development.<sup>14–18</sup> Furthermore, the impacts of untreated perinatal mood and anxiety disorders are pervasive and can affect the parent and developing child, even with administration of treatments.<sup>7,19–23</sup> While our understanding of how the maternal brain is affected by perinatal mental illness and SSRI treatment is limited,<sup>7,8,24</sup> it is important that a form of intervention, either pharmacotherapy or psychotherapy, is available for the 10% of mothers and parents struggling with these debilitating conditions.<sup>24</sup>

Human studies of the impact of SSRI exposure during developmentally sensitive periods are constrained by “confounding by indication” and distinguishing the impact of SSRIs from the maternal mood disturbances that led to antidepressant treatment remains methodologically challenging. Even with stringent analytic approaches to “confounding by indication”,<sup>23,25</sup> many questions remain about the use of SSRIs for both the mother and developing child. For example, unmeasured variables related to the nature of the maternal illness (i.e., stress, anxiety, depression), variations in symptom severity during pregnancy and differing indications for SSRI treatment have contributed to studies showing negative, positive or no altered effect of SSRIs on childhood outcomes.<sup>19,22,26–29</sup> Understanding why the variance in effects of SSRI treatment exist on mother and developing neonate is needed.<sup>30</sup>

Emerging research points to the impact of the microbiome-gut-brain axis on mental health and child development.<sup>30–35</sup> The gut microbiota is a collection of microorganisms within the bowel that are extensively linked to health and disease.<sup>36</sup> They are also capable of producing metabolites and neurotransmitters that allow communication between the gut and the brain, including via the immune system.<sup>36</sup> Colonization of the infant gut largely begins at birth and current research shows that early life factors, such as maternal stress-related illnesses, impact the maternal brain, microbiome and the developing microbiome-gut-brain axis.<sup>37–45</sup> For example, stress during gestation reduces the quantity of a number of gastrointestinal microbial strains in mouse dams<sup>46</sup> and prenatal stress alters fetal intestinal transcriptome and the adult offspring gut and hypothalamic gene expression (in male rat offspring).<sup>47</sup> In addition, early life stress affects the richness and diversity of serotonin-regulating gut microbiota.<sup>33–35</sup> This suggests that the gut microbiome of the mother and developing neonate may offer a critical link between early adversity and brain plasticity.

The impact of SSRIs on the microbiome-gut-brain axis of the mother and offspring has only recently begun to be explored<sup>48,49</sup> with research investigating the impact of SSRIs on the maternal microbiome and/or neurodevelopment.<sup>50,51</sup> SSRI treatment with fluoxetine alters the maternal fecal microbial structure and relative abundance of a number of operational taxonomic units (OTUs) in the rat dam and

these effects are strongest if the dam has been exposed to early life stress herself.<sup>50</sup> Furthermore, gestational SSRI exposure to non-stressed dams alters the fetal brain transcriptome in mice and these effects are related to maternal gut microbiome influences in response to SSRIs.<sup>51</sup>

Perinatal SSRI exposure and perinatal mental illness also impact the neonatal brain.<sup>52–55</sup> Both prenatal exposure to maternal depression and SSRIs affect neonatal functional brain organization on postnatal day 6<sup>56</sup> and the impact of prenatal SSRI-exposure effects on the neonatal brain are sexually differentiated in some brain regions (i.e., the corpus-callosum microstructure).<sup>52</sup> This research highlights the important early impact that SSRIs and perinatal mood symptoms can have on neurobehavioral outcomes; adding to the growing literature of perinatal SSRI exposure on the developing offspring later in life.<sup>15,16,26,57–60</sup> In turn, we are also seeing an impact of SSRIs on the maternal brain.<sup>61,62</sup> Understanding how the maternal gut bacteria may contribute to the effects of SSRIs on mother and offspring will aid in our understanding of the microbiome-gut-brain axis in perinatal mental health.

The present study aimed to understand the impact of perinatal SSRIs and gestational stress on key components of the maternal and neonatal microbiome-gut-brain axis. An animal model of gestational stress and SSRI treatment was used to disentangle the effects of stress-related disorders and SSRIs on mother and offspring. This resulted in four groups of mother-offspring dyads: (1) Ctr\_Veh—unstressed and treated with vehicle; (2) Ctr\_Flx: unstressed and treated with fluoxetine; (3) GS\_Veh—gestationally stressed and treated with vehicle; (4) GS\_Flx—gestationally stressed and treated with fluoxetine. Both male and female offspring were used to determine if sex had an impact on any measures. We hypothesized that gestational stress exposure would impact the maternal microbiome-gut-brain axis during the peripartum period, that these alterations would be similar to effects seen in the neonate, and that SSRIs would prevent some of the effects of gestational stress.

## 2 | MATERIALS AND METHODS

### 2.1 | Animals

The present study used 54 Sprague-Dawley female rats (average weight 310 g) and 15 male rats for breeding from the Laboratoires Janvier (Le Genest-Saint-Isle, France). Animals were kept under standard laboratory conditions in a 12:12 h light/dark schedule (lights on at 7:00 am) with access to standard rat chow and tap water ad libitum. All experiments were approved by the Ethics Committee of the French Ministry of Research (authorization number APAFIS#2959-2015120114544585). The animal facility is licensed by the French Ministry of Agriculture (agreement D35-238-19). Animals were group housed in transparent polyurethane cages upon arrival at the facility and singly housed after breeding.

For breeding, two females and one male were paired. Daily vaginal smears were done to determine if copulation had taken

place as evidenced by sperm in the vaginal smears (gestation Day 1; GD1). Once sperm was evident in a vaginal smear the female was removed from the cage with the male and singly housed. Fifteen females did not have sperm in the vagina or did not maintain a pregnancy (lack of weight gain) and were removed from the study. This left 39 pregnant females randomly divided into four groups: (1) Ctr\_Veh—unstressed and treated with vehicle ( $n = 8$ ); (2) Ctr\_Flx: unstressed and treated with fluoxetine ( $n = 7$ ); (3) GS\_Veh—gestationally stressed and treated with vehicle ( $n = 11$ ); (4) GS\_Flx—gestationally stressed and treated with fluoxetine ( $n = 13$ ). Weight and litter characteristics were recorded. For a timeline of the experimental design, see Figure 1. In brief, confirmed pregnant females were randomly assigned to the four treatment groups. Maternal fecal samples were collected prior to treatment on GD2, and then on GD22. Affective behaviors were assessed from GD16–GD19. Two days after giving birth (postpartum Day 2: PD2), offspring-retrieval tests were performed after which maternal and offspring brains, offspring colon tissue and maternal fecal samples were collected.

## 2.2 | Gestational stress

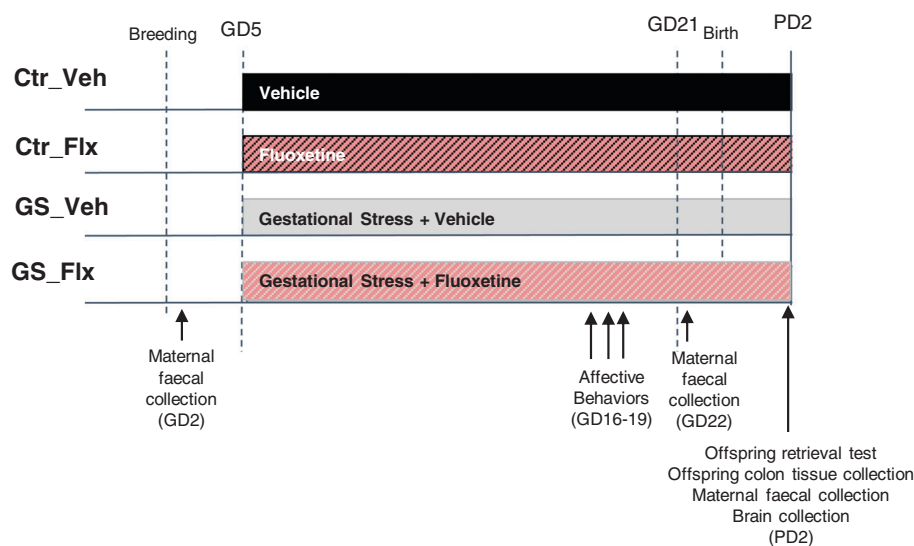
Repeated unpredictable stress during pregnancy was carried out between GD 5 and GD21 according to a previously described procedure.<sup>63,64</sup> The repeated stress consisted of 0–2 stressors per day. Stressors included restraint under bright light for 1 h; overnight exposure to damp bedding; 12 h of food deprivation; or 5 min of forced swimming at 26°C.

## 2.3 | Fluoxetine administration

Treatment with vehicle (saline) or fluoxetine (10 mg/kg/day—Fagron, Belgium) was administered to the pregnant rats as previously described.<sup>64–66</sup> Females were fed 1/9th of a vanilla wafer biscuit (Crousti fondante, Delacre, Belgium) once per day in the morning between 8:00 and 10:00 am from GD5 until sacrifice on postnatal day 2 (PD2). Biscuit portions were injected with vehicle (saline) or fluoxetine dissolved in vehicle. After administration of the biscuit to the home cage, females were monitored to ensure that biscuits were eaten. Biscuits were consistently eaten by all females in less than 5 min.

## 2.4 | Affective measures during gestation

All pregnant females were subject to brief behavioral tests during late pregnancy (GD16–19) to measure anxiety and depressive-like behaviors and to determine the affective state of the females after stress. On GD16–17 the sucrose preference test (SPT) was carried as previously described.<sup>67</sup> Rats were acclimated to the taste of a 1% sucrose solution for 24 h with two bottles in their home cage. For the sucrose preference test animals were presented with two bottles for 24 h, one bottle of 1% sucrose solution and one bottle of water. The weights of both bottles were recorded prior to and after each 24 h period. Preference for sucrose was determined by the quotient of sucrose consumption after 24 h to total liquid consumption of the combined water and sucrose solutions and corrected for bodyweight. On GD18, the open field test (OFT) took



**FIGURE 1** Experimental design. After pregnancy was determined, female rats were randomly assigned to four treatment groups: (1) Ctr\_Veh—unstressed and treated with vehicle ( $n = 8$ ); (2) Ctr\_Flx—unstressed and treated with fluoxetine ( $n = 7$ ); (3) GS\_Veh—gestationally stressed and treated with vehicle ( $n = 11$ ); (4) GS\_Flx—gestationally stressed and treated with fluoxetine ( $n = 13$ ). Maternal fecal samples were collected prior to treatment on gestation day 2 (GD2). Affective behaviors were assessed from GD16–GD19 with the sucrose preference test, open field test and elevated plus maze. Feces from the mothers were collected on GD22. On postnatal day 2 (PD2), pup-retrieval tests were performed after which maternal and offspring brains, offspring colon tissue and maternal fecal samples were collected. For further details of the methodology refer to the Materials and Methods section.

place as previously described<sup>68</sup> between 8:00 and 11:00 am. In brief, dams were placed in a 60 cm × 60 cm × 40 cm box in a dimly lit room for 5 min. Sessions were scored with Ethovision 12 (Noldus) tracking software. Time and crossings into the center and periphery were recorded. On GD 19 the elevated plus maze (EPM) was carried out as previously described<sup>68</sup> between 8:00 am and noon. In brief, females were placed in the center of the maze and left to explore for 5 min. Time, entries and distance traveled in the open and closed arms was recorded using Ethovision 12 (Noldus) tracking software. Scores of the primary affective measures of each test (time immobile, time in the open arms, and sucrose consumption) were z-transformed and added together to give an overall affective state score for each dam.

## 2.5 | Offspring retrieval test

In order to investigate aspects of maternal behavior a test of offspring retrieval was performed on PD2. In brief, offspring were separated from their mother for 5 min and when reunited, offspring were put in the opposite corner to the original nest site in the home cage. Video recordings of maternal behaviors were taken for the duration of 15 min. The following maternal behaviors were scored from video recordings; retrieval off pups to the original nest site, mouthing (picked up a pup but not retrieved to the original nest site), sniffing offspring, licking offspring, nursing, nest building and self-grooming. The duration and number of bouts of each behavior were recorded. The time that all offspring were retrieved was also recorded. Stopwatch+ (developed by Kim Wallen, Emory University, USA) was used to score the maternal behaviors. Data from one dam in the GS\_Flx group was not available due to technical error. Ctr\_Veh ( $n = 8$ ), Ctr\_Flx ( $n = 7$ ), GS\_Veh ( $n = 11$ ) and GS\_Flx ( $n = 13$ ).

## 2.6 | Mother fecal collection

In addition to standard cage changing, the dams were transferred to new cages with clean bedding weekly and the following day feces were collected with sterilized tweezers. Feces were stored in sterile 1.5-mL tubes and frozen at  $-80^{\circ}\text{C}$  until analysis. Two feces were taken each week and stored separately. At the time of terminal anesthesia on PD2, feces were collected from the colon to ensure no contamination of fecal matter from offspring.

## 2.7 | Analysis of maternal microbiome on GD22 with 16S rRNA techniques

### 2.7.1 | DNA extraction

DNA extraction was performed using the QIAmp Fast DNA Stool Mini Kit (Qiagen, Sussex, UK) coupled with an initial bead-beating step.

Briefly, each sample was vortex-mixed in a 2 mL screw-cap tubes (Sarstedt, Wexford, Ireland) containing 0.25 g of a 1:1 mix of 0.1 mm and 1.0 mm sterile zirconia beads plus a single 3.5 mm diameter bead (BioSpec Products, Bartlesville, USA) with 1 mL of Qiagen InhibitEX buffer. DNA was quantified using the Qubit 3.0 Fluorometer (Bio-Sciences, Dublin, Ireland) and the Qubit dsDNA HS Assay Kit (Life Technologies, Oregon). Extracted DNA was kept frozen at  $-20^{\circ}\text{C}$  until further analysis.

### 2.7.2 | 16S sequencing

**16S rRNA Gene Sequence-based microbiota analysis** The V3–V4 hypervariable region of the 16S rRNA gene were amplified and prepared for sequencing as outlined in the Illumina 16S Metagenomic Sequencing Library Protocol ([http://www.illumina.com/content/dam/illumina-support/documents/documentation/chemistry\\_documentation/16s/16s-metagenomic-library-prep-guide-15044223-b.pdf](http://www.illumina.com/content/dam/illumina-support/documents/documentation/chemistry_documentation/16s/16s-metagenomic-library-prep-guide-15044223-b.pdf)). Briefly, first PCR was done using forward primer (5'-TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCCTACGGGNGGCWGCAG-3') and reverse primer (5'-GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGACTA CHVGGGTATCTAATCC-3'). Each 25  $\mu\text{L}$  PCR reaction contained 5 ng/ $\mu\text{L}$  microbial genomic DNA, 1  $\mu\text{M}$  of each primer and 12.5  $\mu\text{L}$  2X Kapa HiFi Hotstart ReadyMix (Kapa Biosystems Ltd., UK). The PCR conditions follow as: initial denaturation at  $95^{\circ}\text{C} \times 3$  min; 25 cycles of  $95^{\circ}\text{C} \times 30$  s,  $55^{\circ}\text{C} \times 30$  s,  $72^{\circ}\text{C} \times 30$  s; and  $72^{\circ}\text{C} \times 5$  min for final extension. PCR products were purified with Agencourt AMPure XP system (Beckman Coulter Genomics, Takeley, UK). In the next step, dual indices and Illumina sequencing adapters were attached to PCR products using the Nextera XT Index Kit (Illumina, San Diego, California, USA). Each 50  $\mu\text{L}$  PCR reaction contained 5  $\mu\text{L}$  purified DNA, 5  $\mu\text{L}$  index primer 1 (N7xx), 5  $\mu\text{L}$  index primer 2 (S5xx), 25  $\mu\text{L}$  2X Kapa HiFi Hot Start Ready mix and 10  $\mu\text{L}$  PCR grade water. PCR amplification was completed using the previous program but with only eight amplification cycles instead of 25. Following this, a second clean-up step with the Agencourt AMPure XP system was done. PCR products were quantified, normalized and pooled in an equimolar fashion using the Qubit dsDNA HS Assay Kit (Life Technologies, Oregon, USA). Next steps in the library preparation were carried out by Clinical-Microbiomics (Copenhagen, Denmark) prior to  $2 \times 300$  (bp) paired-end sequencing on the Illumina MiSeq platform, using standard Illumina sequencing protocols.

### 2.7.3 | Bioinformatic sequence analysis

Bioinformatic sequence analysis was performed as previously described.<sup>69</sup> Briefly, paired-end sequences were assembled using FLASH<sup>70</sup> and analyzed using QIIME version 1.8.0 (Quantitative Insights into Microbial Ecology).<sup>71</sup> Sequences were quality checked and the remaining sequences were clustered into OTUs using USEARCH (version 7-64bit).<sup>72</sup> Taxonomic ranks were assigned with a BLAST search against the SILVA SSURef

database release 116.<sup>73</sup> Alpha diversity indices were generated in QIIME. Beta-diversity based on Bray-Curtis distance matrices was analyzed using R (version 3.3.2), R Studio (version 1.0.136), and the *adonis* function in the *vegan* package (version 2.3.2). Relative abundance of bacterial taxa was expressed as percentage of identified sequences.

#### 2.7.4 | Heatmap construction

Log<sub>2</sub> ratios relative to Ctr\_Veh group were calculated from group medians using R (version 3.3.2), R Studio (version 1.0.136), and the *tools* package (version 3.5.0). Heatmaps were created in R.

#### 2.7.5 | 16S Statistical analysis

Data are presented as median with IQR. The median values between groups were compared with Mann-Whitney U test.  $p < .05$  were deemed significant in all cases.

#### 2.7.6 | Maternal fecal microbiota composition of *Bacteroidetes*, *Firmicutes* and *Actinobacteria*

Based on the 16S RNA data which found significant effects of gestational stress or fluoxetine on *Firmicutes* and *Bacteroidetes* (Figure 3A), we chose to focus on these two phyla with *Actinobacteria* as a control. Fecal samples from GD2, GD 22, PD2 were used to investigate any changes in *Bacteroidetes*, *Firmicutes*, and *Actinobacteria* in dams during pregnancy and the early postpartum period as a function of gestational stress or fluoxetine treatment.

Macherey-Nagel bacterial DNA extraction kits were used for DNA extraction from the feces (60–80 mg per sample). The amounts of extracted DNA were quantified by Nanodrop 8000 spectrophotometer (ThermoFisher). Each sample was then diluted to 1 ng/μL. Primers for the bacterial families were used based on a previous study<sup>74</sup> (Table S1). The amounts of phyla *Actinobacteria*, *Bacteroidetes* and *Firmicutes* and the total bacteria 16S RNA gene for normalization were investigated by quantitative PCR. A specificity check was performed replacing the sample with sterilized water. The melting curve was performed to verify that only one amplicon of the expected size was obtained, thus determining the specificity of our primers. Cycle thresholds (Ct) were used to analyze the proportions of each phylum in the sample microbiota. The results are presented as  $2e(-\Delta\Delta Ct)$ . Tests of normality were done on each distribution and only measures of *Bacteroidetes* were log transformed in order to fit a normal distribution for further statistical analysis. Group sizes are as follows: Ctr\_Veh ( $n = 8$ ), Ctr\_Flx ( $n = 7$ ), GS\_Veh ( $n = 11$ ) and GS\_Flx ( $n = 13$ ). It should be noted that one sample was missing from the GS\_Flx group for *Actinobacteria* at T4 resulting in  $n = 12$  for that group.

### 2.8 | Maternal brain histology

On PD2, approximately 90 min after the offspring retrieval tests, the dams were deeply anesthetized via an intraperitoneal injection. Within minutes dams were subjected to intracardiac perfusion with saline and then 4% paraformaldehyde with picric acid. Brains of dams were extracted and post-fixed in 4% paraformaldehyde for 24 h. Brains were then cryoprotected up to 1 week into a phosphate-saline solution containing 30% sucrose, frozen on dry ice and kept at  $-80^{\circ}\text{C}$ . Brain tissue was sliced using a cryostat (Leica) in 40 μm sections in series of 10 and were stored in antifreeze solution and maintained at  $-15^{\circ}\text{C}$ .

To investigate effects on immature neurons and microglia cells in the dentate gyrus of the hippocampus the endogenous markers for doublecortin (to label immature neurons) and Iba1 (to label microglia) were used. Glucocorticoid density was also assessed in the CA1, CA3 and dentate gyrus (DG) regions of the hippocampus.

For immunohistochemistry, tissue (1 series of brain sections) was used from dams for staining of immature neurons (doublecortin; DCX), microglia (Iba1) and the glucocorticoid receptor (GR) immunoreactivity. Sections were rinsed between steps in PBS and PBS plus 0.01% Triton X-100 (PBST). Tissue was incubated for 30 min at room temperature in 0.6% H<sub>2</sub>O<sub>2</sub> followed by blocking for 30 min at room temperature in 5% normal donkey or goat serum for 30 min. Tissue was incubated overnight at 4°C in rabbit anti-Iba1 1:5000 (Wako #19-19741) or goat anti-DCX 1:300 (Santa Cruz, sc-8066) or rabbit anti-GR antibody (1:500, Abcam3578, Cambridge, Massachusetts, USA—for two nights). Sections were incubated in an appropriate secondary antibody (1:500 biotinylated donkey anti-rabbit or 1:500 biotinylated rabbit anti-goat) for 2 h at room temperature followed by processing using the avidin-biotin-peroxydase complex (VECTASTAIN Elite ABC HRP Kit #PK-6100; 1:1000; Vector Laboratories) and DAB (DAB kit; Vector Laboratories). Sections were mounted on Superfrost Plus slides (Fischer Scientific, Pittsburgh, Pennsylvania, USA), dried, dehydrated, and coverslipped with Permount (Fischer Scientific). For representative photomicrographs see Figures S4 and S5.

For quantification of DCX-immunoreactive (-ir) cells, the number of DCX-ir cells were counted under 40× objective using an Olympus BX60 microscope by an experimenter blind to treatment groups as previously described.<sup>75</sup> Cells were counted in the granule cell layer and subgranular zone (GCL/SGZ) of the hippocampus in both the dorsal (up to  $-4.92$  bregma) and ventral regions. Cells were considered DCX-ir if cell bodies were clearly stained, relatively circular, and located in the area of interest. DCX-ir cells were also quantified and characterized based on their morphology in 150 cells of the dorsal and ventral areas (75 cells per each area). DCX-ir cells were categorized as either proliferative (no process or short process), intermediate (medium process with no branching) or postmitotic neuron.<sup>76</sup>

For quantification of Iba1-ir cells in the dorsal (up to  $-4.92$  bregma) and ventral dentate gyrus of the hippocampus, photomicrographs were taken with Olympus AX70 microscope and the software Cell^F software (Olympus) under 10× objective by an experimenter blind to treatment groups. Three photomicrographs of the GCL/SVZ

of the dentate were taken in three slices of the dorsal hippocampus and two slices of the ventral hippocampus. Photomicrographs were analyzed using ImageJ in order to determine the optical density and the number of Iba1-ir cells (by means of the plugin cell counter) in the GCL/SGZ.<sup>77</sup> Briefly, the density of the region of interest was calculated by subtracting the total density from the density of an adjacent background region. Cells were quantified if they were darkly stained, relatively circular and in the region of interest. One dam from the Ctr\_Veh group was removed from analysis due to lack of staining for DCX and Iba1.

For quantification of GR-ir cells the images were taken at the level of the Bregma  $-2.64$  and  $-4.92$  for the dorsal region, and  $-5.64$  and  $-6.70$  for the ventral region by an experimenter blind to treatment groups. Images were taken from each of the three main regions of the hippocampus: CA1, CA3 and dentate gyrus. In each region, two images were acquired on an Olympus AX70 microscope using the Cell<sup>^</sup>F software (Olympus) (six photos per region in total) for each hippocampal region. GR expression analysis was performed using ImageJ software, previously calibrated using the Stouffer calibration scale (Stouffer Graphic Arts Equipment Co., Mishawaka, Indiana, USA). Briefly, the images were modified to a grey scale and then optical density measurements were made based on the calibration. Background images of adjacent areas were used to normalize the optical density. Outliers were identified as being excluded from the calculated range by the formula: Mean  $\pm$  2.5 times the standard deviation (SD). This occurred once for the DG region of the ventral hippocampus.

## 2.9 | Neonatal offspring brain and colon collection on PD2

On PD2, at the time the dams were perfused, a maximum of two males and two females from each litter of each dam were rapidly decapitated, brains and colons were dissected, frozen on dry ice and stored at  $-80^{\circ}\text{C}$  until further analysis. In total 106 pups were used. The number of offspring used in the four treatment groups were as follows: Ctr\_Veh ( $n = 25$ ), Ctr\_Flx ( $n = 28$ ), GS\_Veh ( $n = 20$ ) and GS\_Flx ( $n = 33$ ) with  $n = 8-14$  offspring per sex per group.

## 2.10 | Offspring colon microbiota composition of *Bacteroidetes*, *Firmicutes* and *Actinobacteria*

Bacterial DNA from colon tissue collected on PD2 was extracted following the same protocol as was used on the mothers' feces, and the same steps were followed to identify the proportions of the phyla of interest. Outliers  $\pm 2.5$  SD ( $2e(-\Delta\Delta\text{Ct})$ ) were removed from the data. This resulted in one sample being removed of *Actinobacteria* (1 male from Ctr\_Veh) and *Firmicutes* (1 male from Ctr\_Veh) and five samples of *Bacteroidetes* (2 males and 2 females from Ctr\_Veh; 1 male from Ctr\_Flx). Final group sizes ranged between eight and 16 per sex per group.

## 2.11 | Offspring brain plasticity

In order to define the potential impact of gestational stress or treatment with fluoxetine on brain development, Western blot analysis was performed on all brains collected from offspring on PD2 ( $n = 20-33$  per group). Western blot analysis was used to quantify the expression of doublecortin and Iba1 by an experimenter blinded to the groups. Prior to Western blot analysis, the brains were brought to a temperature of  $-20^{\circ}\text{C}$  and divided into two parts: anterior and posterior based on the optic chiasm. The cerebellum was removed and not analyzed. Brain tissue was homogenized by grinding in a "Dounce" type homogenizer in RIPA buffer (50 mM Tris-HCL-pH 7.2, 0.1 mM EDTA, 5 mM *N*-ethylmaleimide, 0.2% NP40 and complete protease inhibitor, one pellet per 50 mL). The tissue samples were then centrifuged at 10,000 *g* for 5 min at  $4^{\circ}\text{C}$ . The supernatant was collected, and the protein concentration of each sample was measured by the Bradford method (BioRad, according to the manufacturer's instructions), using bovine serum albumin as a standard. The concentration is measured at 595 nm on a BioRad iMark microplate absorbance reader.

A total of 10  $\mu\text{g}$  of protein diluted in Laemmli (BioRad, #161-0747) was plated on SDS-PAGE gels (8% acrylamide for DCX and 12% acrylamide for Iba1 based on molecular weight). BenchMark Prestained (Novex, #10748-010) was used as a marker for molecular weight. Migration was performed by electrophoresis at 120 Volt for 90 min in TGS buffer (BioRad, #161-0732). After migration, proteins were transferred to PVDF (polyvinylidene fluoride) membranes using BioRad's Trans-Blot SD Semi-Dry Transfer Cell for 1 h at 100 Volts. Following transfer, the nonspecific binding sites of the membranes were blocked in TBS-T (1% Tris buffered-saline and 0.1% Tween) buffer containing 5% skim milk for 1 h.

The membranes were then incubated with the primary anti-DCX antibody (Abcam, #ab18723) diluted 1:5000 or anti-Iba1 (Wako, #016-20001) diluted 1:500 in the blocking solution overnight at  $4^{\circ}\text{C}$ . After rinsing, the membranes were incubated for 1 h at room temperature with agitation in horseradish peroxidase (HRP)-associated anti-rabbit secondary antibody diluted 1:5000 in TBS-T. After rinsing, the HRP was revealed by Millipore's HRP Immobilon Western Substrate (#WBKLS0500). Images were taken with the ChemidocTM XRS+ Scan Molecular Imager and analyzed with BioRad Image Lab software. The membranes were then stripped (0.2 M glycine, pH 2.5, 30 min at  $60^{\circ}\text{C}$ ) extensively washed in TBST, incubated in blocking solution for 2 h then with primary anti- $\beta$ -actin antibody (AC-54 Sigma) diluted 1:150,000 in TBS-T and the secondary HRP-associated anti-mouse antibody diluted 1:5000 in TBS-T, for a 1 h incubation at room temperature for both antibodies. Revelation and analysis were similar as above. Data is expressed as the ratio between the target density and the normalizer  $\beta$ -actin. Outliers more than  $\pm 2.5$  SD from the mean were removed. This resulted in removal of three forebrain and five hindbrain samples of Iba1 and two forebrain and two hindbrain DCX samples resulting in  $n = 9-14$  per sex per group. For representative western blot results see Figure S6.

## 2.12 | Statistical analysis

Statistical analysis was performed using the software Statistica 13 (Tibco Software). For the dam weight and litter characteristics factorial analysis of variance tests (ANOVA) were carried out with condition (control/gestational stress) and treatment (vehicle/fluoxetine) as between subjects' factor. Litter size was controlled for using an analysis of covariance.

For analysis of maternal affective behaviors in late pregnancy as well as the offspring retrieval test Kruskal–Wallis tests were used with four groups (Ctr\_Veh, Ctr\_Flx, GS\_Veh and GS\_Flx). Measures investigated were measures of time, distance and latency in the open and closed arms (EPM) or center and peripheral areas (OFT) and the sucrose preference in the sucrose preference test (SPT). During the offspring retrieval test that following measures were also investigated; time spent licking, nursing, nest building, time to first retrieval, time all offspring retrieved and time retrieving offspring. Post hoc tests used multiple comparisons.

For the analysis of measures of fecal microbiota in dams repeated-measures factorial ANOVAs were used with stage of perinatal period as the repeated measure (pregnancy vs. postpartum) with condition (control/gestational stress) and treatment (vehicle/fluoxetine) as between subject factors.

Measures of hippocampal plasticity in dams were analyzed using a three-way factorial ANOVAs with region (dorsal/ventral), condition (control/gestational stress) and treatment (vehicle/fluoxetine) as factors.

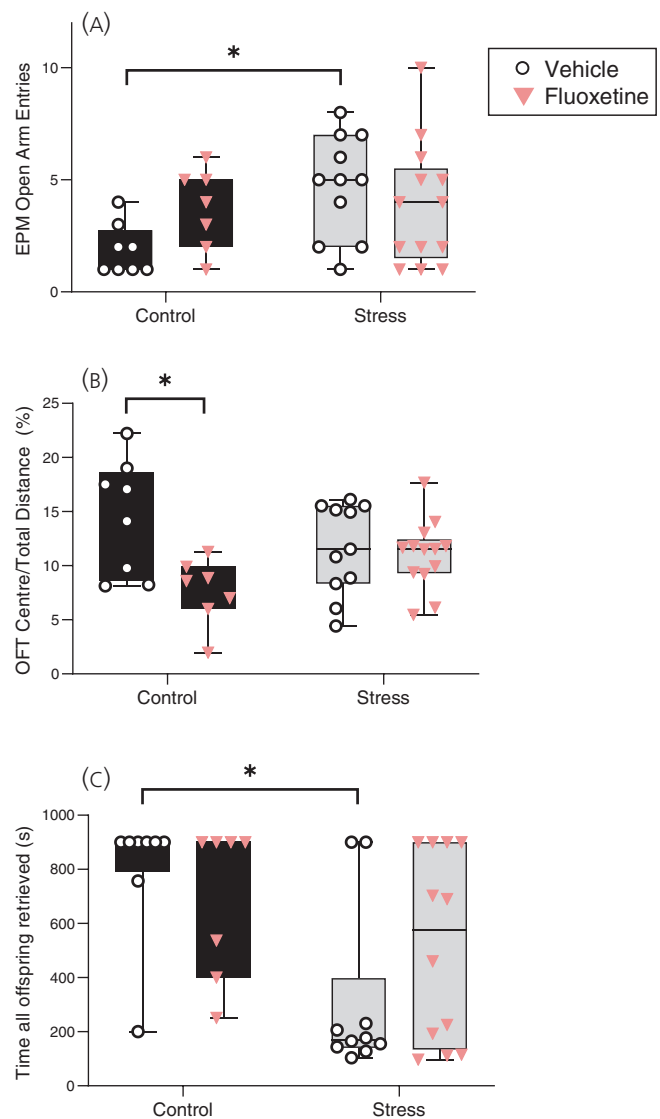
Factorial ANOVAs were also carried out on offspring brain and colon bacterial measures with condition (control/gestational stress), treatment (vehicle/fluoxetine), and offspring sex (male/female) as between subject factors.

Pearson correlations were conducted between maternal fecal microbiota and brain measures on PD2, offspring colon microbiota on PD2, and offspring brain measurements of DCX and Iba1. Post hoc tests for ANOVAs utilized Tukey's test. Significance was set at  $p < .05$ . The data that support the findings of this study are available on request from the corresponding author. The data are not publicly available due to privacy or ethical restrictions.

## 3 | RESULTS

### 3.1 | Gestational stress and fluoxetine impact maternal affective state during late pregnancy

GS\_Veh dams made significantly more open arm entries on the EPM compared to Ctr\_Veh ( $p = .0057$ , multiple comparisons [2-tailed];  $H [3, N = 39] = 11.39786$   $p = .0098$ ) (Figure 2A). Ctr\_Flx dams traveled less in the center of the OFT (center/total distance) compared to Ctr\_Veh dams ( $p = .0236$ , multiple comparisons [2-tailed];  $H [3, N = 39] = 8.361$   $p = .0391$ ) (Figure 2B). There were no other significant effects of gestational stress or fluoxetine on measures of affective behavior in late pregnancy ( $p > .08$ ).



**FIGURE 2** Box plot (min to max) of (A) open arm entries on the EPM, (B) ratio of central distance over total distance traveled on the OFT, and (C) time all offspring were retrieved (s). (A) GS\_Veh dams made significantly more open arm entries on the EPM compared to Ctr\_Veh ( $p = .0057$ ). (B) Ctr\_Flx dams traveled less in the center of the OFT (center/total distance) compared to Ctr\_Veh dams ( $p = .024$ ). (C) GS\_Veh dams took significantly less time retrieving all offspring than Ctr\_Veh dams ( $p = .0057$ ). ( $n = 7$ – $13$ /group) \*  $p < .05$ .

### 3.2 | Gestational stress and fluoxetine administration significantly impact the maternal microbiome in late pregnancy

#### 3.2.1 | Alpha and beta diversity

We detected on average 150 bacterial genera of eight different phyla in the fecal material of each experimental group. There were no differences in the alpha-diversity (Chao1 and Shannon indices) of microbiota communities across groups (Figure S1). Principal component



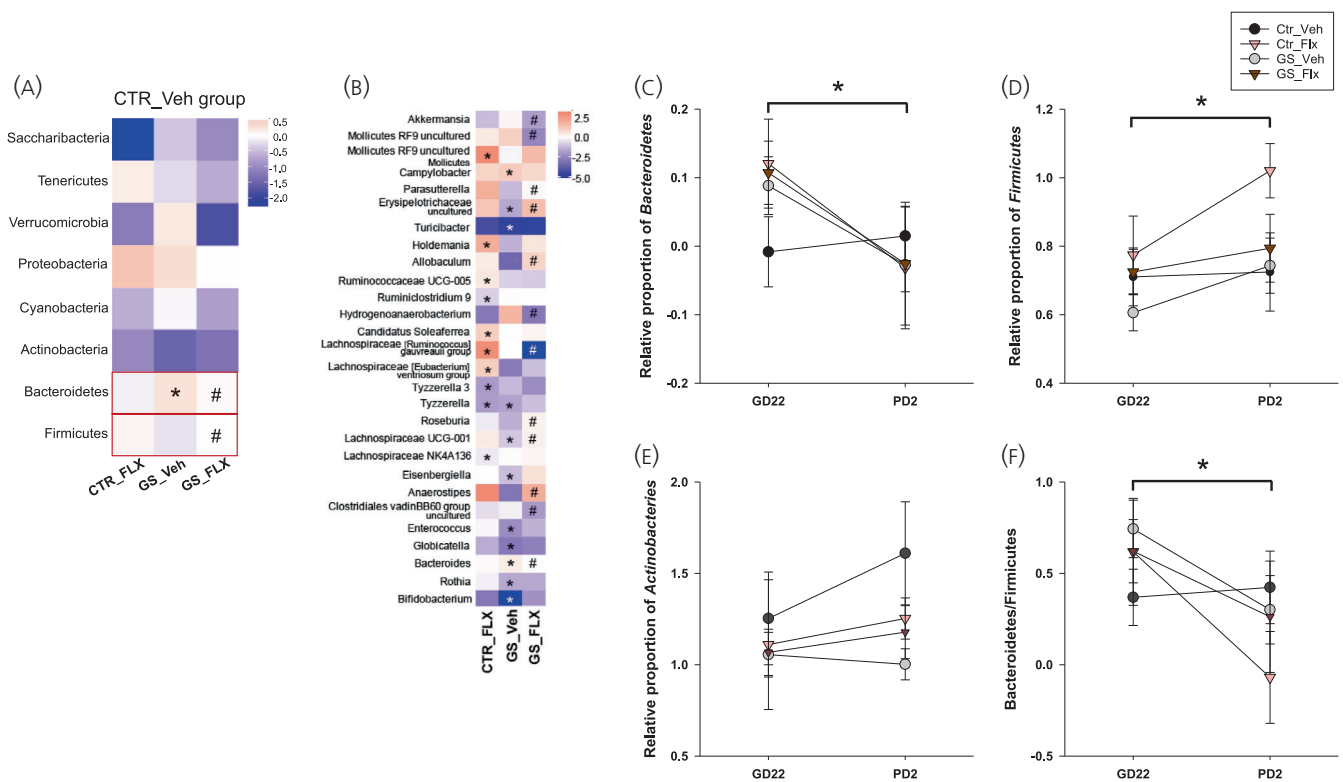
analysis did not show significant separation of gut microbiota across groups as well (Figure S2).

### 3.2.2 | Phylum level

On the phylum level, the two dominant phyla—Bacteroidetes and Firmicutes—comprised more than 95% of all bacterial taxa in all experimental groups (Figure S3). For better visualization of changes occurring within the minor phyla, we constructed a heatmap depicting Log<sub>2</sub> change ratios in the Ctr\_Flx, GS\_Veh and GS\_Flx groups relative to the “reference” Ctr\_Veh group (Figure 3A). Gestational stress induced a significant increase in the abundance of the Bacteroidetes phylum (56.6% vs. 47.4% in Ctr\_Veh group), administration of fluoxetine during the gestational stress ameliorated the impact of stress, significantly decreasing Bacteroidetes and increasing Firmicutes bacterial species in the GS\_Flx group to the control levels (Figure 3A). Yet, fluoxetine administration alone did not affect the microbiome (Ctr\_Flx group, Figure 3A).

### 3.2.3 | Genus level

We also observed differential changes in various bacterial genera in all experimental groups (depicted on Figure 3B). Interestingly, fluoxetine administration to unstressed (Ctr\_Flx) and stressed (GS\_Veh) dams induced very different patterns of increase and decrease in the abundance of bacterial genera. The Ctr\_Flx group had mainly *Lachnospiraceae* and *Ruminococcaceae* families of the Firmicutes phylum affected, while the GS\_Veh group showed significant changes in the *Bifidobacterium* (Actinobacteria), *Bacteroides* (Bacteroidetes), *Enterococcus* (*Lactobacillaceae* family of Firmicutes) and *Turicibacter* (*Erysipelotrichaceae* family of Firmicutes) genera. Administration of fluoxetine throughout the period of gestational stress counteracted the stress-induced changes in some bacterial taxa (*Bifidobacterium*, *Bacteroides*, *Erysipelotrichaceae* uncultured species), had no effect on stress-induced changes in other taxa (*Enterococcus*, *Turicibacter*), as well as induced stress-independent effects similarly observed in the Ctr\_Flx group (*Anaerostipes*, *Allobaculum*) (Figure 3B) (note traditional nomenclature of prokaryotes used as this nomenclature is changing<sup>78</sup>).



**FIGURE 3** Gestational stress and fluoxetine administration effects on the maternal microbiome. (A) Heatmap of log<sub>2</sub> fold change ratios showing increases (red) or decreases (blue) in the abundance of bacterial phyla relative to the Ctr\_Veh group. (B) Heatmap of log<sub>2</sub> fold change ratios showing increases (red) or decreases (blue) in the abundance of bacterial genera relative to the Ctr\_Veh group. Mean (±SEM) proportion in maternal feces during late gestation (GD22) and postpartum day 2 (PD2) of (C) *Bacteroidetes* (D) *Firmicutes* (E) *Actinobacteria*, and (F) *Bacteroidetes/Firmicutes*. (C) There was a significant decrease from late pregnancy to the early postpartum period ( $p = .038$ ) in the relative proportion of *Bacteroidetes* (log). (D) There was a significant increase in the relative proportion of *Firmicutes* from late pregnancy to the early postpartum period ( $p = .040$ ). (F) There was a significant decrease in the ratio of *Bacteroidetes* to *Firmicutes* from late pregnancy to the postpartum period ( $p = .025$ ). ( $n = 7-13/\text{group}$ ) \*  $p < .05$  for Ctr\_Flx versus Ctr\_Veh and for GS\_Veh versus CTR\_Veh, # $p < .05$  for GS\_Flx versus GS\_Veh (Mann-Whitney test).

### 3.3 | Maternal gut bacteria are altered from late pregnancy to the early postpartum

On GD2, prior to stress or fluoxetine treatment there were no baseline differences in fecal abundance of *Bacteroidetes*, *Firmicutes* or *Actinobacteria* of the dams randomly assigned to each of the four groups ( $p > .08$ ). There was a significant decrease in the proportion of *Bacteroidetes* in maternal feces from late gestation (GD22) to PD2 ( $F[1, 36] = 4.6208, p = .03838$ ; Figure 3C) and a significant increase in the abundance of *Firmicutes* from late gestation to PD2 ( $F[1, 36] = 4.5644, p = .03951$ ), regardless of fluoxetine treatment or stress (Figure 3D). This led to a significant decrease in the ratio of *Bacteroidetes* to *Firmicutes* from late gestation to PD2 in dams ( $F[1, 36] = 5.4168, p = .02568$ ) (Figure 3F). There were no other significant main or interaction effects for the *Bacteroidetes*-to-*Firmicutes* ratio, *Bacteroidetes*, *Firmicutes* or *Actinobacteria* in maternal feces ( $p > .200$ ).

### 3.4 | Gestational stress decreases litter weight

There was a main effect of stress on litter weight with stressed dams having smaller litters ( $F[1, 35] = 4.5789, p = .03942$ ); (mean  $\pm$  SEM) Ctr\_Veh (108.7  $\pm$  5.02 g), Ctr\_Flx (94.25  $\pm$  8.77 g), GS\_Veh (86.98  $\pm$  9.15 g) and GS\_Flx (76.98  $\pm$  9.07 g). There were no significant differences between groups in dam weight gain across gestation or at the time of sacrifice ( $p > .1$ ).

### 3.5 | Gestational stress increases offspring retrieval time on PD2

GS\_Veh dams took significantly less time retrieving all offspring than Ctr\_Veh dams on PD2 ( $p = .005734$ ;  $H[3] = 13.87705, p = .0031$ ; Figure 2C). To ensure that a reduced litter size in the stressed females did not have a significant impact on retrieval time an ANCOVA was carried controlling for litter size. Results of this ANCOVA confirmed that indeed retrieval time is enhanced in GS\_Veh dams ( $F[3, 33] = 4.1321, p = .01360$ ; controlling for differences in litter size). During the offspring retrieval test gestational stress or fluoxetine treatment did not affect the duration of offspring-directed care such as sniffing, licking or nursing pups ( $p > .4$ ). There were also no significant differences between groups in nest-building behaviors or self-grooming or correlations between maternal behaviors and fecal bacteria measured in the dam ( $p > .2$ ).

### 3.6 | Maternal glucocorticoid receptor (GR) density in the hippocampus is decreased with fluoxetine treatment (Ctr-Flx) and is associated with changes in the maternal gut microbiome

For GR density there was a significant interaction effect between region (dorsal vs ventral), stress and treatment in the dentate gyrus ( $F[1, 34] = 5.1394, p = .02986$ ) with the GR density in Ctr\_Veh dams being significantly greater than in Ctr\_Flx dams ( $p = .027$ ) and the GR

density in the dorsal dentate gyrus being significantly greater than in the ventral dentate gyrus ( $p < .018$ ) (Figure 4A). There were significant main effects of region (dorsal and ventral) on GR density in the CA1 ( $F[1, 34] = 99.027, p = 0.000001$ ) and CA3 ( $F[1, 34] = 33.862, p = .000001$ ) of the hippocampus. There were significant positive correlations between GR optical density in the ventral CA3 and DG regions of the hippocampus of the dam and the ratio of *Bacteroidetes* to *Firmicutes* in maternal feces on PD2 (CA3:  $r = .38720, p = .012$ ; DG:  $r = .3430, p = .033$ . Figure 4B).

### 3.7 | Maternal immature neuron morphology in the hippocampus is not altered with gestational stress or fluoxetine treatment on PD2

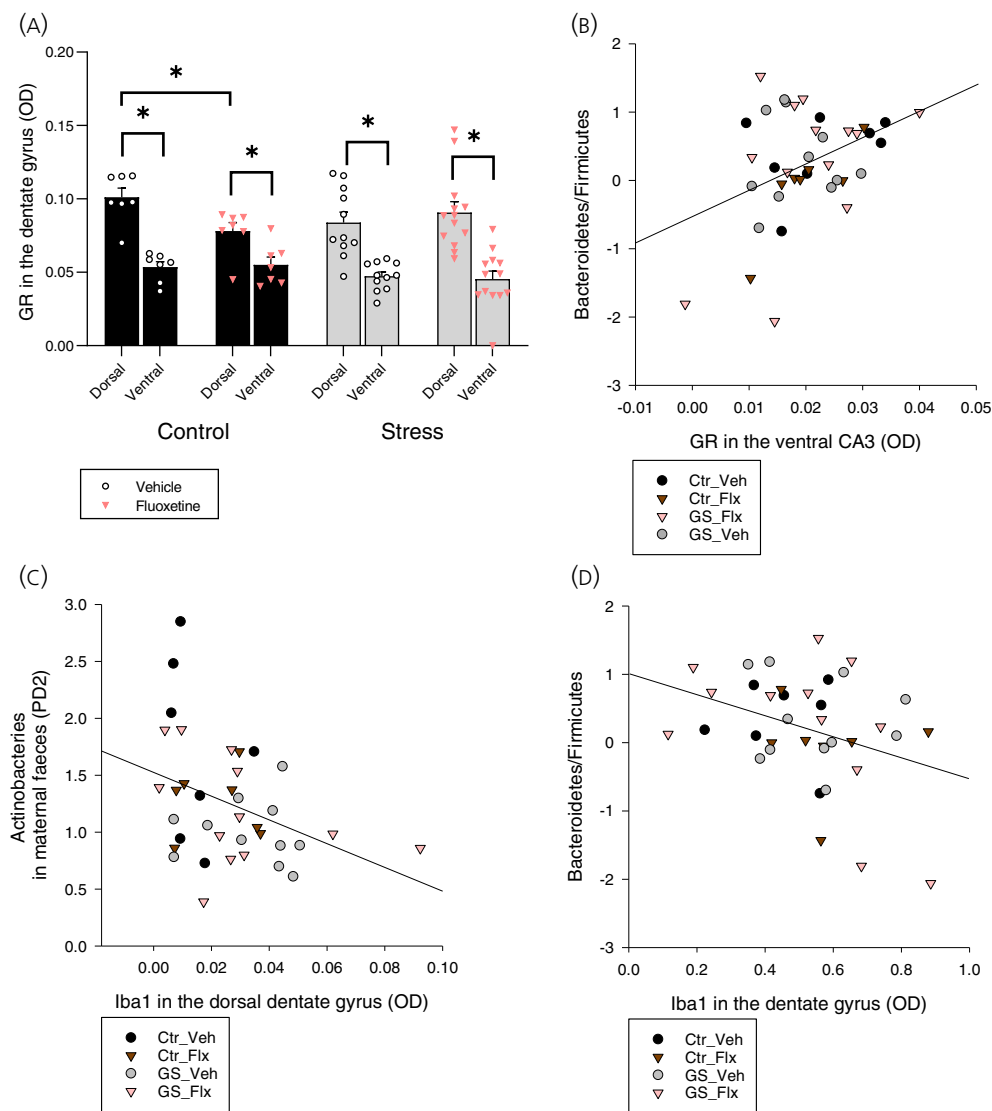
For doublecortin immunoreactive (DCX-ir) cell types there was a significant interaction effect between region, stress and treatment for proliferative ( $F[1, 34] = 4.2233, p = .04762$ ) and postmitotic ( $F[1, 34] = 4.5817, p = .03957$ ) cell types with post hoc tests revealing no significant differences between groups. There were significantly more DCX-ir cells in the ventral than dorsal hippocampus ( $F[1, 34] = 7.9278, p = .00804$ ) (Table 1).

### 3.8 | Maternal Iba1 density in the hippocampus is correlated with abundance of bacteria in maternal feces

For Iba1-ir there was a greater optical density and number of cells in the ventral than dorsal dentate gyrus (OD:  $F[1, 33] = 91.585, p = .000001$ ; cell number:  $F[1, 33] = 37.999, p = .000001$ ) (Table 1). There were significant negative correlations between Iba1 density in the dorsal hippocampus of the dam and the abundance of *Actinobacteria* in maternal feces on PD2 ( $r = -.3724, p = .023$ ; Figure 4C) and Iba1 density in the hippocampus of the dam and the ratio of *Bacteroidetes* to *Firmicutes* ( $r = -.3482, p = .035$ ; Figure 4D).

### 3.9 | Neonatal offspring gut microbiota composition is decreased with gestational stress

For the relative proportion of *Bacteroidetes* and *Actinobacteria* in colon tissue of offspring on PD 2 there was a significant main effect of gestational stress ( $F[1, 80] = 10.759, p = .00154$ ); ( $F(1, 84) = 5.0161, p = .02775$ ), with gestational stress decreasing the content of *Bacteroidetes* (Figure 5A) and *Actinobacteria* (Figure 5C) in offspring colon, regardless of sex. There was a significant interaction between fluoxetine exposure and gestational stress with respect to the abundance of *Firmicutes* ( $F[1, 84] = 5.9640, p = .01670$ ) with post hoc tests showing that Ctr\_Flx neonatal offspring had increased content of *Firmicutes* in the colon compared to Ctr\_Veh offspring ( $p = .035$ ) and GS\_Flx offspring ( $p = .034$ ) (Figure 5B). There was a significant positive correlation between *Bacteroidetes* content in offspring colon and in maternal feces on PD2 ( $r = .2277, p = .035$ ; Figure 5E).



**FIGURE 4** (A) Mean ( $\pm$ SEM) glucocorticoid receptor (GR) density in the dentate gyrus of rat dams. Ctr\_Veh dams had significantly greater GR density than Ctr\_Flx dams ( $p = .027$ ) and the GR density in the dorsal dentate gyrus was significantly greater than in the ventral dentate gyrus ( $p < .018$ ; 3-way ANOVA). (B) There was a significant positive correlation between GR optical density in the ventral CA3 of the dam and the ratio of *Bacteroidetes* to *Firmicutes* in maternal feces on PD2 (CA3:  $r = .38720$ ,  $p = .012$ ). There were significant negative correlations between Iba1 density in the dorsal hippocampus of the dam and the (C) abundance of *Actinobacteria* in maternal feces on PD2 ( $r = -.3724$ ,  $p = .023$ ) and (D) the ratio of *Bacteroidetes* to *Firmicutes* ( $r = -.3482$ ,  $p = .035$ ). ( $n = 7-12$ /group) \* $p < .05$ .

Cell type	Ctr_Veh	Ctr_Flx	GS_Veh	GS_Flx
DCX-ir total counts				
Dorsal	794.14 $\pm$ 108.65	614.29 $\pm$ 57.42	771.00 $\pm$ 81.54	692.77 $\pm$ 72.02
Ventral	887.71 $\pm$ 89.78	983.71 $\pm$ 94.66	817.73 $\pm$ 60.81	850.31 $\pm$ 49.50
Iba1-ir density				
Dorsal	0.0143 $\pm$ 0.0038	0.0222 $\pm$ 0.0050	0.0331 $\pm$ 0.0048	0.0295 $\pm$ 0.0073
Ventral	0.0284 $\pm$ 0.0087	0.0505 $\pm$ 0.0118	0.0513 $\pm$ 0.0090	0.0533 $\pm$ 0.0092

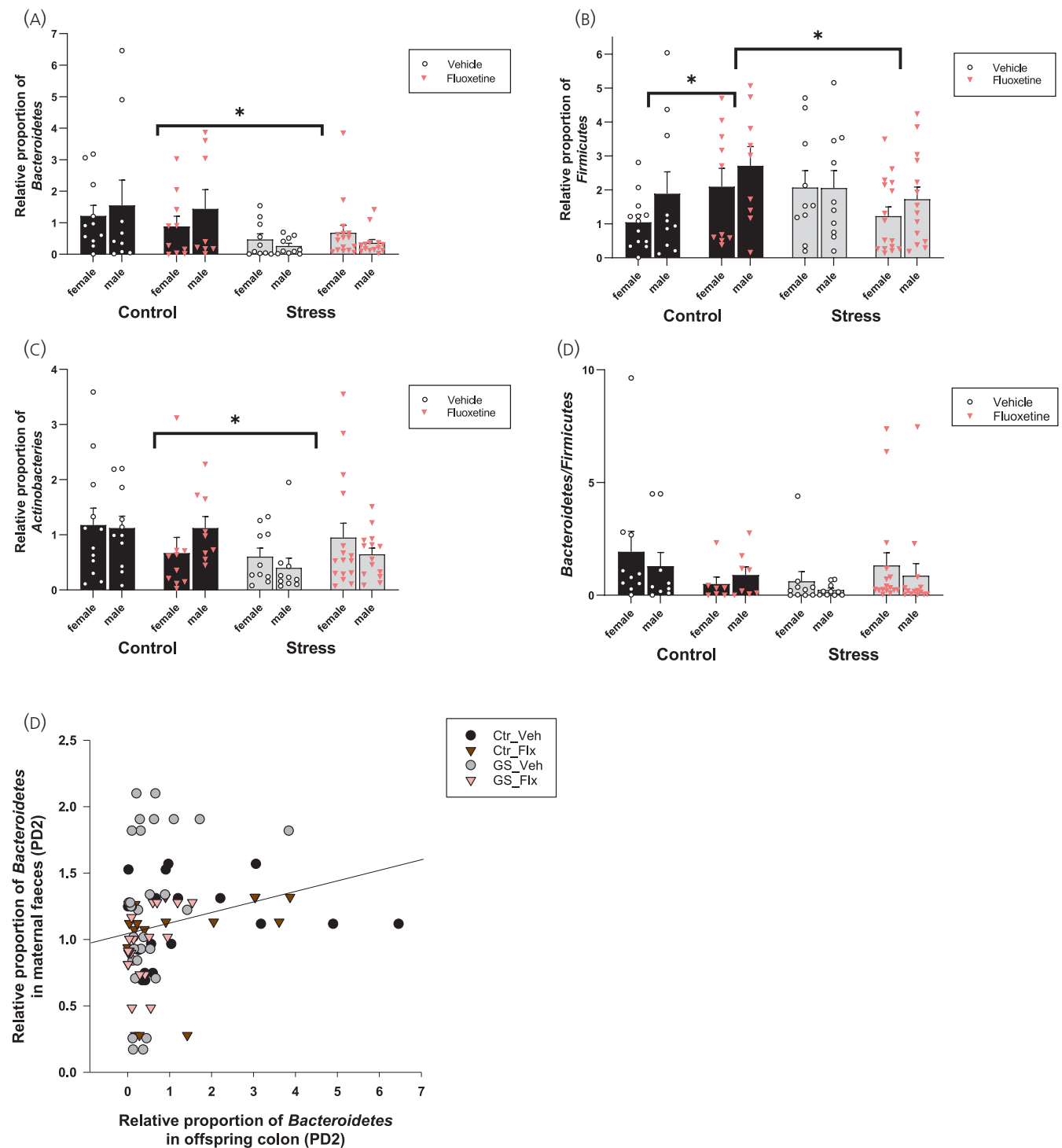
**TABLE 1** Mean ( $\pm$  SEM) for DCX-ir cell counts and Iba1-ir optical density (OD) in the dentate gyrus of the dam ( $n = 7-13$  per group; 3-way ANOVA).

### 3.10 | Offspring brain Iba1 content is decreased by gestational stress and increased by fluoxetine exposure

In the neonatal hindbrain, there was a significant decrease in Iba1 density induced by gestational stress (GS\_Veh and GS\_Flx) (main effect:  $F[1, 73] = 5.2981$ ,  $p = .02420$ ; Figure 6A) and a significant increase in Iba1 density in offspring exposed to fluoxetine (Ctr\_Flx and GS\_Flx) (main effect:  $F[1, 73] = 9.6123$ ,  $p = .00275$ ; Figure 6B).

### 3.11 | Offspring brain immature neuron content is negatively correlated with offspring gut microbiota on PD2

There was a significant negative correlation between offspring colon *Bacteroidetes* content and DCX/actin in the forebrain ( $r = -.2877$ ,  $p = .014$ ; Figure 6C). The main findings are summarized in Table S2.

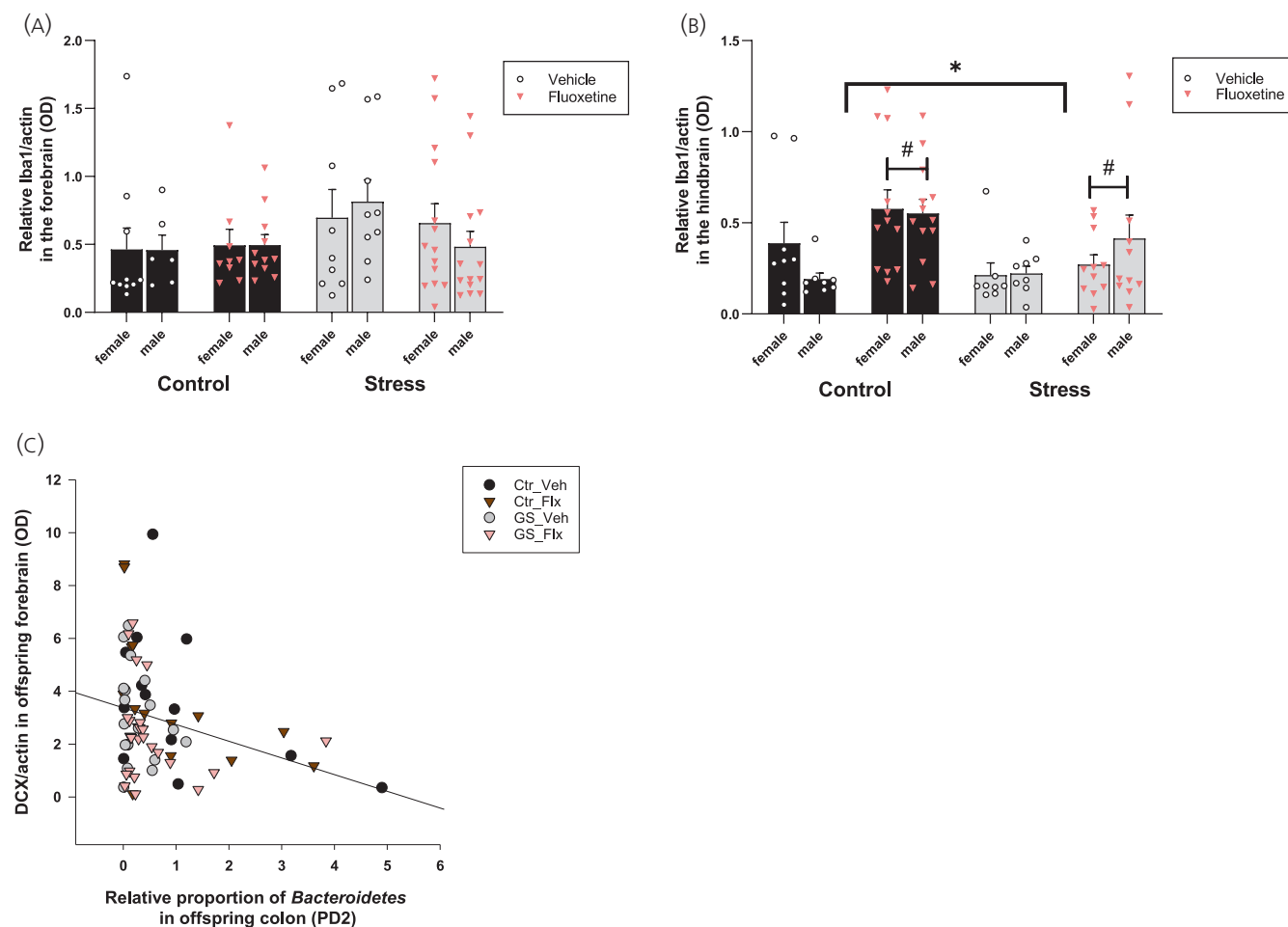


**FIGURE 5** Mean ( $\pm$ SEM) relative proportion of (A) *Bacteroidetes*, (B) *Firmicutes* and (C) *Actinobacteria* and (D) *Bacteroidetes/Firmicutes* in the colon of offspring on postnatal day 2. There was a significant main effect of gestational stress on (A) the relative proportion of *Bacteroidetes* ( $p = .00154$ ) and (C) the relative proportion of *Actinobacteria* ( $p = .02775$ ), regardless of offspring sex. (B) Ctr\_Flx neonatal offspring had increased content of *Firmicutes* compared to Ctr\_Veh offspring ( $p = .035$ ) and GS\_Flx offspring ( $p = .034$ ). (D) Ratio of *Bacteroidetes* to *Firmicutes*. Three-way ANOVA. (E) There was a significant positive correlation between *Bacteroidetes* content in offspring colon and in maternal feces on PD2 ( $r = .2277$ ,  $p = .035$ ). ( $n = 8$ – $16$  offspring per sex per group) ( $n = 7$ – $12$  dams per group) \* $p < .05$ .

## 4 | DISCUSSION

The maternal and neonatal gut microbiome and brain undergo a number of dynamic changes during the perinatal period as a consequence

of stress.<sup>50,79</sup> The main findings from our study show that gestational stress and fluoxetine treatment can differentially impact the maternal microbiome-gut-brain axis. These effects may play a fundamental role in mediating plasticity in the maternal hippocampus and possibly



**FIGURE 6** Mean ( $\pm$ SEM) relative optical density (OD) of Iba1/actin in the neonatal (A) forebrain and (B) hindbrain. (B) In the neonatal hindbrain, there was a significant decrease in Iba1 density induced by gestational stress (GS\_Veh and GS\_Flx) ( $p = .02420$ ) and a significant increase in Iba1 density in offspring exposed to fluoxetine (Ctr\_Flx and GS\_Flx) ( $p = .00275$ ). Three-way ANOVA. (C) There was a significant negative correlation between offspring colon *Bacteroidetes* and DCX/actin optical density in offspring forebrain ( $r = -.2877$ ,  $p = .014$ ). ( $n = 8-14$  offspring per sex per group) \*Main effect of gestational stress. #Denotes main effect of fluoxetine,  $p < .05$ .

maternal mental health. In addition, we found effects of both gestational stress and fluoxetine exposure on neonatal gut microbiota and brain plasticity.

#### 4.1 | Gestational stress effects on the microbiome-gut-brain axis of the mother

Our work shows that the maternal gut microbiome is significantly impacted by gestational stress, fluoxetine and reproductive state (pregnant vs. postpartum) and can impact plasticity in the hippocampus of the mother. We found that gestational stress significantly alters the gut microbiome during late pregnancy with increases and decreases in abundance of a number of bacterial genera (Figure 3A,B). These findings are in line with previous research showing that gestational stress reduces the bacterial abundance in the feces of rat dams during late pregnancy<sup>46</sup> and shifts gut bacterial abundance during pregnancy in mice.<sup>80</sup> In women, high levels of psychosocial stress also

result in a shift in the abundance of fecal microbiota during pregnancy further demonstrating the impact of maternal mental health on gut microbial composition during reproduction.<sup>81</sup>

In addition to the effects of gestational stress on the maternal gut microbiome in late pregnancy, we found that the abundance of prominent gut microbiota in the rat dam was altered with the transition to motherhood such that there was a significant decrease in the proportion of *Bacteroidetes* and an increase in the proportion of *Firmicutes* in the maternal gut from late pregnancy to the early postpartum period. Given these findings, and the well known relationship between the microbiome and the brain, it is not surprising that the microbiome-gut-brain axis has begun to be a focus for intervention in perinatal mental health research.<sup>31</sup> In the current study we found that maternal gut microbiota abundance was associated with changes in the hippocampus of the dam such that the ratio of *Bacteroidetes* to *Firmicutes* was positively associated with glucocorticoid receptor density in the CA3 region of the maternal hippocampus. This suggests an important role between the maternal gut bacteria

and the HPA axis. In addition, we found that microglia density in the hippocampus increased with decreasing ratio of *Bacteroidetes* to *Firmicutes* and with the abundance of *Actinobacteria*. Previous work has not explored the relationship between the maternal gut microbiome and the maternal brain<sup>77,82,83</sup> but the associations reported here suggest a role of the gut microbiome on key factors in the maternal hippocampus that are important for stress regulation and neuroplasticity.<sup>84</sup>

#### 4.2 | Gestational stress decreases abundance of key bacteria in the neonatal colon

In neonatal offspring, at 2 days of age, we found a predominant effect of gestational stress in decreasing the abundance of *Bacteroidetes* and *Actinobacteria* in the colon, regardless of sex. Previous research has shown that gestational stress has enduring effects on the gut microbiota of male and female offspring later in life. Prenatally stressed adult male mouse offspring differ in abundance of commensal microbes, including decreases in *Bacteroides* and *Parabacteroides*, compared to nonstressed adult male offspring and prenatally stressed adult female mouse offspring show a decrease in the relative abundance of gut *Bacteroidetes* and *Firmicutes*.<sup>42</sup> In juvenile offspring (20 days of age) prenatal stress results in an increase in the abundance of *Bacteroidetes* and decreased *Firmicutes*,<sup>85</sup> particularly in juvenile female offspring. These findings show that not only are gestational stress effects on the offspring microbiome enduring through most of life, but that after the postnatal period, sex of the offspring is also important.

#### 4.3 | Gestational stress impacts microglia density in neonatal offspring

When looking at the neonatal brain, we found that gestational stress decreases the density of microglia on postnatal day 2. We know that microglia have receptors that respond to stress hormones in early development<sup>86,87</sup> and on embryonic day 14 prenatal restraint stress results in increased microglia density in the embryonic neocortical primordium of mice<sup>88</sup> while during the early postnatal period prenatal stress reduces the number of immature microglia in the corpus callosum and internal capsule, and promotes an accelerated microglial differentiation in brain regions such as the entorhinal cortex, parietal lobe neocortex, thalamus and septum in the neonatal rat.<sup>89</sup> In addition, the impact of stress on microbiota could also impact microglia via the release of various metabolites such as short-chain fatty acids (SCFAs) or aryl hydrocarbon receptor (AhR) ligands.<sup>90</sup> This research points to effects of gestational stress on microglial content and differentiation in the neonatal brain. Further investigation into the timing and duration of these gestational stress effects on the neonate throughout the perinatal period and beyond is needed.

#### 4.4 | SSRI effects on the microbiome-gut-brain axis of mother and neonate

SSRIs are prescribed to 3.0% of pregnant women globally and are often used as a first line of treatment to manage perinatal mental illness.<sup>1</sup> One of the main questions of the present study was how SSRIs may alter the effects of gestational stress on the microbiome-gut-brain axis and how these effects compare to the effects of gestational stress alone on both the mother and neonate. When looking at the maternal gut microbiome, we found that fluoxetine treatment during gestation prevented the stress-induced increase of *Bacteroidetes* abundance and prevented the stress-induced changes in bacterial taxa (*Bifidobacterium*, *Bacteroides*, *Erysipelotrichaceae* uncultured species) during late gestation suggesting a protective effect of fluoxetine against the effects of stress on certain microbes of the maternal microbiome. These findings are in line with previous research showing that dams exposed to early life stress and treated with fluoxetine during pregnancy have a decrease in the relative abundance of *Bacteroides*.<sup>50</sup> A recent study in male Sprague-Dawley rats shows that long-term treatment with fluoxetine after weeks of chronic unpredictable stress significantly enhanced *Bacteroidetes* and reduced *Firmicutes* relative abundance in the gut.<sup>34</sup> This is opposite of what has been reported in pregnant female rats. These differences may be due to the length of the treatment and stress exposure but also the significant impact that pregnancy can have on the gut microbiome,<sup>50,91,92</sup> as well as the impact of gonadal hormones on gut microbiota composition.<sup>93</sup>

In neonatal offspring we found minimal effects of SSRIs on measures of the microbiome-gut-brain axis. However, when looking at brain microglial content we found that fluoxetine exposure, regardless of gestational stress, increased the density of microglia in the neonatal brain. We know that perinatal SSRIs and stress have an enduring impact on offspring brain plasticity later in life with the majority of research focusing on neural and not glial plasticity.<sup>94-96</sup> Research has found that SSRI administration to stressed adult male mice increases the spacing and cell body areas of microglia and decreases the arborization of these cells<sup>97</sup> but further research is needed to understand how SSRIs can alter the effects of gestational stress on the developing brain.

#### 4.5 | SSRI effects in the absence of stress

It should be noted that treatment with SSRIs such as fluoxetine, in the absence of stress, often results in a different pattern of effects on the brain and gut as found in the present study and previous research.<sup>15</sup> SSRIs and stress affect a number of physiological systems<sup>97,98</sup> and, at times, in opposite directions or at least in significantly different ways (i.e., the 5-HT system<sup>99</sup>). Therefore, it is important that translational research use appropriate models of stress-related illnesses in order to determine the full impact of SSRI medications on mother and offspring.

## 4.6 | Limitations and future directions

The present study offers a basis for a number of future studies to further investigate the mechanisms and outcomes of the effects reported here. For example, future studies would benefit from understanding the link between the maternal microbiome-gut-brain axis and postpartum maternal affective state as well as maternal offspring-directed care. Understanding in more detail the link between the maternal and neonatal gut would be beneficial, particularly with regards to other forms of stress (i.e., pregestational) as well as other antidepressant medications commonly prescribed during the perinatal period (i.e., sertraline). One key finding in the present study was the impact of gestational stress on the density of microglia in the neonatal brain. Future studies detailing the changes in microglia content specific to different brain areas in the developing brain would aid to our understanding of how early life adversity affects these cells that are essential to brain function.

## 5 | CONCLUSIONS

There is a growing body of research investigating how gestational stress and SSRIs can affect offspring outcomes,<sup>26,57,95</sup> more recently pointing to the important impact of perinatal mental health on these outcomes.<sup>19</sup> We now know that untreated depression and stress-related disorders can have a more significant impact on the mother and offspring than perinatal SSRI medication.<sup>8,19</sup> The present study further demonstrated these findings and is the first study to begin to understand key components of the maternal-neonatal microbiome-gut-brain axis, potentially reflecting a nonplacental gestational stress transfer mechanism. Exploring the dyadic relationship of this axis in the mother and neonate in the context of perinatal mental illness and medication exposure will better elucidate the extent to which this biological system can be used as a biomarker and treatment target to improve parent and offspring development.

### AUTHOR CONTRIBUTIONS

**Jodi Pawluski:** Conceptualization; data curation; funding acquisition; methodology; project administration; supervision; writing – original draft; writing – review and editing. **Pauline Murail:** Data curation; investigation; methodology; writing – original draft; writing – review and editing. **Florine Grudet:** Data curation; investigation; methodology; writing – original draft. **Lena Bys:** Data curation; investigation; writing – original draft. **Anna Golubeva:** Data curation; methodology. **Thomaz Bastiaanssen:** Data curation; investigation. **Tim Oberlander:** Investigation; methodology; writing – review and editing. **John F Cryan:** Methodology; resources; supervision; visualization. **Siobhain M. O'Mahony:** Methodology; supervision; validation; writing – review and editing. **Thierry Charlier:** Conceptualization; data curation; methodology; writing – review and editing.

### FUNDING INFORMATION

J. L. Pawluski received funding from the Institut de Neurosciences Clinique de Rennes and Bretagne Atlantique Ambition. APC

Microbiome Ireland is supported by Science Foundation Ireland (SFI), SFI/12/RC/2273.

### CONFLICT OF INTEREST STATEMENT

The authors declare no conflicts of interest.

### PEER REVIEW

The peer review history for this article is available at <https://www.webofscience.com/api/gateway/wos/peer-review/10.1111/jne.13261>.

### DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available on request from the corresponding author. The data are not publicly available due to privacy or ethical restrictions.

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### SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

**How to cite this article:** Pawluski JL, Murail P, Grudet F, et al. Gestational stress and perinatal SSRIs differentially impact the maternal and neonatal microbiome-gut-brain axis. *J Neuroendocrinol.* 2023;e13261. doi:[10.1111/jne.13261](https://doi.org/10.1111/jne.13261)