Neurobiology of Stress 15 (2021) 100333

Contents lists available at ScienceDirect

Neurobiology of Stress

journal homepage: www.elsevier.com/locate/ynstr

Prenatal stress leads to deficits in brain development, mood related behaviors and gut microbiota in offspring

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ARTICLE INFO

Keywords:
Prenatal stress
Cerebral cortex
Neurogenesis
Mood related disorders
Gut microbiota

ABSTRACT

Early exposure to stressful and adverse life events at fetal and neonatal stages is one of crucial risk factors for mood disorders such as anxiety and depressive disorder in adulthood. Intergenerational effects of prenatal stress on offspring are still not fully understood. We here uncover a significant negative impact of prenatal stress on brain development in embryos and newborns, and on mood-related behaviors and gut microbiota in adult offspring. Prenatal stress leads to reduced numbers in neural progenitors and newborn neurons, and altered gene expression profiles in the mouse embryonic cerebral cortex. Adult mouse offspring exposed to prenatal stress displays altered gene expression in the cortex and elevated responses in anxiety- and depression-like behaviors. Interestingly, prenatal stress has an enduring effect on gut microbiota, as specific microbial community structure is altered in adult F1 offspring treated with prenatal stress, compared to that of the control. Our results highlight the essential impact of prenatal stress on cortical neurogenesis, gene expression patterns, mood-related behaviors, and even gut microbiota in the next generation.

1. Introduction

Mental health is becoming a worldwide issue and affects many people at different life situations, including pregnant women (Biaggi et al., 2016; Wainberg et al., 2017). Studies have shown that prenatal stress and early life psychological stress affect infant development and health across the lifespan, which in turn results in a high risk for anxiety, depression, and even alcohol addiction and schizophrenia in offspring (Campbell et al., 2009; Rice et al., 2007b; Tegethoff et al., 2011). Rodent models with exposure to stress during pregnancy, in particular postnatal periods also display elevated levels of anxiety, depression and emotional deficits (Bronson and Bale, 2014; Franklin et al., 2010; Jiao et al., 2018).

The timing of prenatal stress exposure is a critical factor that affects fetal development, in particular the brain (Charil et al., 2010; Class et al., 2011; Weinstock, 2017). In the mouse cerebral cortex, neural progenitors are actively expanded in the ventricular zone (VZ) and subventricular zone (SVZ) at early embryonic stages, and newborn neurons differentiate and migrate into the cortical plate (CP) and form a six-layered structure subsequently (Phillips et al., 2003; Shim et al., 2012). Prenatal stress, maternal separation and breastfeeding in animal models have shown significant impacts on altering development of specific brain regions, and causing anxiety- and depression-like behaviors in postnatal and adult offspring (Anderson et al., 1985; Franklin et al., 2010; Kraszpulski et al., 2006; Lemaire et al., 2000; Pope and Mazmanian, 2016; Wei et al., 2010). However, the molecular associations of prenatal stress, cortical development and mood disorders in offspring remain underexplored.

Furthermore, intriguing studies have shown significant connections of gut microbiota and neurological functions such as regulations in brain development, mental health and immunity (Belkaid and Harrison, 2017; Gur et al., 2017; Valles-Colomer et al., 2019; Zheng et al., 2016). Microbes associated with neurotransmitter production are perturbed in patients with mental disorders (Terry and Margolis, 2017). Low representation of *Prevotella* genus is found in autistic patients, and overexpression of *Bacteroidales* and low representation of the family *Lachnospiraceae* are shown in depressive patients (Kang et al., 2013; Naseribafrouei et al., 2014). Mice raised in pathogen-free environment or treated with antibiotics display alterations in neurogenesis or

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https://doi.org/10.1016/j.ynstr.2021.100333
Received 30 December 2020; Received in revised form 27 April 2021; Accepted 27 April 2021
Available online 3 May 2021

dendrite morphology in hippocampus and behavioral responses, suggesting that normal gut microbiota is essential for brain development and normal behaviors (Heijtz et al., 2011; Luczynski et al., 2016; Luo et al., 2018; Zheng et al., 2016). Comparative analyses of metabolic pathways between the brain and gut will help uncover the underlining mechanisms of gut microbiota and behaviors.

In this study, we investigate the effect of prenatal stress on brain development, gene expression pattern and gut microbiota in offspring. We show that prenatal stress leads to abnormal early cortical neurogenesis, and altered gene expression profiles in both embryonic and adult mouse cortices. Moreover, prenatal stress results in anxiety- and depression-like behaviors in adult F1 offspring. Interestingly, specific microbiota colonization and distinct diversity are detected in fecal samples of adult F1 mice in control and stress-induced groups. Our results demonstrate that prenatal stress has an instant effect on cortical neurogenesis, and an enduring impact on behaviors and gut microbiota in offspring.

2. Materials and methods

2.1. Animal experiments

8 weeks-old naïve female C57BL/6 mice were housed at 22 ± 2 °C with humidity of 50 ± 10% and at a 12 h light/12 h dark cycle. After timed mating, pregnant females were randomly assigned to either the control or stress-induced group, n = 30 mice for each group. In either the control or stress-induced group, for brain tissue collection at embryonic day 13.5 (E13.5) and E15.5, at least 7 litters (2 embryos from one litter) were collected. For brain tissue collection at postnatal day 0 (P0), at least 7 litters at the stage and 1 pup from each litter were collected. For brain tissue collection at P14 and adult, at least 7 litters at each stage and 2 pups from each litter were collected (totally 7 female and 7 male pups were tested). For behavioral tests, at least 12 litters and 1–3 female or male pups from each litter were randomly chosen (totally 12–24 female and 12–24 male pups were tested). For fecal sample collection and 16S rDNA sequencing, at least 4 litters and 3 female pups from each litter were used (totally 12 female samples were tested).

The animal protocol was reviewed and approved by the Animal Care Committee of Shanghai Jiao Tong University.

2.2. Prenatal stress

Prenatal stress was conducted right after timed mating. Briefly, control pregnant females were removed from the housing cage and handled for 5 min per day, while stress-induced pregnant females were subjected to immobilization stress in a 50 ml conical centrifuge tube for 2 h per day from 19:00 to 21:00 o'clock in a day from E0.5 to E19.5/P0 before pup delivery. For females after 13.5 days pregnancy, the 50 ml conical centrifuge tube was replaced with an 80 ml conical centrifuge tube.

To eliminate potential effects of stressed mothers on pups at the postnatal stage, all the dams were cross-fostered immediately after delivery without further stress induction. Pups from both control and stress-induced groups were cross-fostered by naïve C57BL/6 mothers. To avoid infanticide drive after delivery (P0) by stressed mothers and by cross-fostered naïve mothers, we inhabited the cross-fostered naïve mothers (1–5 days after delivery) with control and stress-induced pregnant females in one cage 2–3 days before their delivery.

2.3. Tissue preparation and immunohistochemistry

Mouse brains were rapidly dissected in cold phosphate-buffered saline (PBS) from mice after euthanasia by cervical dislocation under isoflurane inhalation anesthesia. For RNA extraction, brain tissues were homogenized and stored in Trizol at −80 °C. For brain sectioning, tissues were fixed in 4% paraformaldehyde (PFA) in PBS overnight, incubated in 30% sucrose in PBS, embedded in OCT and then stored at −80 °C until use.

Brain tissues were sectioned and collected using a cryostat. For BrdU labeling in embryos, 50 mg/kg of BrdU (Sigma) was injected into pregnant females 30 min before sacrifice (Chenn and Walsh, 2002; Jin et al., 2016; Pollock et al., 2014).

Coronal sections were collected from the medial cortical region at levels between the anterior commissure and the anterior hippocampus of a brain. All analyzed sections were selected from a similar medial point on the anterior-posterior axis in the brain among groups. At least four sections from each brain and 5–6 brains from different litters were chosen for antibody labeling. 14 μm serial coronal sections were taken from E13.5 and E15.5 brains, and 40 μm serial coronal sections were taken from postnatal brains. Immunohistochemistry was performed on sections by using the following antibodies: mouse anti-BrdU (G3G4, 1:200, DSHB), mouse anti-Sox2 (sc-365823, 1:100, Santa Cruz Biotechnology), rabbit anti-Pax6 (PRB-278P, 1:200, BioLegend), rabbit anti-ki67 (AB9260, 1:100, Millipore), rabbit anti-Tbr1 (ab31940, 1:200, Abcam), rabbit anti-Tbr2 (ab23345, 1:200, Abcam), rabbit anti-Satb2 (ab34735, 1:200, Abcam), mouse anti-NeuN (1:150, Millipore), Caspase3 (1:100, Abcam) and DAPI (D9542, 1:1000, Sigma). For antibody co-staining, BrdU/Tbr2, Sox2/Tbr2, BrdU/Sox2 were co-stained as combinations.

Briefly, frozen sections were air dried, and fixed in 4% PFA for 15 min. Then antigen retrieval was performed with Tris-EDTA (1 mM EDTA, 5 mM Tris, pH = 8.0). 10% goat serum in 1xPBS with 0.1% Triton-X was used to block for 1 h, and then the primary antibody was added in 10% goat serum in 1xPBS with 1% Triton-X. Sections were incubated at 4 °C overnight. After washing with PBS, sections were incubated with the secondary antibodies such as Alexa Flour 488 (103-545-155, 1:300, Jackson ImmunoResearch), Cy3 (711-165-152, 1:300, Jackson ImmunoResearch), 647 (115-605-003, 1:300, Jackson ImmunoResearch) for 1 h. DAPI staining was carried out for 10 min. Images of brain sections were captured under a Leica & TCS SP8 confocal microscope with a 20× or 40× objective through three channels: 488 nm, 594 nm, 647 nm, and UV as separated files, and merged later on for analyses.

2.4. Cell number quantification in brain sections

At least 4–6 sections from each brain, and 3–4 brains from different littermates were chosen for antibody labelling. For each brain, positive cells from at least 4–6 sections were counted and quantified. For cell quantification in brain sections, positive cells were quantified in width of 100 μm for E13.5 cortices, 200 μm for E15.5, and width of 400 μm for P1 cortices in a view. Cell counting was performed in minimal three chosen areas in each brain section, and at least 5–6 individual brains were analyzed in each group. Cell counting in each chosen area in a brain section was repeated at least three times and a mean was obtained.

2.5. Fecal sample collection

Fecal samples were collected from F1 adult female mice at 6-8 weeks-old, in total, 12 mice for each group (n = 3 mice from each litter and 4 different litters), and transferred to liquid nitrogen immediately. Fecal samples were then stored at −80 °C until use. 12 mice from each control and prenatal stress-induced group were analyzed, and at least 4 pieces of fecal samples were collected from each mouse.

2.6. Mouse behavioral tests

Investigators were blinded to control and stress-induced mice in individual experiments. Female and male mice at 8-12 weeks-old were subjected to behavioral tests in the following order: open field test, elevated plus maze test, light-dark transition test, sucrose preference test and forced swim test. Behavioral tests were performed from 19:00 to 24:00 o'clock in a day. We used a lighting of 60 lux, which was

supported by 4 bulbs of 60 W and placed at 1 m distance in the animal facility. After each test, all equipment was cleaned using 70% ethanol and left to air dry. Mice were given 2–3 days interval between each test. All videos were recorded and analyzed by using the Anymaze tracking system.

2.6.1. Open field test

Mice were placed in the center of Plexiglas box (35 cm × 35 cm × 35 cm). During the 10 min trial, animals were recorded for the distance travelled.

2.6.2. Elevated plus maze test

Mice were placed on a four-arms plus maze made of two open and two closed arms (gray PVC, 40 cm × 40 cm). The maze was raised 50 cm above the ground. The time spent in the open and closed arms were recorded by the Anymaze video tracking system over a 10 min trial.

2.6.3. Light-dark transition test

Light-dark transition box consists of two Plexiglas chambers of equal size (45 cm × 45 cm): one black and one transparent. Mice were placed in the box, the time spent in the light and dark zone was recorded and analyzed over a 10 min trial.

2.6.4. Forced swim test

Mice were individually placed in Plexiglas cylinders (20 cm diameter, 50 cm height) containing water of 20 cm height (23 ± 1 °C) and videotaped for 10 min. Immobility behaviors were recorded in each 10 min trial. After the test, mice were dried and placed in a cage surrounded by a heating pad. Water was changed between each animal.

2.6.5. Sucrose preference test

Mice were first habituated to 1% sucrose and plain water for 72 h, during which the positions of the sucrose or water bottles were switched daily. Then after 6 h of deprivation of water from 00:00 to 6:00 a.m., mice were exposed to 1% sucrose and plain water with two identical bottles. Total consumption of each fluid was measured after 2 h. Sucrose preference was defined as the ratio of the volume of sucrose versus total liquid intake.

2.7. RNA-sequencing (RNA-seq) and bioinformatic analyses

Total RNA was isolated from the dorsal cortex of E15.5 embryos and 8 weeks-old adult female mice by using Trizol (Ambion) according to the manufacturer's instructions. RNA was stored at −80 °C until use. Total RNA samples were quantified using a NanoDrop ND-1000 instrument. 2–3 μg of total RNA was enriched by oligo (dT) magnetic beads (rRNA removed), and RNA library was prepared using a KAPA stranded RNA-seq library Prep Kit (Illumina), and sequenced using the Illumina Hiseq 4000 instrument according to the manufacturer's protocol. Differentially expressed genes (DEGs) with a threshold FDR adjusted and DEGs with a $p < 0.05$ cutoff for FDR was selected for further RNA-seq analysis.

Moreover, we used David for Gene ontology analysis (https://david.ncifcrf.gov/summary.jsp) with Benjamini-Hochberg correction to estimate the statistical significance of such enrichment of terms between the two groups. p value ≤ 0.05 was recommended for filtering statistically significant GO terms.

2.8. Quantitative reverse transcription PCR (qRT-PCR)

Reverse Transcription were performed using PrimerScript RT reagent Kit with gDNA eraser (Takara). The qRT-PCR was performed using SYBR green qPCR kit (Takara) according to the protocol. The relative expression of the results was shown by using the 2-ΔΔC (t) method, and the relative expression were normalized by housekeeping gene Actin and shown as fold change. The specific primers were listed (Table S1).

2.9. Fecal sample DNA extraction

Total genome DNA from fecal samples was extracted using the Soil DNA Kit according to manufacturer's protocols. DNA concentration was quantitated by Qubit3.0 fluorometer.

2.10. Amplicon generation and library preparation

20–30 ng of DNA was used to generate amplicons. V3 and V4 hypervariable regions of prokaryotic 16S rDNA were selected for generating amplicons following taxonomy analysis. A panel of proprietary primers aimed at relatively conserved regions bordering the V3 and V4 hypervariable regions of bacteria and Archaea 16S rDNA were used. The V3 and V4 regions were amplified using forward primers containing the sequence of "CCTACGGRRBGCASCAGKVRVGAAT" and reverse primers containing the sequence of "GGACTACNVGGGTWTCTAATCC". At the same time, indexed adapters were added to the ends of the 16S rDNA amplicons to generate indexed libraries ready for sequencing. PCR reactions were performed in triplicates of 25 μL mixture containing 2.5 μL of TransStart Buffer, 2 μL of dNTPs, 1 μL of each primer, and 20 ng of template DNA.

2.11. 16S rDNA sequencing

Concentration of DNA libraries was validated using the Qubit3.0 Fluorometer. DNA libraries were quantified to 10 nM and multiplexed and loaded on an Illumina MiSeq instrument according to manufacturer's instructions (Illumina, San Diego, CA, USA). Sequencing was performed using paired-end; and image analysis and base calling were conducted by the Control Software in the instrument.

2.12. 16S rDNA sequencing data analyses

Paired-end sequencing of positive and negative reads were joined together by QIIME 2. Sequences were filtered with a quality score >30 with Cutadapt 1.9.6, followed by discarding sequences containing the bases ('N'). Sequences with length larger than 200 bp were retained. After filtering chimeric sequences, all remaining sequences were clustered into Amplicon sequence variant with a 97% sequence similarity through VSEARCH clustering 1.9.6 (Gloor et al., 2017; Rognes et al., 2016). ASVs were mapped against the 16S rRNA reference database Silva 132 (Quast et al., 2013). Then we used the Ribosomal Database Program (RDP) classifier 2.2 for ASV taxonomy classification (Wang et al., 2007). Shannon, Chao1, and ACE were used to calculate Alpha-diversity (Morris et al., 2014). Meanwhile, Beta-diversity analysis based on unweighted UniFrac metric were used to visualize the dissimilarity between control and stress-induced groups using four parameters: Nonmetric multidimensional scaling (NMDS), principal component analysis (PCA), Principal Coordinates Analysis (PCoA), and Analysis of similarities (Anosim) (Baselga and Orme, 2012). At deeper taxonomic levels (from phylum to genus level), we performed linear discriminant analysis (LDA) effect size (LEfSe) method based on PICRUSt for biomarker discovery, with a $p < 0.05$ and LDA score > 2.0 (Segata et al., 2011).

2.13. Statistical analyses

Statistical significance was analyzed between groups by Statistical Package for the Social Sciences (SPSS). The normality of the data was analyzed by Shapiro-Wilk test, followed by Levene test to determine if variances were homogeneous. Behavioral results were analyzed by performing a model of two-way ANOVA, with the stress and sex as independent variables. Cortical cell counting and qRT-PCR were analyzed by Student's t-test. All data are presented as mean ± s.e.m. Statistical significance was defined as * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

3. Results

3.1. Prenatal stress affects proliferation of embryonic neural progenitors in the cortex

Previous studies have shown significant effects of stress on brain development and functions (Bale, 2015; Gandal et al., 2018; Snyder et al., 2011). To investigate impacts of prenatal stress on brain development such as neurogenesis, and behavioral conditions in offspring, we conducted restraint stress on timely mated naïve female C57BL/6 mice (Fig. S1A). Pregnant mice, termed as F0, were randomly divided into two groups. The stress group was exposed to restraint stress 2 h per day for 13, 15 and 19 days to induce mild and enduring prenatal stress, and the control group was removed from cages 5 min per day for 13, 15 and 19 days.

To determine whether prenatal stress disturbs proliferation of cortical neural progenitors, we collected embryos from control and stress-induced pregnant mice at embryonic day 13.5 (E13.5) (Fig. S1A). To analyze development of neural progenitors in the cortex, we first applied a 30 min BrdU pulse to label proliferative cells in the S phase, and used anti-Ki67 antibodies to label cells in all active phases in a cell cycle. We detected significantly decreased percentages of $BrdU^+$ and $Ki67^+$ cells in E13.5 stress-induced cortices, compared to those in the control (Fig. S2A-D). The reduction of $BrdU^+$ and $Ki67^+$ cells was not due to alterations of total cell numbers, as the number of $DAPI^+$ cells was compatible between control and stress-induced cortices (Fig. S2I). We next quantified proportions of $Sox2^+$ radial glial cells (RGCs) ($Sox2^+$ versus $DAPI^+$ cells), and $Tbr2^+$ intermediate progenitors (IPs) ($Tbr2^+$ versus $DAPI^+$ cells) in E13.5 (Fig. S2E and F). While the proportion of $Tbr2^+$ IPs was significantly decreased in the stress-induced cortex, the proportion of $Sox2^+$ RGCs was unchanged (Fig. S2G and H). In addition, apoptotic cells labeled by anti-Caspase3 antibodies also didn't show difference between control and stress-induced cortices (Fig. S2J and K). These results indicate that prenatal stress causes an early reduction of neural progenitors, in particular IPs, in the embryonic cortex.

Moreover, similar to E13.5 cortices, we detected a reduction of percentages of $BrdU^+$ and $Ki67^+$ cells in E15.5 stress-induced cortices, compared to those in the control (Fig. 1A, B, E and F). We observed reduced proportion of $Tbr2^+$ IPs, and didn't detect obvious changes in the proportion of $Sox2^+$ RGCs (Fig. 1 C, D, G, and H). In addition, we tested a transition from RGCs to IPs by co-labeling Sox2 and Tbr2 antibodies, and found a reduced number of $Sox2^-/Tbr2^+$ cells, and unchanged number of $Sox2^+/Tbr2^+$ cells, indicating that prenatal stress does not affect the transition from RGCs to IPs (Fig. 1I, L and M). Moreover, we examined proliferation status of RGCs and IPs by co-labelling BrdU with Sox2 and Tbr2, respectively. While the number of $Sox2^+/BrdU^+$ cells did not change, the number of $Tbr2^+/BrdU^+$ cells was significantly reduced in stress-induced cortex, compared to their controls (Fig. 1J, K, N, and O). These data suggest a reduced proliferation of IPs in the stress induced embryonic cortex.

3.2. Prenatal stress causes reduced neuronal production in embryonic and newborn cortices

As proliferative neural progenitors were reduced in stress-induced embryonic cortices, we next examined whether neuronal production also was affected. We used anti-Tbr1 and anti-Satb2 antibodies to label newborn neurons residing in the deep layers and upper layers, respectively. We found that percentages of $Tbr1^+$ and $Satb2^+$ ($Tbr1^+$, $Satb2^+$ versus $DAPI^+$) cells are significantly reduced in E15.5 cortices of stress-induced mice, compared to controls (Fig. 2A and B). However, apoptotic cells labeled by anti-Caspase3 antibodies didn't show difference between control and stress-induced cortices, suggesting that reduced neurons are not due to apoptosis (Fig. 2A and B). Moreover, after 19 days prenatal stress treatment to the pregnant females, we examined enduring effects of prenatal stress on neonatal neuronal production by analyzing the cortex of postnatal day 0 (P0) newborn mice. Numbers of $Tbr1^+$, $Satb2^+$, and $NeuN^+$ neurons also were reduced in the P0 cortex of stress-induced mice (Fig. 2C and D). These results suggest that prenatal stress causes a reduction of early born neurons, and these effects persist into neonatal periods.

Moreover, we analyzed neuronal production in cortices of P14 and 8 weeks old female adult mice. Interestingly, numbers of $Tbr1^+$, $Satb2^+$ and $NeuN^+$ cells were compatible in cortices of control and stress-induced mice (Fig. 2E and F, and Fig. S3). These results indicate that while prenatal stress causes reduced neuronal production in embryonic and newborn brains, there is a recovery in the number of neurons in postnatal and adult brains.

3.3. Prenatal stress leads to anxiety- and depression-like behaviors in adult F1 offspring

Previous studies have shown that prenatal stress has an impact on behaviors and emotional outcomes in offspring (Campbell et al., 2009; Rice et al., 2007a). We thus bred control and stress-induced offspring into adulthood (8–10 weeks old), termed as F1 offspring, and performed anxiety- and depression-like behavioral tests (Fig. S1B).

We first conducted an open field test, and did not detect significant changes in total distances traveled between control and stress-induced F1 offspring by two-way between subjects (sex × stress) ANOVA ($(F_{(1,67)} = 0.095, p = 0.759)$) (Fig. 3A). For anxiety-like behaviors, we performed an elevated plus maze test, and found that both stress-induced female and male offspring show a significantly increased trend to stay in the closed arm ($F_{(1,72)} = 24.842, p < 0.0001$), and significant latency to stay away from the open arm compared to the control offspring ($F_{(1,73)} = 11.797, p < 0.001$) (Fig. 3B). In a light-dark box test, stress-induced F1 offspring spent more time in the dark area, and showed a greater tendency to avoid the bright area than the control ($F_{(1,52)} = 17.97, p < 0.001$) (Fig. 3C). These results suggest that prenatal stress leads to elevated anxiety-like behaviors in F1 offspring.

For depression-like behaviors, we first performed a sucrose preference test to assess anhedonia-like effect. While stress-induced F1 females showed significantly reduced preference for sucrose compared to control female offspring, F1 males displayed no significant preference between two groups (Fig. 3D). In a forced swim test, both stress-induced F1 females and males displayed decreased mobility, indicating elevated depression compared to the control group ($F_{(1,50)} = 18.457, p < 0.001$) (Fig. 3E). These data indicate that prenatal stress causes persisting effects on anxiety- and depression-like behaviors in offspring.

3.4. Altered cortical transcriptome in stress-induced embryonic and adult F1 offspring

Because prenatal stress-induced mice showed neurogenesis defects in the developing cortex, we next examined whether gene expression profiles are altered by performing RNA sequencing (RNA-seq) analyses in F1 mice. Total RNA was extracted from E15.5 cortices and adult cortices of female control and prenatal stress-induced mice, and overall changes in RNA profiles were detected in both E15.5 and adult cortices. We identified 401 up-regulated and 521 down-regulated genes, and 480 up-regulated and 967 down-regulated genes in E15.5 and adult stress-induced cortices, respectively, compared to control ones (FPKM $\geq$ 0.5 mean in each group, cutoff: $p < 0.05$), indicating an early and ongoing effects of prenatal stress on cortical gene expression (Fig. 4A–D).

Previous studies have demonstrated a repertoire of RNA transcripts in neural progenitors and distinct neuronal subtypes (Ayoub et al., 2011). We thus aligned differentially expressed genes in stress-induced E15.5 cortices with known genes that are specifically expressed in the developing cortex (Table S2). We found that 58 genes such as *Fzd9*, *Hap 1* and *Gprc5b*, and 117 genes such as *Grin 1*, *Fzd9*, *Nrxn1, Nef 1* and *NPY*, which are normally expressed in the ventricular zone (VZ) and cortical plate (CP), respectively, displayed significant differential expression in

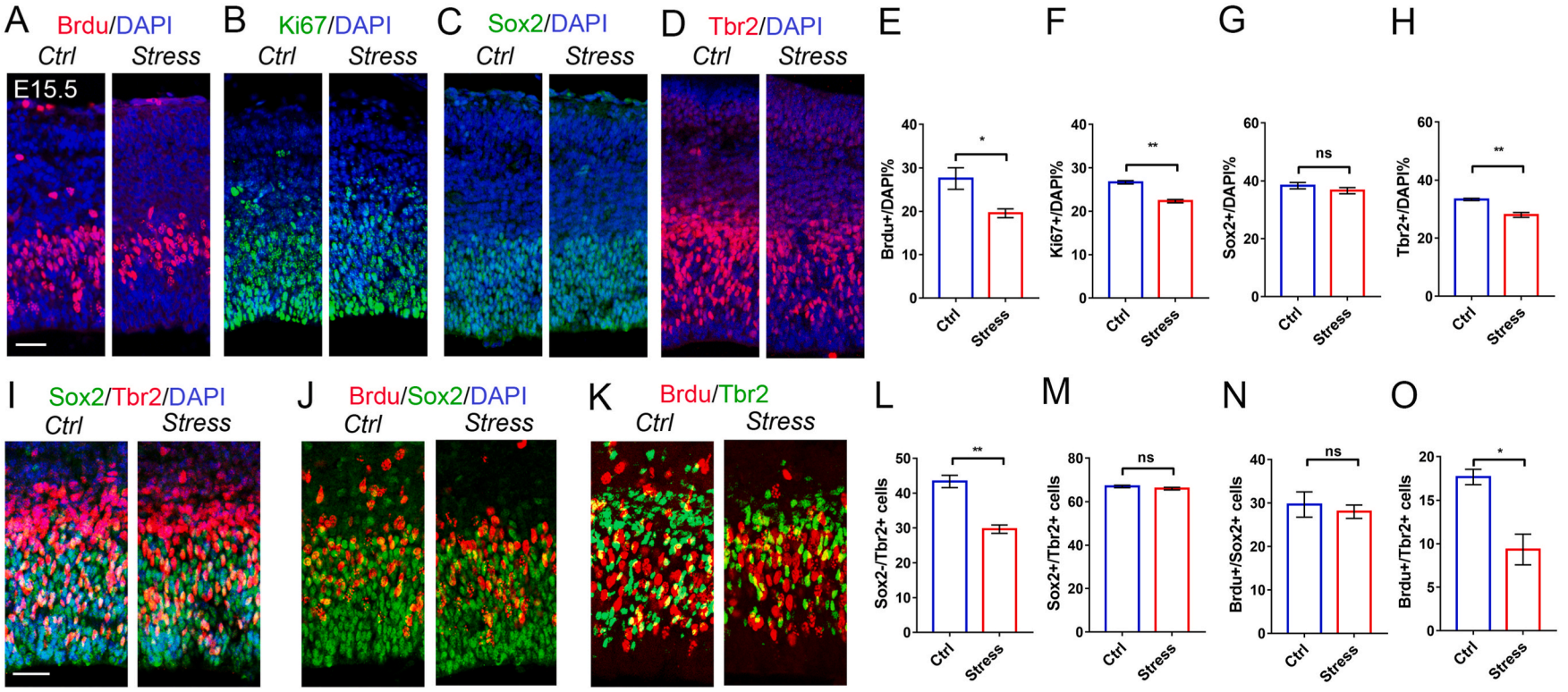

Fig. 1. Prenatal stress affects proliferation of embryonic neural progenitors in the cortex. **(A–D)** Representative images of BrdU (red), Ki67 (green), Sox2 (green), and Tbr2 (red) expressing cells in the E15.5 cortex of control (*Ctrl*) and prenatal stress-induced (*Stress*) embryos. DAPI was used to label the nucleus. **(E, F)** Percentages of $BrdU^+$ and $Ki67^+$ cells were significantly reduced in prenatal stress-induced E15.5 cortices. **(G)** The proportion of $Sox2^+$ neural progenitor cells was unchanged in the stress-induced cortex. **(H)** The percentage of $Tbr2^+$ intermediate progenitors was decreased in the stress-induced cortex. **(I–K)** Representative images of Sox2 (green), Tbr2 (red), and BrdU (red) expressing cells in the E15.5 cortex of *Ctrl* and *Stress* embryos. **(L, M)** Numbers of $Sox2^-/Tbr2^+$ cells were reduced, and numbers of $Sox2^+/Tbr2^+$ were not changed in the stress-induced cortex. **(N, O)** Numbers of $BrdU^+/Sox2^+$ cells were not altered, and numbers of $BrdU^+/Tbr2^+$ were reduced in the stress-induced cortex. Data are presented as means ± s.e.m, Student's t-test was used, n = 4–6 brains for each group, and 4–6 sections in each brain. *: $p < 0.05$, **: $p < 0.01$, ***: $p < 0.001$, ns: none significance. Scale bar = 100 μm. . (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

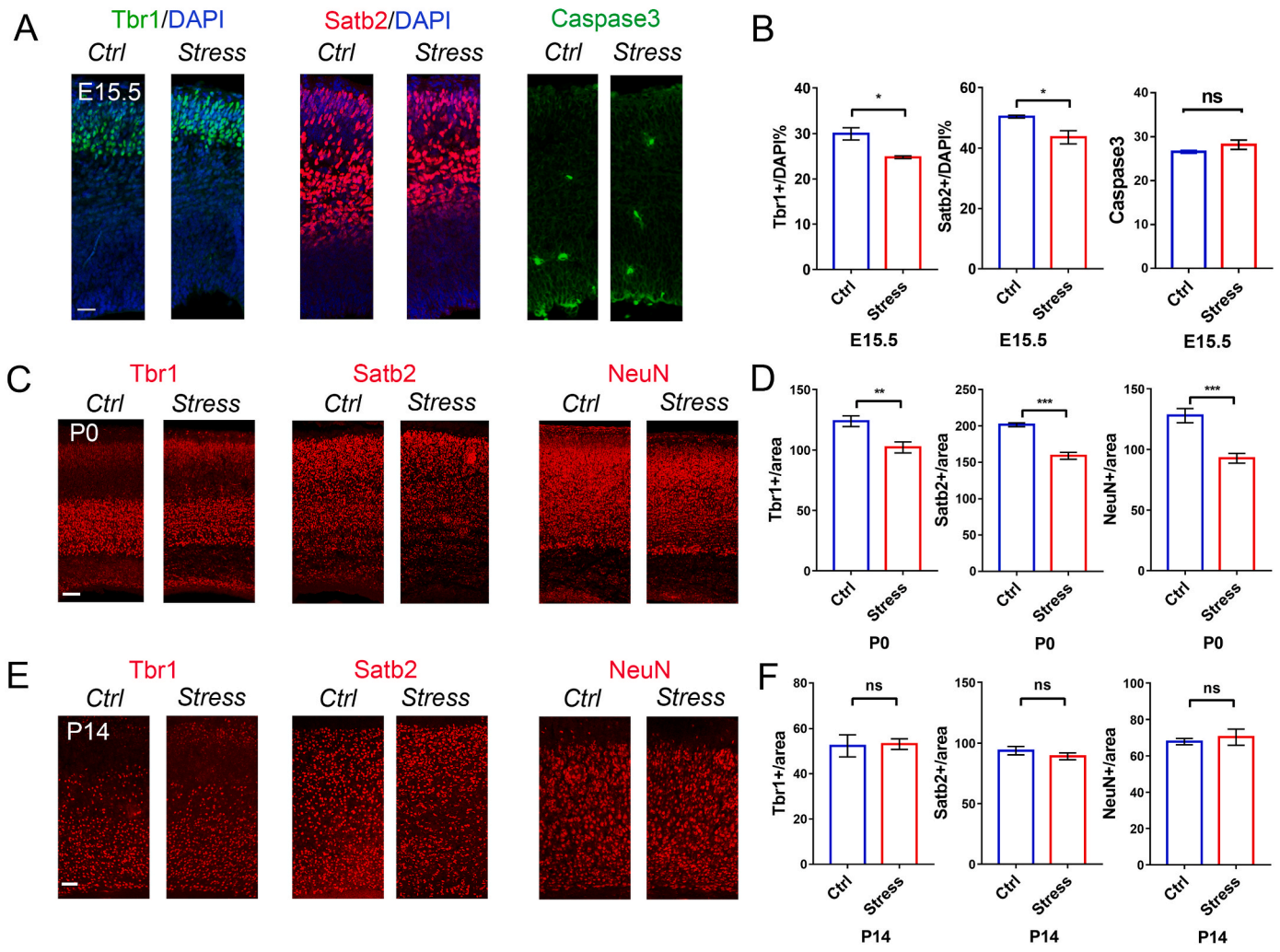

Fig. 2. Prenatal stress causes reduced neuronal production in embryonic and newborn cortices. **(A)** Representative images of Tbr1 (green), Satb2 (red) and Caspase3 (green) expressing cells in the E15.5 cortex of control (*Ctrl*) and prenatal stress-induced (*Stress*) embryos. **(B)** Percentages of Tbr1-, Satb2-positive cells, but not Caspase3-positive cells, were significantly decreased in the E15.5 stress-induced cortex. **(C, E)** Representative images of Tbr1-, Satb2-and NeuN-expressing neurons in P0 and P14 *Ctrl* and *Stress* cortices. **(D)** Numbers of Tbr1-, Satb2-and NeuN-expressing neurons were significantly reduced in P0 stress-induced cortices. **(F)** Numbers of Tbr1-, Satb2-and NeuN-expressing neurons were unchanged in P14 stress-induced cortices. Data are presented as means ± s.e.m, Student's t-test was used, n = 4–6 brains for each group, and 4–6 sections in each brain. *: $p < 0.05$, **: $p < 0.01$, ***: $p < 0.001$, ns: none significance. Scale bar = 100 μm. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

the stress-induced cortex (Fig. S4). This data suggests that prenatal stress affects a broad range of genes, and may in turn disturb normal neurogenesis and neural function.

Furthermore, we analyzed signaling pathways that might be affected by prenatal stress using Gene Ontology (GO) analyses. Among differentially expressed genes in the prenatal stress-induced E15.5 cortex, up-regulated genes were mostly enriched in terms of cell cycle, cell division, and forebrain development, whereas down-regulated genes were highly enriched in synaptic transmission, synaptic plasticity, and cell adhesion (Fig. 4E). These data indicate a significant alteration of genes that function in regulating cell cycle and synaptic plasticity by the prenatal stress. Moreover, among differentially expressed genes in the stress-induced adult cortex, up-regulated genes were mostly enriched in terms of regulation of transcription, learning, and adult behavior, and down-regulated genes were highly enriched in translation, transport, ATP, and apoptotic process (Fig. 4G). In addition, we validated expression levels of some differentially expressed genes using quantitative reverse transcription PCR (qRT-PCR). Most of genes displayed up- or down-regulation that is consistent with those detected by RNA-seq (Fig. 4F, H). In particular, genes related to microglial cells displayed altered expression, for instance *Mafb* and *Apoe* expressions were significantly down-regulated in E15.5 and adult cortices, respectively, in the stress-induced group (Fig. 4I).

In summary, our RNA-seq analyses indicate that prenatal stress has an early and an enduring effect on expression of genes in embryonic and adult cortices, and in turn affect neural function in F1 offspring.

3.5. *Community structure alterations in gut microbiota of prenatal stress induced adult F1 offspring*

Accumulating evidence indicates an association of maternal stress and depressive behaviors with alterations in gut microbiota (Valles--Colomer et al., 2019). To determine a potential impact of prenatal stress on gut microbiota in offspring, fecal samples were collected from 6 to 8 weeks old female control and stress-induced adult F1 mice. We analyzed gut microbiota composition using 16S rDNA sequencing and obtained 250 bp paired-end DNA reads for data analysis (Fig. S5A). We detected an average of 32,786 reads (32,786 ± 1740) per sample in the control group, and an average of 26,944 reads (26,944 ± 1324) per sample in the stress-induced group (Fig. S5B).

Moreover, 16S rDNA sequences with >97% similarity were assigned to the same Amplicon sequence variant (ASVs) (Fig. 5A). We identified

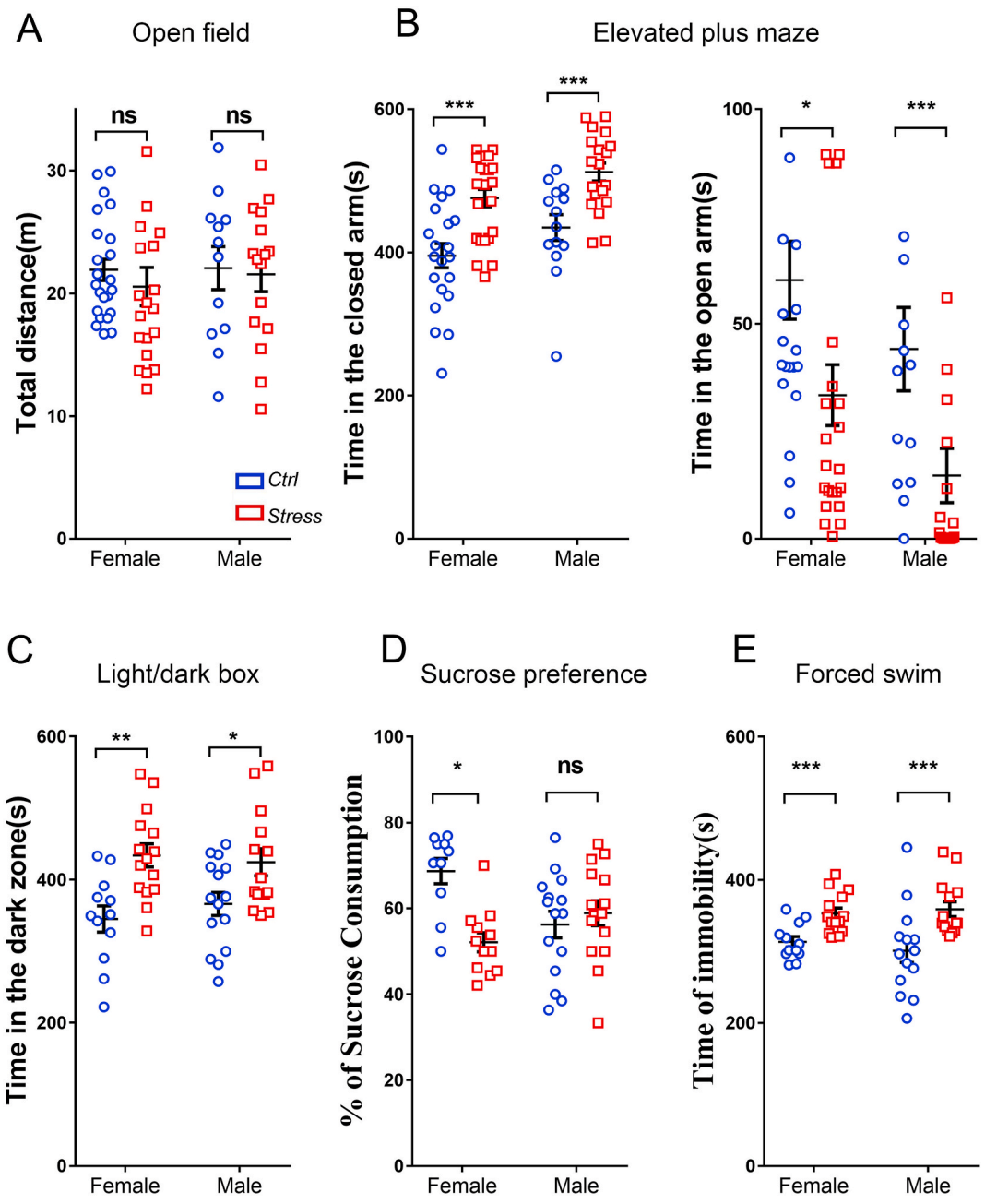

Fig. 3. Prenatal stress causes elevated anxiety- and depression-like behaviors in adult F1 offspring. (**A**) Open field test for control (*Ctrl*) and prenatal stress-induced (*Stress*) adult F1 mice. There were no significant changes in total distances between *Ctrl* and *Stress* offspring. **(B)** Elevated plus maze test: both stress-induced female and male offspring showed significantly increased trend to stay in the closed arm and stay away from the open arm. **(C)** Light-dark box test: stress-induced F1 offspring spent more time in the dark area. **(D)** Sucrose preference test: stress-induced F1 females, not males, showed significantly reduced preference for sucrose compared to controls. **(E)** Forced swim test: stress-induced F1 mice displayed decreased mobility, compared to the control group. Two-ways ANOVA between subjects (sex × stress) was conducted. Data are presented as means ± s.e.m, n = 10–24 mice for each group. *: $p < 0.05$, **: $p < 0.01$, ***: $p < 0.001$, ns: none significance.

7477 and 4339 unique ASVs in control and stress-induced groups, respectively, and 1195 common ASVs shared by both groups (Fig. 5B). The overall ASV distribution and 30 altered ASVs ($p < 0.05$) indicate significant changes in richness of gut microbiota between two groups (Fig. 5C and Fig. S5A).

To further evaluate microbial structural alteration induced by prenatal stress, we performed alpha-diversity analysis, including Chao1, Shannon, and Abundance-based Coverage Estimator (ACE), and we detected significant difference in community richness and diversity between two groups (Fig. S5C-E). To identify overall profiles of microbial community structure, we performed beta-diversity analysis, including Nonmetric Multidimensional Scaling (NMDS), Principal Component Analysis (PCA) and Principal Coordinates Analysis (PCoA) based on weighted unifrac distances. We found a consistent and distinct separation in beta-diversity analysis between control and stress-induced groups (Fig. S5F and G). In addition, analysis of similarities (Anosim) based on relative abundance, which provides an insight into the degree of separation, also displayed a significant difference ($R = 0.206$, $p = 0.001$) between two groups (Fig. S5H). These data indicate that both the community richness and specific microbial community structure is altered in F1 offspring exposed to prenatal stress.

Furthermore, we performed taxonomic cladogram analysis and found that the phylogenetic distributions of microbiota are different between control and stress-induced groups (Fig. S5I). We next

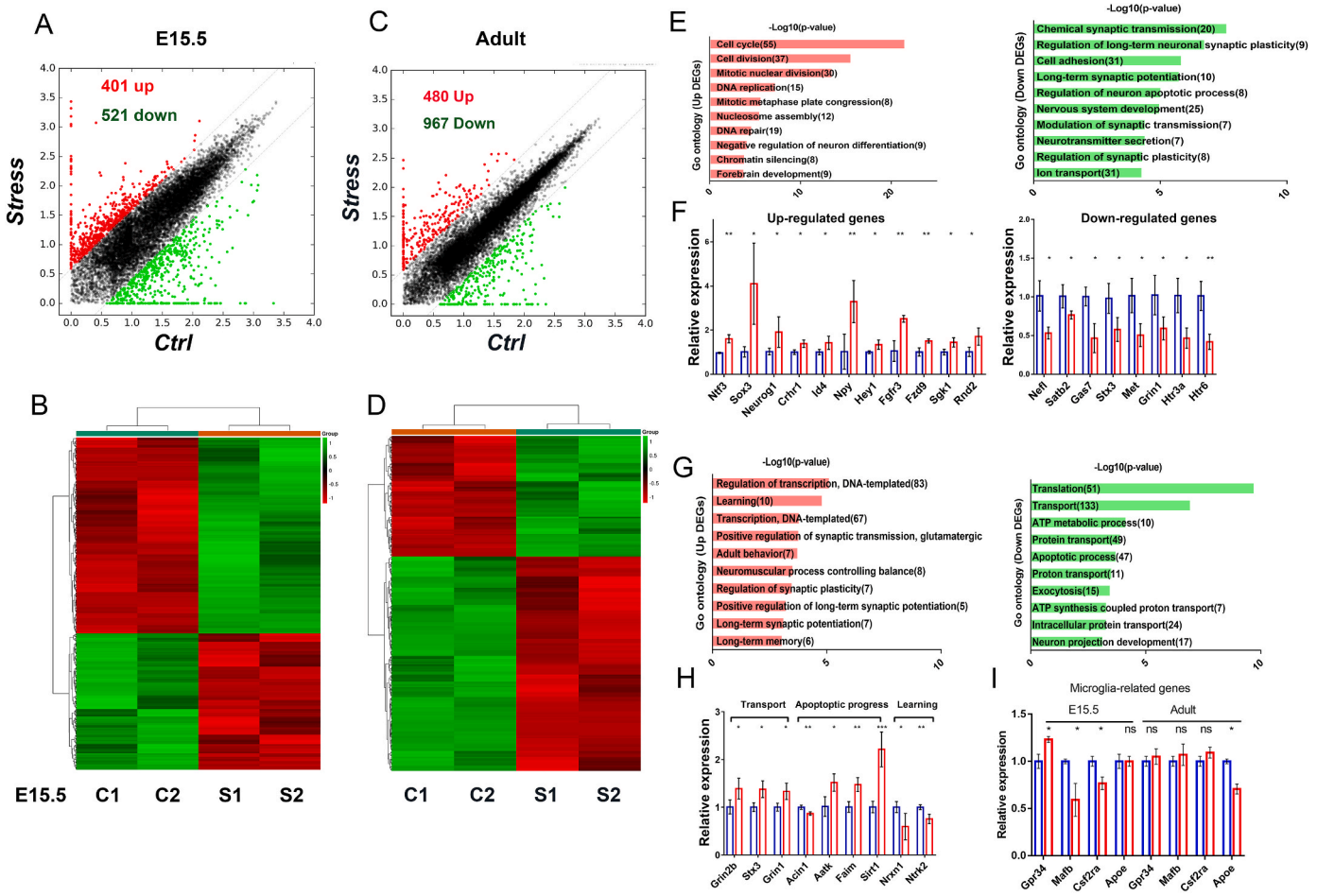

Fig. 4. Prenatal stress alters gene expressions in stress-induced E15.5 and adult cortices. (**A**) Scatter plot of 401 up-regulated genes (red) and 521 down-regulated genes (green) detected by RNA-Sequencing in E15.5 control (*Ctrl*) and stress-induced (*Stress*) cortices. Values of X and Y axes in the scatter plot are averaged FPKM values of each group (log2 scaled). **(B)** Heatmap of relative expression levels (log2 scaled FPKM) of differentially expressed genes in control (C) and stress-treated (S) E15.5 cortices. **(C)** Scatter plot of 480 up-regulated genes (red) and 967 down-regulated genes (green) detected by RNA-Sequencing in adult *Ctrl* and stress-induced cortices. **(D)** Heatmap of relative expression levels (log2 scaled FPKM) of differentially expressed genes in control (C) and stress-treated (S) adult cortices. **(E)** Gene ontology (GO) analysis identified significant enrichment of up-/down-regulated gene sets in control and stress-treated E15.5 cortices. **(F)** qRT-PCR verification of up- and down-regulated genes in E15.5 cortices of control (blue bars) and stress-induced (red bars) mice. **(G)** GO analysis of up- and down-regulated genes in adult cortices. **(H)** qRT-PCR verification of up- and down-regulated genes in adult cortices of control (blue bars) and stress-induced (red bars) mice. **(I)** qRT-PCR verification of microglia-related genes in E15.5 and adult cortices of control (blue bars) and stress-induced (red bars) mice. Data are presented as means ± s.e.m, Student's t-test was used, n = 4–6 samples for each group. *: $p < 0.05$, **: $p < 0.01$, ***: $p < 0.001$, ns: none significance. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

conducted Linear discriminant analysis Effect Size (LEfSE) to determine taxonomic biomarkers. We identified 8 different features with the Linear discriminant analysis (LDA) score (log 10) > 2 (Fig. S5J). In particular, *Muribaculaceae* was predominantly enriched at the family level, and *Verrucomicrobia* and *Verrucomicrobiae* were more abundant at the class and phylum level in the control group. *Prevotellaceae* and *Bacteroidaceae* were highly enriched at the family level, *Bacteroides*, *Alloprevotella* and *Butyricicoccus* were top genus-level biomarkers in the stress-induced group (Fig. S5J). These analyses further indicate a perturbation of gut microbiota in prenatal stress-induced adult F1 offspring.

Finally, to examine whether prenatal stress affects specific microbial communities, we compared relative abundance in taxonomic distribution (taxa containing 1% or more) between control and stress-induced groups. At the genus level, 7 out of 87 genera showed significant difference in relative abundance (cut-off of 1%) between two groups (Fig. 5D). In particular, the most abundant genera included *Muribaculaceae* with a relative abundance of 50.57% versus 40.16% (control versus stress-induced), *Alleprevotella* of 3.79% versus 6.90%, and *Bacteroides* of 1.91% versus 6.51% (Fig. 5D). These results indicate that *Murbaculaceae* is more abundant, while *Alleprevotella* and *Bacteroides* are less abundant in the control than the stress-induced group. In addition, at the family level, the relative abundance of *Muribaculaceae* was 52.07% versus 41.66% (control versus stress-induced), *Prevotellaceae* was 4.71% versus 9.41%, and *Bacteroidaceae* was 3.243% versus 6.51% (Table S3). At the species level, 81 species were identified and 9 were determined to be significantly altered in the prenatal stress-induced group (Table S3). Of which, *Lactobacillus* and *Bacteroides* showed higher abundance, while *Alloprevotella* showed less abundance in the stress-induced group, compared to the control. Taken together, the taxonomic characterization of gut microbiota at different levels is altered in prenatal stress-induced adult F1 offspring, compared to those in the control.

3.6. Comparative analysis of metabolic pathways in adult F1 cortex and gut microbiota

Microorganism-derived compounds can directly or indirectly signal to the brain, and affect host physiological responses (Yang and Chiu, 2017). To explore associations between metabolic pathways in the gut microbiota and in the cortex, we compared metagenome functional

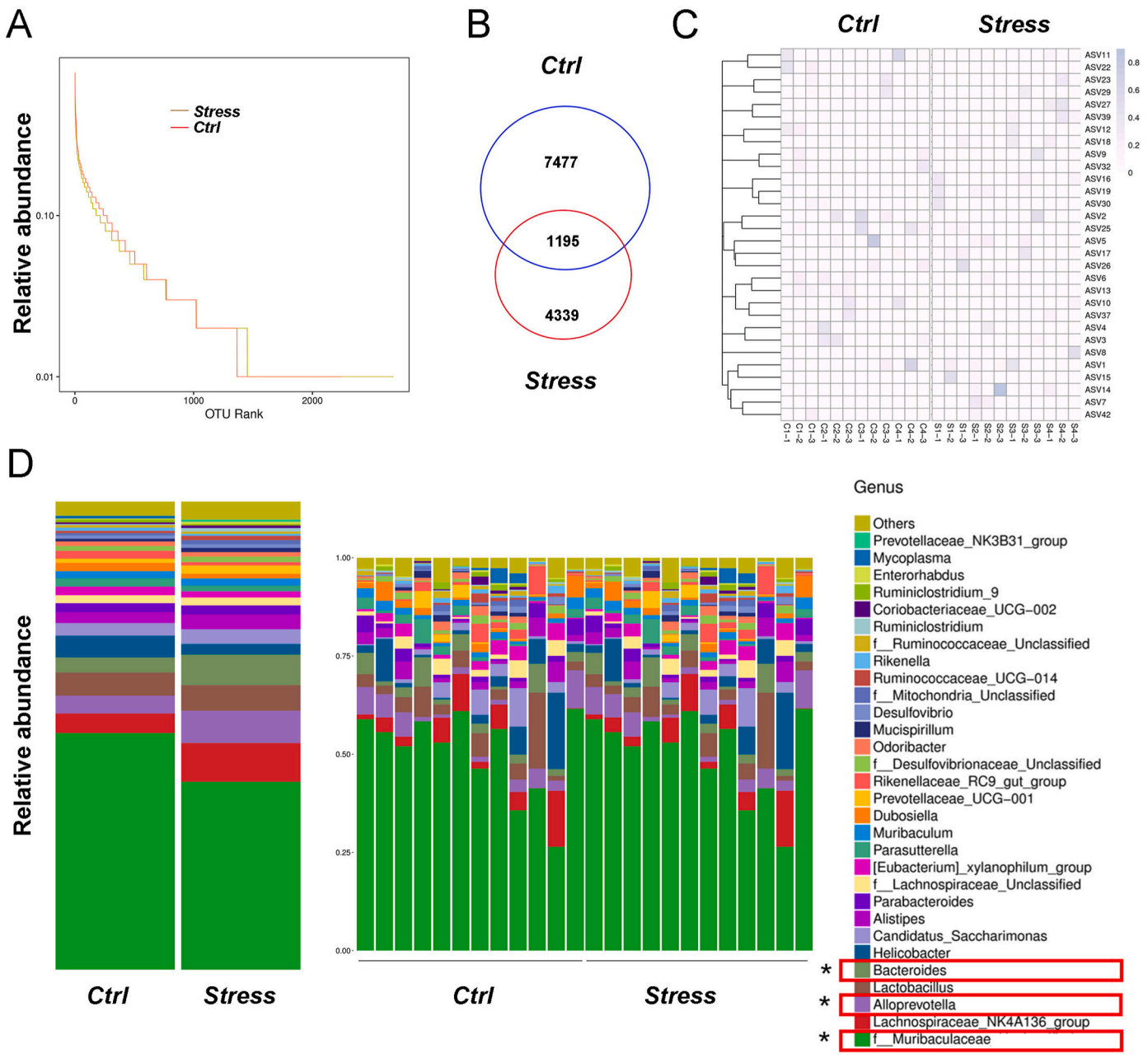

Fig. 5. Prenatal stress causes changes in gut microbiota of adult F1 offspring. (A) Relative abundance of Amplicon sequence variant (ASVs) in control (*Ctrl*) and prenatal stress-induced (*Stress*) adult F1 mice. **(B)** Venn diagram of the numbers of ASVs in *Ctrl* and *Stress* groups. **(C)** Heatmap of 30 significantly altered ASVs in *Ctrl* (C) and *Stress* (S) groups. **(D)** Relative abundance analysis at the genus level in *Ctrl* and *Stress* groups. Student's t-test was used, n = 12 samples for each group. *: $p < 0.05$.

content of F1 gut microbiota detected by Phylogenetic Investigation of Communities by Reconstruction of Unobserved States (PICRUSt) with Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analyses based on adult F1 cortical transcriptome. In fecal samples, using PICRUSt analyses, we identified three levels of KEGG enrichment such as human diseases in KEGG level 1, energy metabolism and amino acid metabolism in KEGG level 2, and steroid hormone biosynthesis, beta-alanine metabolism, flavone and flavonol biosynthesis, and glycan biosynthesis and metabolism in KEGG level 3 (Fig. 6A and Fig. S6). Moreover, in stress-induced adult F1 cortices, we identified 113 differentially expressed genes associated with metabolic pathways, and detected KEGG pathway enrichment in metabolism of lipids, cholesterol biosynthesis, and fatty acid metabolism (Fig. 6B and C).

We next conducted comparative analyses of metabolic pathways between the gut microbiota and cortex. Notably, among metabolic pathways based on stress-induced F1 cortex, common pathways such as purine metabolism, steroid hormone biosynthesis and lipid metabolism also were identified in those from fecal samples (Fig. 6D). Moreover, because corticosterone is an important intermediate in the steroidogenic pathway from pregnenolone to aldosterone, we next validated serum corticosterone expression level and detected elevated expression in stress-induced adult F1 mice (Fig. 6E and F). These results suggest a high association of altered metabolism between the gut microbiota and brain in responding to prenatal stress, in particular the steroid metabolism.

4. Discussion

Accumulating evidence has demonstrated a significant impact of

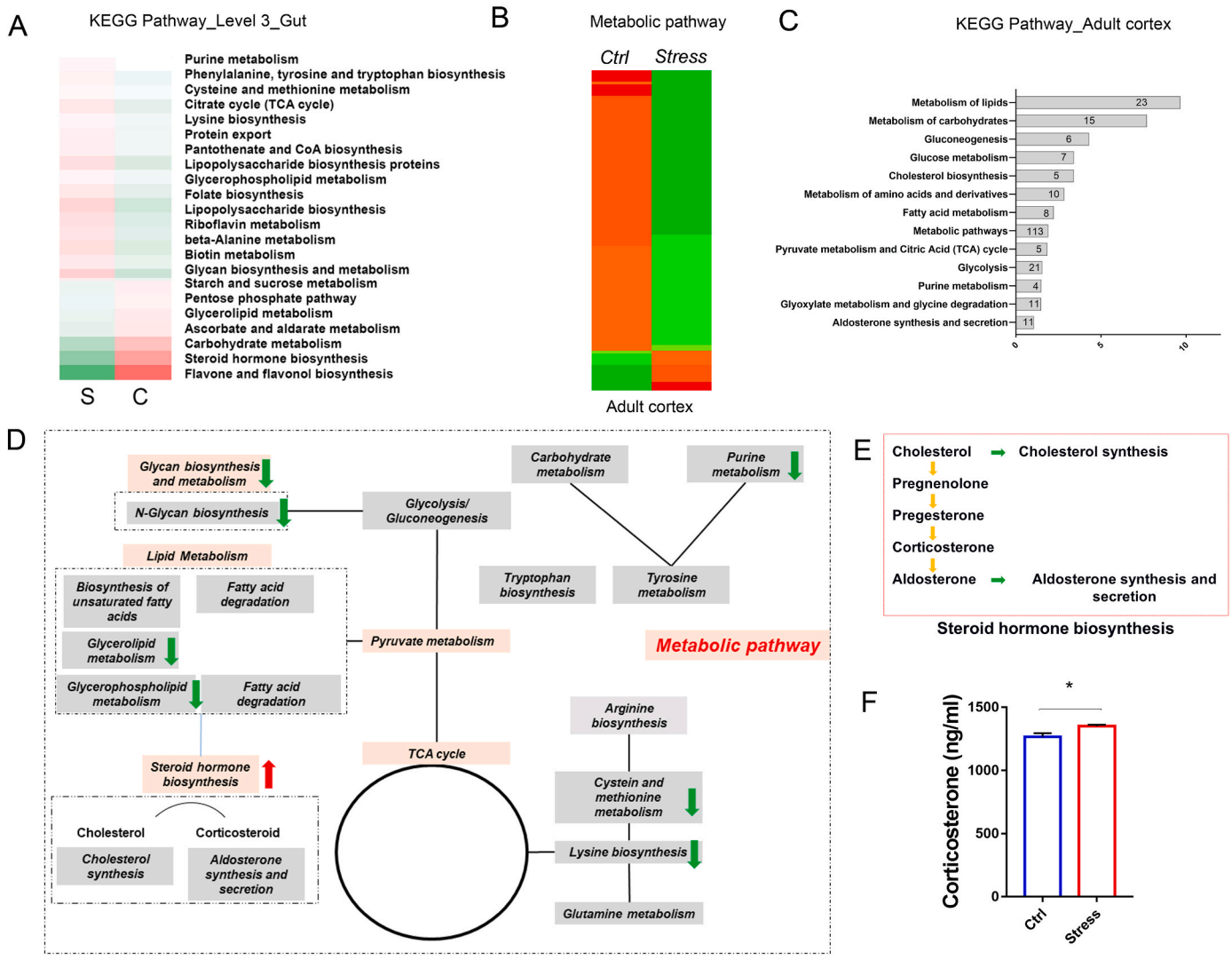

Fig. 6. Comparative analysis of metabolic pathways in gut microbiota and cortices of prenatal stress induced adult F1 mice. (A) Major metabolic pathways identified in gut microbiota. **(B)** Heatmap of differentially expressed 113 genes associated with metabolic pathways in the adult cortex of control (*Ctrl*) and prenatal stress-induced (*Stress*) offspring. **(C)** KEGG analyses of metabolic pathways for differentially expressed genes in the adult cortex. **(D)** Common metabolic pathways (highlighted in brown) identified in gut microbiota and cortices of adult F1 mice. Red and green arrows represent up- and down-regulated pathways, respectively. **(E)** A scheme of steroid hormone pathway. **(F)** Elevated serum concentration of corticosteroid in stress-induced adult F1 mice. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

prenatal stress on mental health. How prenatal stress affects brain development, gene expression and gut microbiota in offspring is unclear. In this study, we find that prenatal stress causes abnormal development of the embryonic cerebral cortex, changes cortical gene expression profiles, and results in anxiety- and depression-like behaviors in the adult F1 offspring. The adult F1 offspring also displays altered gut microbiota between control and prenatal stress-induced groups. Our study indicates substantial influences of prenatal stress on normal brain development and mood-related behaviors. Our results further demonstrate the tight relationship between prenatal stress and gut microbiota in offspring.

Previous reports have proposed an association between brain neurogenesis and mood-related disorders (Drevets et al., 2008; Kang et al.). In particular, altered neurogenesis in hippocampus is associated with behavioral abnormalities (Anacker et al., 2013; Urban and Guillemot, 2014). The connection between normal cortical development and behaviors is becoming appreciated. The mammalian cortex consists of many neuronal subtypes and is involved in sophisticated motor, sensory, cognitive, and emotional functions (Lodato and Arlotta, 2015; Temple, 2001). For example, prenatal protein restriction and late stage stress cause reduction in proliferation of neural progenitors and neuronal production (Buss et al., 2012; Gould et al., 2018). In this study, we have examined the impact of early life stress on brain development and behaviors by conducting prenatal stress right after pregnancy. Indeed, we find that prenatal stress leads to an early reduction of neural progenitors in the embryonic cortex, with a strong effect on intermediate progenitors, and results in reduced neuronal production in embryonic and newborn brains. Moreover, we have detected altered gene expression profiles in stress-induced embryonic cortices, compared to those in controls. Our results have demonstrated a significant negative impact of prenatal stress on early cortical neurogenesis, and these cortical deficits are likely caused by altered gene expression. The future work will be to explore how prenatal stress affects early cortical gene expression.

Previous studies have shown that cortical development persists into postnatal stages, which also is essential for mental health (Austin and Constance, 1990; Drevets et al., 2008; Glantz and Lewis, 2000). In this study, we have found a reduction in the number of neurons in stress-induced embryonic and postnatal cortices, and a recovery in adult brains. Interestingly, even though neuronal production is recovered, the adult F1 offspring still shows deficits in cortical gene expression, and

anxiety- and depression-like behaviors. It appears that prenatal stress has an enduring effect on cortical gene expression and behaviors in offspring mice. Our results suggest that prenatal stress might transmit risk factors that affect brain gene expression and mood-related behaviors to the next generation.

Furthermore, increasing evidence has pointed out influence of pregnant conditions on gastrointestinal dysfunction in offspring (Golubeva et al., 2015; Jašarević et al., 2015; Martin et al., 2018). Pregnant mothers with adverse events, such as maternal stress, drug usage and dietary structure changes, may have long-term effects on offspring, especially through re-shaping the gut microbiome, and leading to phenotypes similar to anxiety, depression and autism (Calatayud et al., 2019; Edwards et al., 2017). Animal model studies have shown that pups exposed to prenatal stress show reduced *Lactobacilli* abundance (Jašarević et al., 2017), and pups with postnatal stress induced by maternal separation display decrease in *Lactobacillus* abundance (Bailey and Coe, 1999). In our study, we have found decreased total reads of 16S rDNA in fecal samples of prenatal stress-induced adult F1, which indicates an overall change in gut microbiota. In particular, we have found that *Muribaculaceae*, a relative abundance of 20–30% among total mouse gut microbiota, is significantly less abundant in prenatal stress-induced mice. Our study suggests that prenatal stress also has an enduring effect on gut microbiota in adult offspring. Interestingly, adult F1 offspring exposed to prenatal stress also displays anxiety- and depression-like behaviors. Moreover, in addition to vaginal microbiome, milk can serve as vertical microbiota delivery system to affect behaviors in the next generation (Korpela et al., 2020; Liu et al., 2014). In this study, we have applied cross-fostering to minimalize effects of milk transmission and postnatal care on the contribution of prenatal stress in behavioral and physiological outcomes in F1 mice. The future study is to decipher the direct and indirect relationship between gut microbiota and mood-related behaviors in adult F1 offspring exposed to prenatal stress, and to better understand abnormal behaviors are the cause of altered gut colonization, or vice versa.

Moreover, we speculate that abnormal intestinal flora might be one of the causes of the onset of depressive disorder. Some OTUs/microbiota have been reported to be positively or negatively connected with depressive behaviors. Which specific taxa is most associated with depression is still unclear (Cheung et al., 2019). Studies in animal models have shown impacts of neurotransmitters produced by gut bacteria, such as serotonin and dopamine, on host physiology through the gut-brain axis (Galland, 2014; Strandwitz, 2018). In our study, we have found high abundance of common metabolic pathways detected in gut microbiota and in cortices of stress-induced adult F1 offspring. In particular, we have identified high enrichment of steroid and hormone production pathways, which are associated with mood disorders and play a multifaceted role in major depression disorder (MDD) (Sanderson, 2006; Tetel et al., 2018).

Taken together, our study has established a significant negative impact of prenatal stress on brain development in embryos, and on mood-related behaviors and gut microbiota in adult F1 offspring. Our results highlight importance of maternal care, in particular prenatal stress, on mental health in the next generation.

Funding sources

This work was supported by the Scientific Research Funds of Huaqiao University (Z16Y0017, T.S.) and the National Natural Science Foundation of China (31771141).

CRediT authorship contribution statement

Conceived and designed the study: T.S.; Sampling, RNA extraction, experiments and result analyses: Z. Z, N.L., R.C., T.L., Y.G., Z.Y., Y.N. and T.S.; Wrote the paper: Z.Z.; Edited paper: Z.Z., T.L. and T.S. All authors read and approved the final manuscript.

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.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ynstr.2021.100333.

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