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Gut Microbiota Mediates Prenatal METH Exposure-Induced Anxiety- and Depression-Like Behaviors by Modulating the Wnt Signaling Pathway

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Abstract

Background: Prenatal methamphetamine (METH) exposure poses a significant threat to offspring health, including anxiety and depression-like behaviors. This study investigated the neurotoxicity of prenatal METH exposure in offspring and explored the underlying mechanisms.

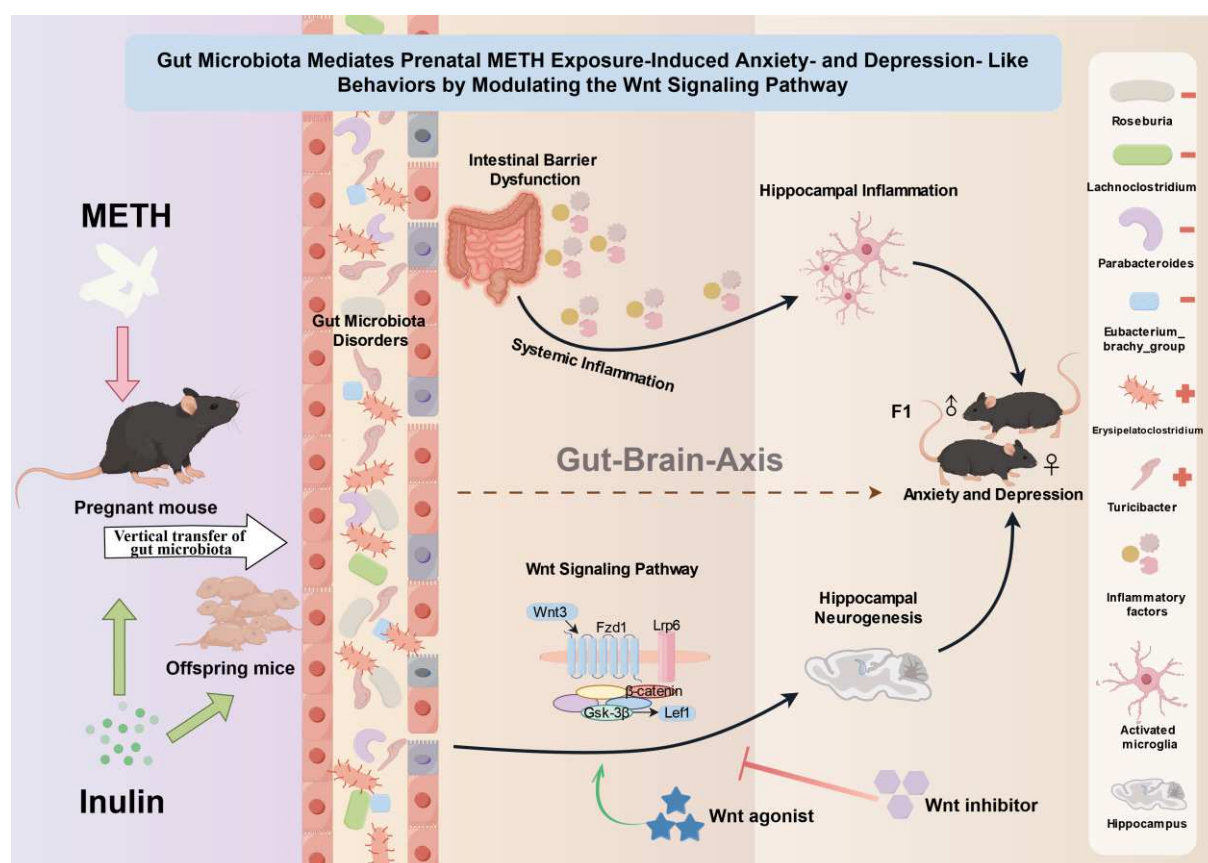
Results: Our results showed that prenatal METH exposure induced anxiety- and depression-like behaviors in offspring, accompanied by increased hippocampal inflammation and decreased hippocampal neurogenesis. Importantly, offspring exposed to prenatal METH exhibited gut microbiota dysbiosis, characterized by a reduced abundance of beneficial bacteria and an increased abundance of pro-inflammatory bacteria. To assess the involvement of the gut microbiota, we conducted cross-fostering and inulin supplementation experiments. Cross-fostering with control dams partially reversed METH-induced behavioral deficits, suggesting a role for maternal microbiota transmission. Inulin supplementation, both during pregnancy and in offspring, effectively remodeled the gut microbiota composition and

alleviated anxiety and depression-like behaviors. This effect was associated with reduced hippocampal inflammation and increased hippocampal neurogenesis, potentially mediated by activation of the canonical Wnt signaling pathway.

Conclusions: Gut microbiota mediates prenatal METH exposure-induced anxiety- and depression-like behaviors by modulating the WNT signaling pathway. This study provides evidence for the critical role of the gut microbiota in mediating the neurodevelopmental consequences of prenatal METH exposure, and highlights the potential of inulin supplementation as a promising therapeutic strategy.

Keywords: Prenatal methamphetamine exposure; Offspring; Anxiety- and depression-like behaviors; Gut microbiota; Inulin.

Graphical abstract



Graphical abstract: Gut microbiota mediates prenatal METH exposure-induced anxiety- and depression-like behaviors by modulating the Wnt signaling pathway.

Prenatal METH exposure induces gut microbiota disorders in offspring, leading to anxiety- and depression-like behaviors by activating hippocampal inflammation and inhibiting hippocampal neurogenesis, potentially mediated by activation of the canonical Wnt signaling pathway. By Figdraw. (<https://www.figdraw.com>).

Running title: Prenatal METH exposure Induced Mental Disorders in Offspring

Short abstract: Prenatal METH exposure induces gut microbiota disorders in offspring, leading to a mental disorders by activating hippocampal inflammation and inhibiting hippocampal neurogenesis.

1. Introduction

The abuse and misuse of methamphetamine (METH) have caused a series of problems due to its remarkable hallucinogenic effect and increased sexual arousal and pleasure^[1]. This has led to an increased risk of unintended pregnancy^[2-3]. Mounting evidence indicates that the number of births associated with METH abuse is increasing^[4-5], raising concerns regarding its adverse effects on the unborn child. Multiple cohort studies have shown that prenatal METH exposure is associated with increased physiological stress, emotional reactions, depression, anxiety, and central nervous system stress in offspring^[6-7].

Prenatal METH exposure has adverse effects on offspring. Children exposed to prenatal METH exposure have a greater incidence of low birth weight, stillbirth, intrauterine developmental delay^[8], and even sudden fetal death^[9]. These adverse effects are closely related to neurodevelopmental impairment^[10]. Brain structural changes, such as a decreased volume of the hippocampus and striatum and decreased spine density in the cortex, have been observed in offspring exposed to prenatal METH^[11-12]. Additionally, genetic alterations or epigenetic changes related to neurodevelopment in the striatum and hippocampus have been reported^[13-14]. Children exposed to METH during pregnancy show an increased risk of psychiatric disorders compared to their peers^[15]. However, the adverse effects and potential mechanisms of prenatal METH exposure in offspring still need to be further explored.

Growing studies have confirmed that the role of the gut microbiota in human health, especially in infants, is becoming increasingly important^[16]. The early infant gut microbiota is mainly contributed by vertical transmission of the parental microbiota^[17]. Offspring inherit certain gut microbial characteristics from their biological mothers, while the early lactation period and the environment also influence it^[18]. The maternal gut microbiome contributes directly and indirectly to offspring neurodevelopment^[19]. Intervening in the gut microbiota during pregnancy can protect offspring from cognitive impairment and synaptic defects induced by stress in pregnant rats^[20]. Depression-like behaviors in offspring caused by maternal separation were reversed after maternal probiotics were administered^[21]. Whether

the gut microbiota is involved in the adverse effects of METH exposure on offspring during pregnancy remains to be clarified.

Known as a natural prebiotic, inulin is a soluble dietary fiber that exerts various effects by stimulating the growth of the gut microbiota, such as exerting anti-inflammatory and immunoregulatory effects and alleviating anxiety and depression^[22-23]. The gut microbiota and its metabolites, such as short-chain fatty acids, neurotransmitters, and various inflammatory mediators, impact brain function. However, the detailed underlying mechanisms remain to be elucidated. Studies have shown that inulin can relieve anxiety and depression-like symptoms caused by disorders such as schizophrenia, post-stroke, and chronic constipation^[24-26]. Intervention in the gut microbiota of offspring through maternal inulin supplementation is considered a feasible strategy for alleviating the intergenerational implications of various substances^[27-29]. However, whether inulin also plays a role in prenatal METH-induced neurotoxicity in offspring and the potential target and related mechanisms involved remain to be investigated.

This study aimed to investigate the neurotoxicity of prenatal METH exposure in offspring and the role of the gut microbiota. We utilized a prenatal METH exposure mouse model and examined anxiety- and depression-like behaviors, hippocampal inflammation and neurogenesis, gut barrier dysfunction, and gut microbiota dysbiosis in offspring. To assess the involvement of the gut microbiota, we conducted cross-fostering and inulin supplementation experiments. Additionally, we explored the effects of the Wnt signaling pathway using a Wnt agonist and an inhibitor. Our study provides evidence for the critical role of the gut microbiota in mediating the neurodevelopmental consequences of prenatal METH exposure and highlights the potential of inulin supplementation as a promising therapeutic strategy. This research enriches our understanding of the intergenerational toxicity of METH and proposes a possible intervention.

2. Materials and methods

2.1. Reagents and antibodies

METH with a purity greater than 99% was kindly provided by the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). Inulin (CAS No: 9005-80-5) was purchased from Sangon Biotech (Shanghai, China). Rosiglitazone (Ros, CAS No: 122320-73-4, HPLC $\geq$ 98%), which was used as a Wnt agonist, was purchased from Sigma-Aldrich (St. Louis, MO, USA). SB216763 (CAS No: 280744-09-4, HPLC $\geq$ 98%),

which was used as a Wnt inhibitor, was purchased from Sigma–Aldrich (St. Louis, MO, USA).

2.2. Animals and treatment

Adult C57BL/6J mice (8–9 weeks old, acquired from Southern Medical University) were used in the experiments. Specific pathogen-free-level laboratory conditions were maintained for the mice during breeding and housing. The Medical Ethics Committee of Southern Medical University approved all animal care and experimental protocols (Approval Code: L2020075).

Prenatal METH exposure

Mating was conducted at a 2:1 ratio between C57/BL6J females and males. When a vaginal plug was detected in the morning, it was considered day 0.5 of pregnancy (PD0.5). A separate housing facility was then provided for female F0 individuals. The mice were randomly divided into two groups: the control group (Con) and prenatal METH exposure group (PME). During the period of pregnancy from PD0.5 to PD17.5, a daily dose of METH (5 mg/mL) was administered to the PME group by intraperitoneal injection. The Con group received the same amount of saline. The doses used were chosen based on previous studies, which revealed that the brain METH concentrations in METH-treated mice were similar to those in METH-abusing mothers^[30-31]. Offspring mice were weaned at the age of 3 weeks (PND21) and housed by gender. Mice feces were collected twice at 3 and 6 weeks of age. Behavioral tests were performed on the offspring mice at the age of 6 weeks. Serum, hippocampus, and colon tissues were collected and stored at –80°C for further analysis after the mice were sacrificed.

Cross-fostering

Based on previous research^[32], the pregnancy status of female rats was detected every day, and cross-fostering was carried out between PD0.5 and PD1.5. All the pups in the PME group, termed the MOCM group, were housed with lactating Con mothers. The same-day born pups of the Con group were housed with the lactating mothers of the PME group as well, termed the COMM group. Cross-fostering lasted for three weeks until the offspring were weaned. The offspring mice were housed in cages by gender until they reached 6 weeks of age, and behavioral tests were performed as described above. Serum, hippocampus, and colon tissues were collected and stored at –80 °C for further analysis after the mice were sacrificed.

Maternal inulin supplementation

There were four groups, Con, PME, Con+I, and PME+I, each consisting of eight mice. PME mice and Con mice were established as described above. The mice were given drinkable water containing inulin (1% in water) ad libitum throughout pregnancy and lactation. It has been reported that this particular inulin dosage does not affect feed or water intake^[33-34]. Those receiving METH and maternal inulin supplementation were referred to as the PME+I group. Those receiving saline treatment and maternal inulin supplementation were referred to as the Con+I group. The offspring mice were fed a normal control diet after birth. After 6 weeks of age, behavioral tests were performed as described above. Serum, hippocampus, and colon tissues were collected and stored at -80 °C for further analysis after the mice were sacrificed.

Inulin supplementation in offspring

There were four groups, Con, PME, 3wC+I, and 3wM+I, each consisting of eight mice. PME mice and Con mice were established as described above. After weaning, the offspring mice were provided normal drinking water or drinking water supplemented with 1% inulin. The treatments lasted for 4 weeks. After 7 weeks of age, at the end of the administration, behavioral tests were performed as described above. Serum, hippocampus, and colon tissues were collected and stored at -80 °C for further analysis after the mice were sacrificed.

SB216763 treatment

SB216763, a GSK-3 β inhibitor, was used to inhibit the Wnt signaling pathway in this study. This experiment was divided into three groups: PME+V, PME+I+V, and PME+I+Y, each consisting of eight mice. The prenatal METH exposure model was established as described above. After weaning, the offspring were given 2 mg/kg SB216763 or an equal dose of solvent V (10% DMSO) once a day for four weeks while drinking 1% inulin or normal drinking water. After the administration period, behavioral tests were performed as described above. Serum, hippocampus, and colon tissues were collected and stored at -80°C for further analysis after the mice were sacrificed.

Ros treatment

Ros is a PPAR γ agonist that has been shown to up-regulate the Wnt signaling pathway^[35]. In this study, it was used as a Wnt pathway agonist. The experiment was divided into three groups: Con+V2, PME+V2, and PME+Ros. As mentioned above, a prenatal METH

exposure model was established. After weaning, 3-week-old offspring mice were given 5 mg/kg Ros or an equal dose of solvent V2 (5% DMSO) once a day for four weeks. After the end of administration, behavioral experiments were carried out, and tissue samples were collected as described above.

2.3. Behavioral experiments

Open field test (OFT)

Based on previous research^[36], the mice were acclimated to the experimental environment for 2 days before the experiment began. The mice were gently placed in the center zone of the OFT platform (40 cm × 40 cm × 30 cm) and allowed to freely explore for 5 minutes. Using a video tracking system, the travel trajectories of the mice were tracked, and the distance in the central zone (%), time in the central zone (in seconds), and total distance were analyzed as indices of anxiety.

Elevated plus maze (EPM)

Based on previous research^[36], the mice were acclimated to the experimental environment for 2 days before the experiment began. The mice were placed in the elevated plus-maze apparatus 60 cm above the ground, which consisted of two open arms (30 cm × 6 cm × 0.5 cm), two closed arms (30 cm × 6 cm × 15 cm), and a central platform (6 cm × 6 cm). The travel track of the mice was recorded using a video tracking system, and the number of open arm entries and time spent in the open arms were analyzed as indices of anxiety.

Light-dark test (LDT)

Based on previous research^[37], the mice were acclimated to the experimental environment for 2 days before the experiment began. The mice were lightly placed in a light-dark shuttle box, which consisted of two equally sized (25 cm × 15 cm × 25 cm) light and dark chambers connected by a freely accessible corridor (5 cm × 5 cm × 25 cm). A video tracking system was used to record the travel track of the mice, and the number of light chamber entries and time spent in the light chamber were analyzed as indices of anxiety.

Forced swimming test (FST)

Based on previous research^[37], two days after the mice had acclimated, they were individually placed in circular glass chambers (diameter 15 cm, height 25 cm), filled with fresh water to a height of 15 cm, and maintained at 25°C. Using a video recorder, the entire experiment, lasting six minutes, was recorded. Afterwards, each mouse's immobility for the last four

minutes was examined. The immobility time (in seconds) refers to the period in which the mice stopped struggling and remained still. The immobility latency refers to the period from the beginning of the test to the first time the mouse was scored as immobile. The immobility times and immobility latency of the mice were then analyzed as indices of depression.

Tail suspension test (TST)

Based on previous research^[37], two days after they had acclimated, the mice were placed on the edge of a shelf 50 cm above a tabletop and taped to it. A video recorder was used to record the entire experiment for six minutes, and each mouse's immobility time was analyzed for the last four minutes. The immobility times and immobility latency of the mice were then analyzed as indices of depression.

2.4. RNA sequencing

Consistent with our previous studies, total RNA was extracted from the hippocampus using a QIAGEN RNeasy Mini Kit (Cat. 74104). All the sequencing works were conducted by Shanghai Majorbio Bio-pharm Biotechnology Co.,Ltd. (Shanghai, China). The whole processes were carried out in strict according to the kit and instrument instructions (Illumina, San Diego, CA). Majorbio Cloud provided the online platform for analyzing the data. The following selection criteria were used for differentially expressed genes (DEGs) expression difference analysis: $FDR < 0.05$ & $|\log_2FC| \geq 1$. Using the Majorbio-Cloud Platform's free online platform (www.majorbio.com), differential gene expression, functional enrichment analyses, variable shear analysis, and other RNA-seq results were performed. The Protein-Protein Interaction network analysis (PPI) of DEGs was performed via STRING database (<http://string-db.org/>).

2.5. 16S rRNA sequencing

Consistent with our previous studies, total microbial genomic DNA was extracted and amplified using the ABI GeneAmp 9700 PCR temperature scales' -actcctacgggagcgag-3' and 806R, 5' -GGACTACHVGGGTWTCTAAT-3'. It was then a pyrosequencing analysis of the microbiomes was performed with the Illumina MiSeq PE300 platform at Shanghai Majorbio Bio-pharm Biotechnology Co., Ltd. (Shanghai, China). The sequencing results were analyzed using the Majorbio Cloud platform (<https://www.majorbio.com>), and various indicators, including α -diversity, β -diversity, principal coordinate analysis (PCoA) maps, and linear discriminant analysis coupling effect size (LEfSe) were obtained.

2.6. HE and PAS staining

Colon tissue was fixed with 4% paraformaldehyde, and then stained with Sigma-Aldrich's periodic acid-Schiff staining (PAS) after dehydration, xylene hyalinization, and embedding in paraffin. According to the guidelines provided by the manufacturer, Goblet cells were counted under the Leica DMI 3000 microscope.

2.7. Fluorescein isothiocyanate-dextran (FITC-D) assay

Intestinal permeability was determined by using a fluorescein isothiocyanate-dextran (FITC-D) assay. After fasting for 4-6 hours, the mice were orally gavaged with 0.6 mg/g body weight of FITC-D (dissolved in normal saline infusion). Whole blood was collected 4 h after FITC-D administration via cardiac puncture. After centrifuging the blood at 3,000 rpm for 10 minutes at 4°C, plasma was obtained. Following the guidelines provided by the manufacturer, 100 µl of serum diluted with PBS was measured in a 96-well plate using a plate reader. At an excitation wavelength of 485 nm and an emission wavelength of 535 nm, the FITC-D standard curve was drawn, and the FITC-D concentration of each sample was calculated.

2.8. Enzyme-linked immunosorbent assay (ELISA)

After cardiac puncture, plasma was obtained by centrifuging whole blood at 3,000 rpm at 4°C for 10 minutes. Inflammatory response indicators, including serum IL-6 (Cat # MM-0163M2), IL-1β (Cat # MM-0040M2), and TNF-α (Cat # MM-0132M2), were quantified using ELISA kits following the manufacturer's instructions (Jiangsu Meimian Industrial Co., Ltd., Jiangsu, China). Serum LPS (Cat #MB-3418A) was also detected according to the manufacturer's instructions (Jiangsu Meibiao Biological Technology Co., Ltd., Jiangsu, China).

2.9. Immunofluorescence (IF)

The IF assay was carried out as described previously. Briefly, brain tissue was isolated and fixed overnight with 4% paraformaldehyde. After dehydration, xylene hyalinization, and embedding in paraffin, the tissue was cut into 5 µm slices using a microtome (Leica Germany). Antigens were repaired by dewaxing paraffin sections and soaking in citric acid antigen repair buffer (120°C for 5 minutes, then cool for 1 hour to room temperature). Following that, sections were inactivated with 3% hydrogen peroxide and blocked with 5% bovine serum albumin (Sigma-Aldrich). Primary antibodies (DCX, 1:500, Abcam, Cat # ab18723; IBA1, 1:500, Abcam, Cat # ab178846) were then incubated overnight at 4°C on the slices. Next, incubation was carried out for 1 hour in the dark at room temperature with the secondary antibody (Alexa Fluor® 594 donkey anti-rabbit immunoglobulin (IgG) (H+L), 1:400, Life Technologies, Cat # A21207). DAPI solution (Solarbio, Cat # C0060) was added to the sections after three washes in PBS. As a final step, capture the figures with laser

confocal microscope. Image J software (Image J) was used to analyze the percentage of IBA1-positive area (positive area/total tissue area) and the mean fluorescence intensity (total fluorescence intensity/total fluorescence area) of DCX in the hippocampal region.

2.10. Western blot analysis

As described previously, the tissues were lysed using RIPA lysis buffer (Beyotime Biotechnology, Jiangsu, China) containing 1% PMSF and 1% phosphatase inhibitor. The protein concentration was measured using a BCA protein assay kit (ThermoFisher Scientific, USA). The proteins were separated on an 8%–12% polyacrylamide gel (PAGE) by Millipore (Billerica, MA, USA), transferred to a PVDF membrane, and blocked with 5% skim milk at room temperature for 2 h. The membranes were incubated with primary antibodies overnight at 4°C, followed by incubation with the appropriate secondary antibodies for 2 h at room temperature. With a Tanon Gel Image System (version 4.2), the target protein bands were visualized. Band intensities were quantified using ImageJ (version 1.8.3), with β -Actin serving as a normalization standard. Primary antibodies were applied as follows: (a) β -Actin monoclonal antibody (1:1000, Proteintech, Cat # 66009-1-Ig); (b) IL6 rabbit pAb (1:1000, ABclonal, Cat No. A0286); (c) TNF- α rabbit mAb (1:1000, ABclonal, Cat No. A24214); (d) IL-1 β rabbit mAb (1:1000, ABclonal, Cat No. A19635); and (e) Occludin polyclonal antibody (1:10 00; Proteintech, Cat # 13409-1-AP).

2.11. RT-qPCR analysis

Using TRIzol reagent (Invitrogen), total RNA was isolated from the hippocampus and colon. After assessing the quality and concentration of RNA using a NanoDrop spectrometer, complementary DNA (cDNA) was synthesized using StarScript III All-in-one RT Mix with gDNA Remover (GenStar, Beijing, China, Cat # A230-10). The amplification reaction was prepared using 2 $\times$ RealStar Fast SYBR qPCR Mix (GenStar, Beijing, China, Cat # A301-10). mRNA expression was quantified using an ABI Q6 Flex system (Applied Biosystems, USA). The $2^{-\Delta\Delta CT}$ method was used to calculate the relative quantification of target genes, and the results were normalized to β -Actin. The primers used are listed in Table S1.

2.12. Statistical analysis

All the data were analyzed using GraphPad Prism version 9.0, except for the 16S rRNA and RNA sequencing data, which were analyzed according to the methods provided by the Majorbio Cloud platform (<https://www.majorbio.com>). In each experiment, at least three technical replicates were conducted, as well as at least three biological replicates. For comparisons between two groups, the student's t-test was conducted, while the one-way ANOVA analysis of variance followed by the post-hoc Tukey test was used for comparisons

among more than two groups. The data are presented as the mean $\pm$ standard error of the mean (SEM), and a P value < 0.05 was considered to indicate statistical significance. (** P < 0.05 , *** P < 0.01 , *N.S.*, no significant difference).

3. Results

3.1 Prenatal METH exposure induced anxiety- and depression-like behaviors in offspring

We utilized a prenatal METH exposure model (Fig. 1A) by administering intraperitoneal injections of METH to female C57BL/6J mice aged eight to nine weeks. This model allowed us to investigate the neurotoxicity effects on the offspring of both sexes. METH exposure during pregnancy significantly increased adverse pregnancy outcomes, including miscarriage, preterm birth, neonatal death, and dystocia (Fig. 1B). Additionally, the litter size was notably decreased (Fig. 1C). Prenatal METH exposure resulted in a marked decrease in maternal body weight (Fig. 1D), but did not significantly affect the body weight of offspring of either sex (Fig. 1E).

Behavioral tests were conducted on offspring of both sexes to explore the effects of prenatal METH exposure on anxiety- and depression-like behaviors in these mice. Compared with those in the unexposed group, the offspring of prenatal METH-exposed dams exhibited shorter residence time and distance in the central zone, as well as shorter total distance in the OFT (Fig. 1F). Prenatal METH exposure also resulted in a significant decrease in the number of entries into open arms in the EPM (Fig. 1G), and a notable reduction in time spent in the light chamber during the LDT (Fig. 1H). In the FST, the offspring of prenatal METH-exposed dams exhibited a significantly shortened latency to immobility compared to the non-exposed group (Fig. 1I). Similar trends were observed in the TST, where prenatal METH exposure resulted in a reduced latency to immobility and increased immobility time in offspring (Fig. 1J). These findings suggest that maternal METH exposure during pregnancy induces anxiety- and depression-like behaviors in offspring, regardless of gender.

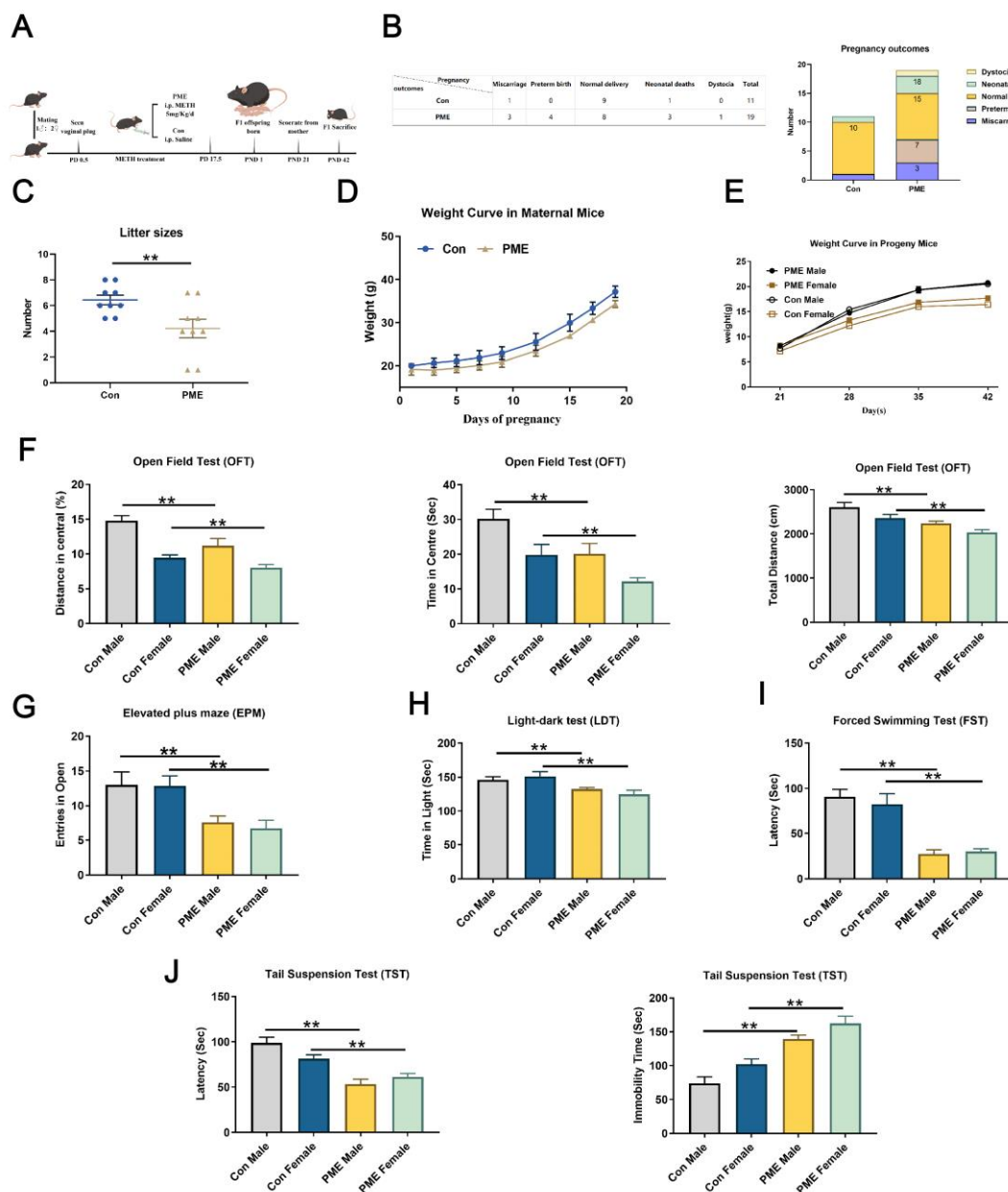


Fig. 1 Prenatal METH exposure induced anxiety- and depression-like behaviors in offspring

(A) Modeling flow chart of the prenatal METH exposure mouse model. By Figdraw.

(https://www.figdraw.com). (B) Statistics table and bar charts of pregnancy outcomes. (C) The number of

per litter. n = 9 per group. (D) Effects of prenatal METH exposure on maternal body weight, n = 6 per

group, and (E) offspring body weight, n = 9 per group. (F) OFT: the residence time and distance in the

central zone, and total distance. n = 7-8 per group. (G) EPM: the number of entries into open arms. n = 7

per group. (H) LDT: the time spent in the light chamber. n = 7 per group. (I) FST: latency to immobility.

n = 7 per group. (J) TST: latency to immobility and immobility time. n = 7 per group. Data are expressed as

mean $\pm$ SEM, ** P < 0.05, N.S.: no significant difference (P > 0.05).

To further explore the mechanism by which prenatal METH exposure causes anxiety- and depression-like behaviors in offspring, RNA sequencing analysis was performed on

hippocampal tissue. PCA differentiated the offspring of the PME group from those of the Con

group, indicating significant alterations in RNA gene expression in both male and female offspring (Fig. 2A, C). In comparison to the Con group, the male offspring of the PME group exhibited a total of 54,270 identified genes, with 634 upregulated and 512 downregulated genes (Fig. 2B), while the female offspring exhibited a total of 55,353 identified genes, including 27 upregulated and 36 downregulated genes (Fig. 2D).

Gene Set Enrichment Analysis (GSEA) revealed three clusters with differentially enriched gene sets (Fig. S1A), with the STARK_PREFRONTAL_CORTEX_22Q11_DELETION_UP gene set being significantly associated with anxiety and depression phenotypes (Fig. 2E, F). Pathways such as dopaminergic synapse, glutamatergic synapse, GABAergic synapse, axon guidance, and Wnt signaling were co-enriched in both groups (Fig. 2G, Fig. S1B). PPI analysis identified GSK-3 β , a pivotal component of the Wnt signaling pathway, as the nucleus of the entire PPI network (Fig. S1C). Differential splicing analysis demonstrated that prenatal METH exposure significantly increased alternative splicing events in offspring, primarily characterized by increased mutually exclusive exons and decreased exon skipping (Fig. S1D, E).

Given the close association between Wnt signaling and hippocampal neurogenesis, a potential mechanism for anxiety- and depression-like behaviors, we further investigated the Wnt signaling pathway. IF was performed to detect the expression of the neurogenesis marker DCX in the hippocampal region. The results showed that METH exposure during pregnancy significantly decreased DCX expression in the hippocampus of male offspring (Fig. 2H, I). Key molecules of the Wnt signaling pathway were also examined, revealing significantly reduced RNA expression levels of Wnt3, Lrp6, Lef1, Gsk-3 β , β -catenin, and Fzd1 (Fig. 2J), indicating significant inhibition of the Wnt signaling pathway. Consistent trends were observed in female mice (Fig. 2K-M). These findings suggest that the Wnt signaling pathway and hippocampal neurogenesis may mediate the anxiety- and depression-like behaviors in offspring induced by prenatal METH exposure.

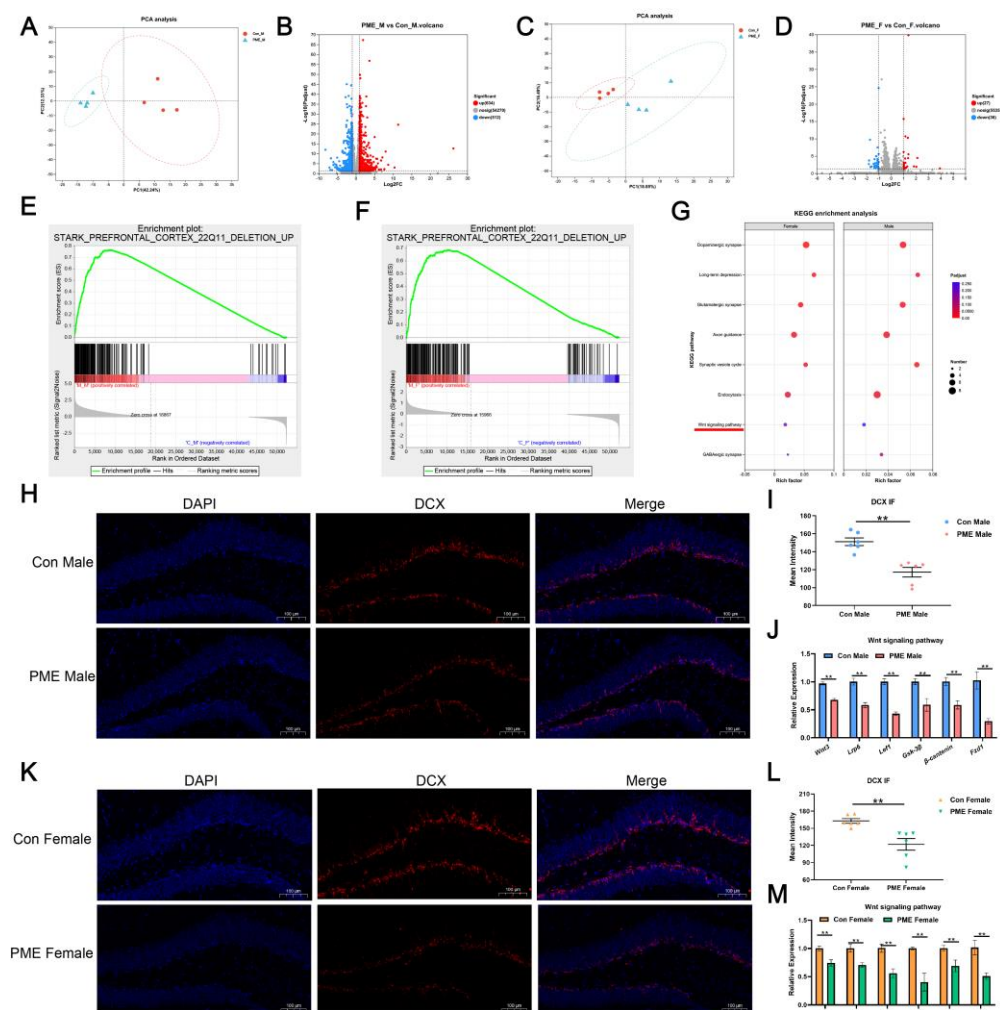


Fig. 2 Prenatal METH exposure induced alterations in gene expression and impaired hippocampal neurogenesis

(A) PCA plot analysis of hippocampal RNA-seq in male offspring. Red represents male offspring of Con group, and blue represents male offspring of PME group. $n = 4$ per group. (B) Volcano map of differential gene expression in male offspring. Red represents upregulated genes, green represents downregulated genes, and white represents no significant change in the gene. $P < 0.05$ and fold change equal to or greater than 2. (C) PCA plot analysis of hippocampal RNA-seq in female offspring. Red represents female offspring of Con group, and blue represents female offspring of PME group. $n = 4$ per group. (D) Volcano map of differential gene expression in female offspring. Fold change equal to or greater than 2. (E) GSEA results for DEGs in male and (F) female offspring. (G) Bubble plot of KEGG enrichment analysis of DEGs. (H) Representative images of DCX IF staining (in red) in male offspring. Nuclei were labeled with DAPI (in blue). Scale bar, 100 μm . (I) Statistical diagrams of DCX mean fluorescence intensity in the DG of the hippocampus. $n = 6$ per group. (J) The relative mRNA expression levels of key molecules of the Wnt signaling pathway by RT-qPCR in male offspring. $n = 3$ per group. (K) Representative images of DCX IF staining (in red) and (L) Statistical diagrams of DCX mean fluorescence intensity in the DG of the hippocampus in female offspring. (M) The relative mRNA expression levels of key molecule of the Wnt signaling pathway by RT-qPCR in female offspring. $n = 3$ per group. Data are expressed as mean $\pm$ SEM, $**P < 0.05$, N.S.: no significant difference ($P > 0.05$).

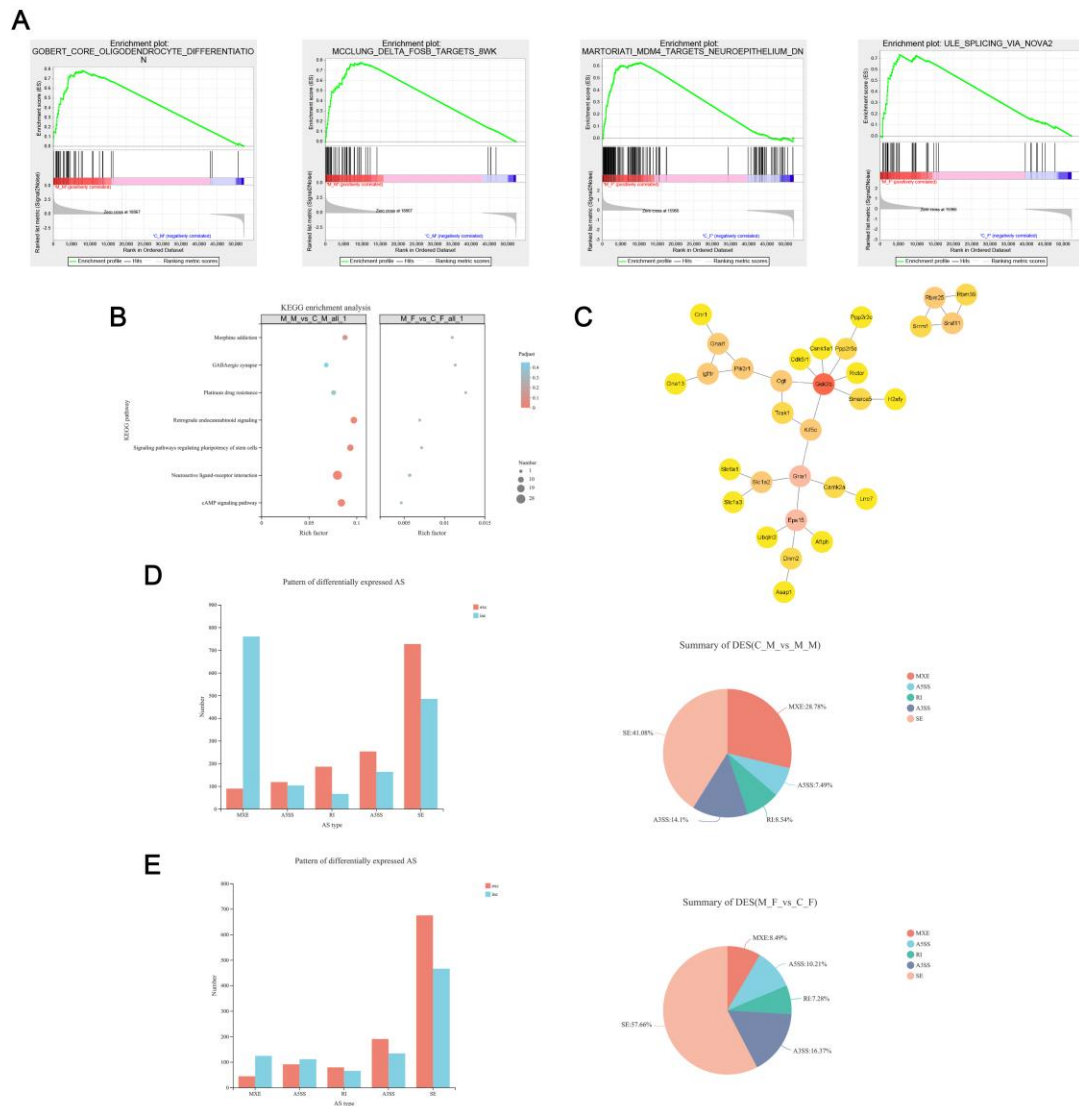


Fig. S1 Prenatal METH exposure induced abnormal gene expression and aberrant alternative splicing events

(A) The other two pathways were enriched according to GSEA. (B) Bubble plot of KEGG enrichment analysis of DEGs identified via GSEA. (C) PPI network analysis of common DEGs in male and female offspring. A redder color indicates greater importance. (D) Analysis of differential alternative splicing events among genes identified by RNA-Seq in male and (E) female offspring.

3.2 Prenatal METH exposure induced dysbiosis of the gut microbiota, intestinal barrier dysfunction, and systemic inflammation

To further investigate the impact of prenatal METH exposure on offspring, we examined changes in the gut microbiota. Analysis of alpha diversity revealed that prenatal METH exposure significantly increased both the Chao and ACE indices (Fig. 3A, B), indicating enhanced microbial richness. Beta diversity analysis, using PCoA, demonstrated distinct clustering of samples from each group, highlighting significant differences in microbiota

profiles (Fig. 3C). Hierarchical clustering further supported these findings (Fig. 3D, F). LEfSe analysis identified differentially enriched microbial taxa from the phylum to the genus level (Fig. 3E, G). Six differentially expressed genera showed consistent trends in both male and female mice: *Roseburia*, *Eubacterium_brachy_group*, *Lachnoclostridium*, and *Parabacteroides* decreased significantly (Fig. 3H-K), whereas *Turicibacter* and *Erysipelatoclostridium* were significantly increased (Fig. 3L, M). Correlation analysis indicated a strong relationship among these gut microbiota changes, anxiety- and depression-like behaviors, and intestinal barrier dysfunction (Fig. S2). These findings suggest that maternal METH exposure during pregnancy results in gut microbiota dysbiosis in offspring, irrespective of gender.

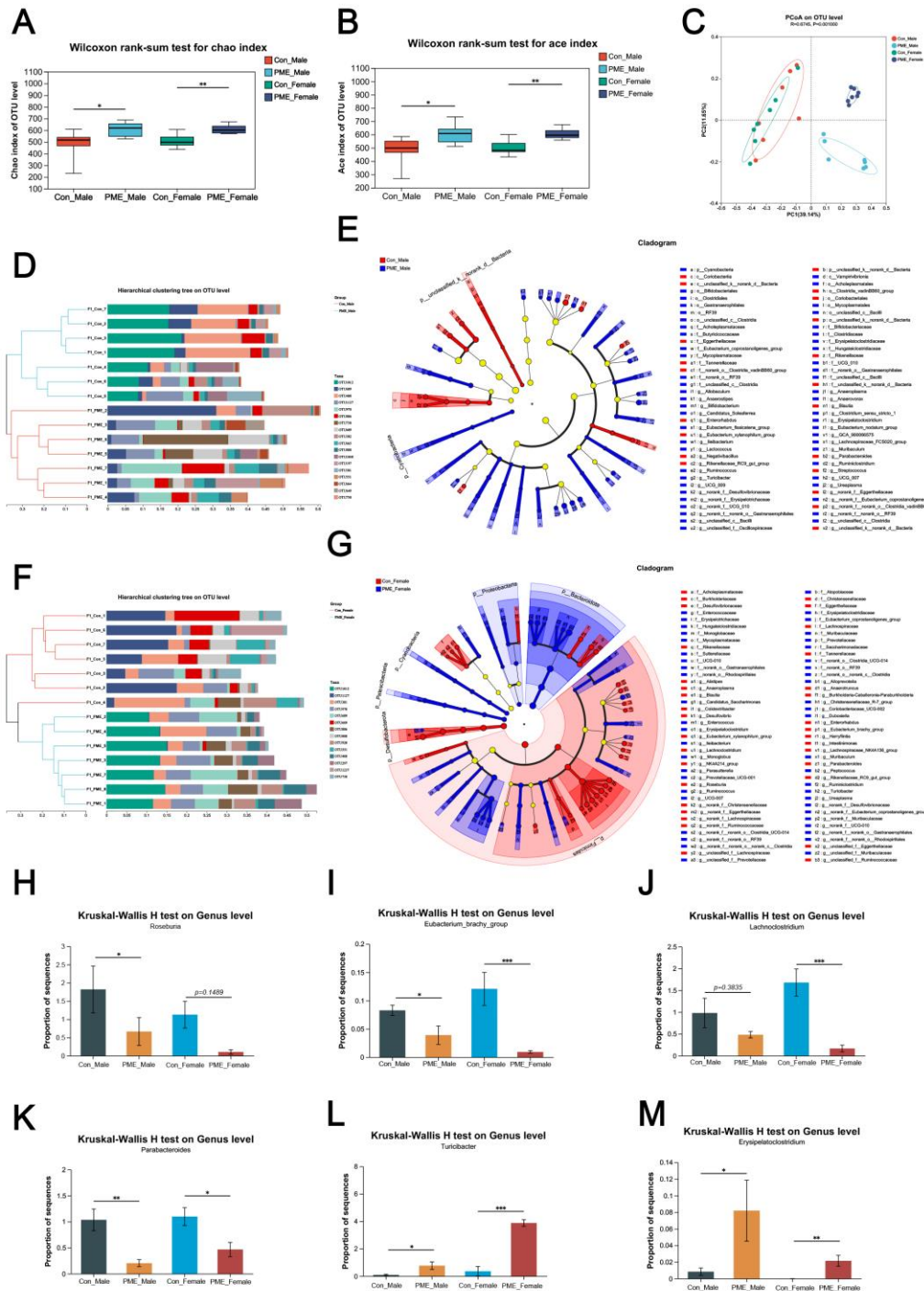


Fig. 3 Prenatal METH exposure induced dysbiosis of the gut microbiota in offspring

(A) Chao index and (B) ACE index of the gut microbiota. (C) PCoA plot analysis of 16S rRNA gene sequences at OUT level. (D) Hierarchical clustering of 16S rRNA gene sequences in male offspring. n = 7 per group. (E) LefSe bar from family to genus levels (LDA fold = 3.5 and $P < 0.05$). (F) Hierarchical clustering of 16S rRNA gene sequences in female offspring. n = 7 per group. (G) LefSe bar from family to genus levels (LDA fold = 3.5 and $P < 0.05$). The abundance of (H) *Roseburia*, (I) *Eubacterium_brachy_group*, (J) *Lachnospiraceae*, (K) *Parabacteroides*, (L) *Turicibacter* and (M) *Erysipelatoclostridium*. n = 7 per group. Data are expressed as mean $\pm$ SEM, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

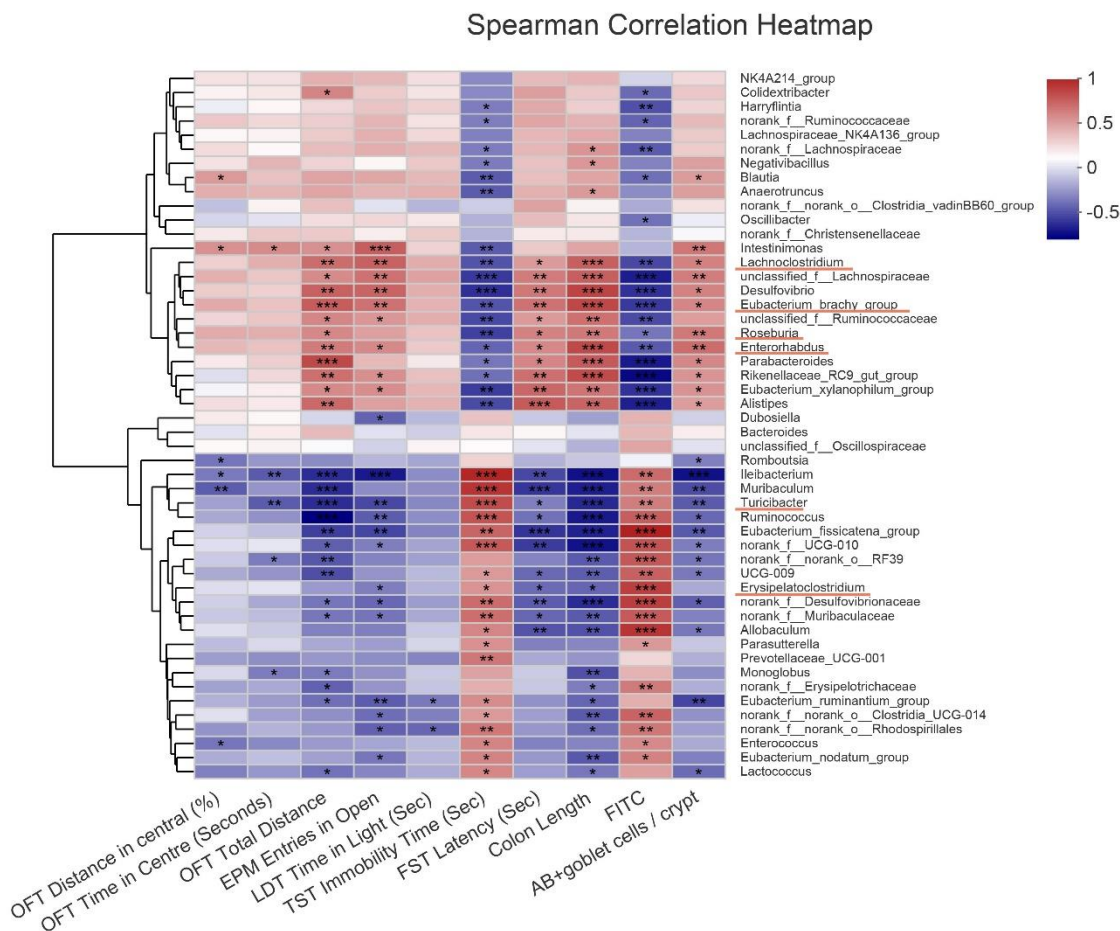


Fig. S2 Correlation analysis of the gut microbiota, anxiety- and depression-like behaviors and intestinal barrier dysfunction

Correlation analysis of the gut microbiota, anxiety- and depression-like behaviors and intestinal barrier dysfunction. *P < 0.05, **P < 0.01, *** P < 0.001.

Subsequent assessments of intestinal damage in offspring revealed that prenatal METH exposure led to colorectal shortening in both sexes (Fig. S3A, B). PAS staining revealed a significant increase in the number of goblet cells in the PME group compared with the control group (Fig. S3C, D). Elevated serum concentrations of dextran-FITC in the PME group indicated impaired intestinal barrier function (Fig. S3E). This was further supported by decreased gene and protein expression levels of tight junction proteins (Fig. S3F, G). ELISA measurements showed increased serum levels of LPS and inflammatory factors (IL-6, IL-1 β , TNF- α) in the PME group compared with the control group (Fig. S3J, K). These trends were consistent in female offspring (Fig. S3H, I, L, M). These results suggest that prenatal METH exposure induces intestinal barrier dysfunction and systemic inflammation.

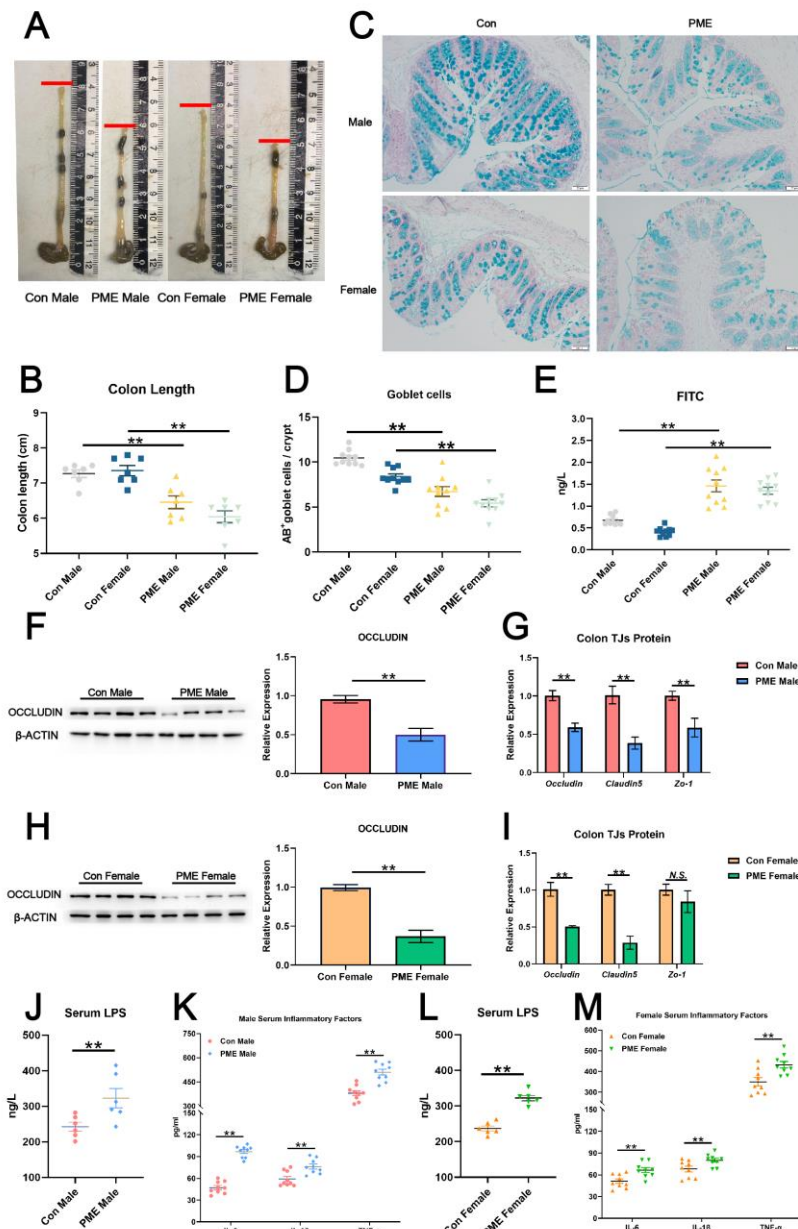


Fig. S3 Prenatal METH exposure induced intestinal barrier dysfunction and systemic inflammation in offspring

(A) Representative images of the colon and (B) statistical diagrams of the colorectal length. $n = 7$ per group. (C) Typical images of PAS-stained colon tissue and (D) quantification of PAS-positive goblet cells per crypt. $n = 10$ per group. (E) The concentration of FITC-dextran in serum. $n = 10$ per group. (F) The protein expression levels of OCCLUDIN and β -ACTIN in male offspring. β -ACTIN served as a loading control. $n = 4$ per group. (G) The relative mRNA expression levels of tight junction proteins (*Occludin*, *Claudin-5*, and *Zo-1*) in male offspring. $n = 3$ per group. (H) The protein expression levels of OCCLUDIN and β -ACTIN in female offspring. $n = 4$ per group. (I) The relative mRNA expression levels of tight junction proteins (*Occludin*, *Claudin-5*, and *Zo-1*) in female offspring. $n = 3$ per group. (J) The concentrations of serum LPS ($n=6$) and (K) serum inflammatory factors ($n=9$), including IL-6, IL-1 β , and TNF- α , in male and (L, M) female offspring. Data are expressed as mean $\pm$ SEM, ** $P < 0.05$. N.S.: no significant difference ($P > 0.05$).

Given the link between systemic inflammation and neuroinflammation, which is closely associated with anxiety and depression, we evaluated neuroinflammation in the hippocampus using IBA1 immunofluorescence. The percentage of IBA1-positive area in the CA1, CA3, and DG regions of the hippocampus was significantly greater in the PME group than in the control group (Fig. S4A, B), indicating prenatal METH-induced neuroinflammation. Additionally, prenatal METH exposure upregulated the gene and protein expression of inflammatory factors (IL-6, IL-1 β , TNF- α) in the hippocampus (Fig. S4C-E). These patterns were also observed in female offspring (Fig. S4F-J). These findings confirm that prenatal METH exposure induces hippocampal inflammation in offspring, regardless of gender.

These results suggest that prenatal METH exposure leads to gut microbiota dysbiosis, intestinal barrier dysfunction, and systemic inflammation, which subsequently induces hippocampal inflammation and may contribute to anxiety- and depression-like behaviors in offspring.

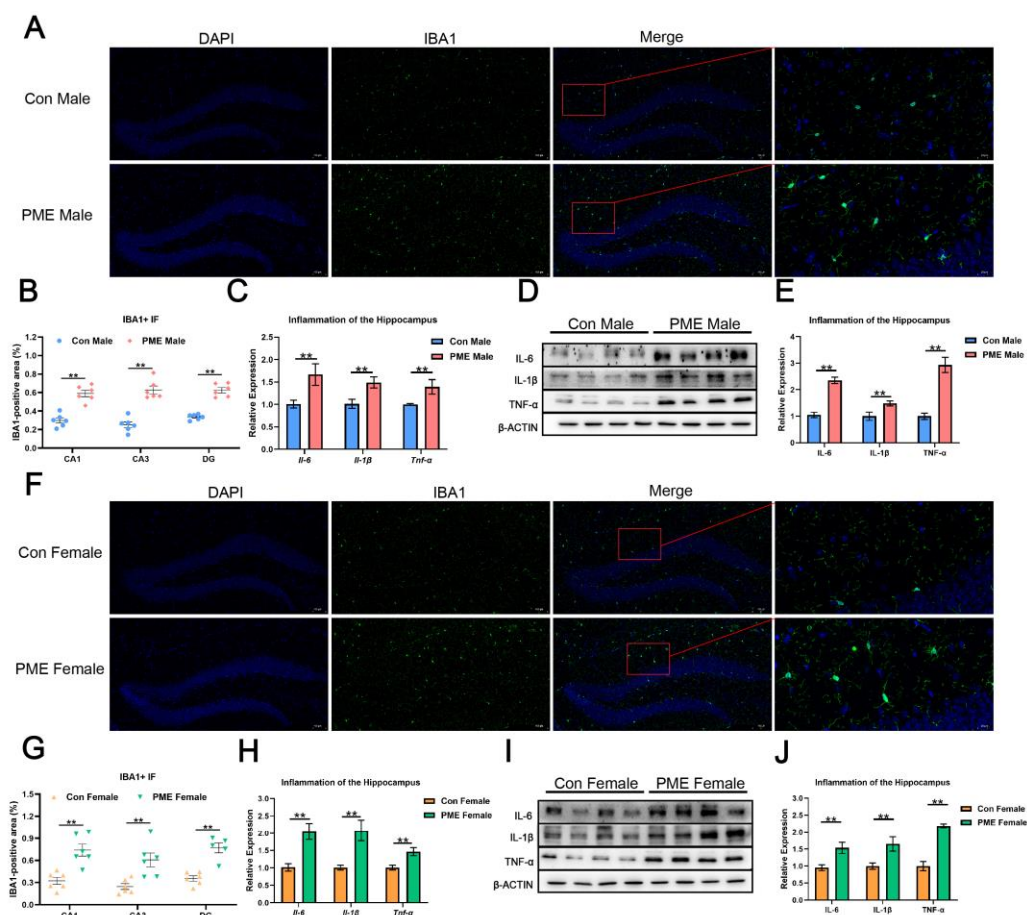


Fig. S4 Prenatal METH exposure induced hippocampal inflammation in offspring

(A) Representative images of IBA1 IF staining (in green) in male offspring. Nuclei were labeled with DAPI (in blue). Scale bar, 100 μ m. (B) The percentage of IBA1-positive area in the CA1, CA3 and DG regions of

the hippocampus in male offspring. n = 6 per group. (C) The relative mRNA expression levels of inflammatory factors (*Il-6*, *Il-1 β* , and *Tnf- α*). n = 3 per group. (D) Representative blots and (E) statistical graphs of inflammatory factors. β -ACTIN served as a loading control. n = 4 per group. (F) Representative images of IBA1 IF staining (in green) in female offspring. (G) The percentage of IBA1-positive area in the CA1, CA3 and DG regions of the hippocampus in female offspring. n = 6 per group. (H) The relative mRNA expression levels of inflammatory factors (*Il-6*, *Il-1 β* , and *Tnf- α*). n = 3 per group. (I) Representative blots and (J) statistical graphs of inflammatory factor expression. n = 4 per group. Data are expressed as mean $\pm$ SEM, ** $P < 0.05$. N.S.: no significant difference ($P > 0.05$).

3.3 Gut microbiota mediated anxiety- and depression-like behaviors induced by prenatal METH exposure

The gut microbiome can be vertically transmitted from mother to offspring, with profound impacts on offspring development. To further clarify the role of the gut microbiota, we sequenced the gut microbiota of 3-week-old (3w) offspring. In the 3w-aged male offspring, the PCoA plots demonstrated that the samples from the 3wM and 3wC groups clustered separately (Fig. 4A). In terms of α diversity, Chao index and ACE index showed an upward trend, although the differences were not statistically significant (Fig. 4B). All results showed a consistent trend in 3w female offspring (Fig. 4D, E). Through community bar plot analysis, we analyzed the different gut microbiota at the genus level (Fig. 4C, F). Notably, the significantly different microbiota observed in the 6-week-old offspring showed the same trend as those in the 3-week-old offspring. Specifically, the abundances of *Eubacterium_brachy_group*, *Lachnoclostridium*, and *Parabacteroides* tended to decrease, while those of *Turicibacter* and *Erysipelatoclostridium* displayed a rising trend (Fig. 4G).

Next, assessment of anxiety-like behaviors was conducted on 3w-aged offspring mice, but depression-like behavior was not evaluated because 3-week-old mice were too small to be suitable for the TST and FST. It was found that, regardless of gender, 3w-aged offspring of prenatal METH-exposed exhibited shorter residence time and distance in the central zone, and shorter total distance in the OFT (Fig. 4H). In the EPM, a significantly shorter distance in the open arm was observed (Fig. 4I), while the time spent in the open arm did not statistically differ from that of 3w-aged Con offspring. There was no difference in the result of LDT between the groups of mice. Still, all results showed a consistent trend in female offspring (Fig. 4J). Taken together, these results indicate that gut microbiota disorders and increased anxiety-like behaviors are already present in the 3w-aged offspring. The effects of gut microbiota are present early in life and persist until adult life.

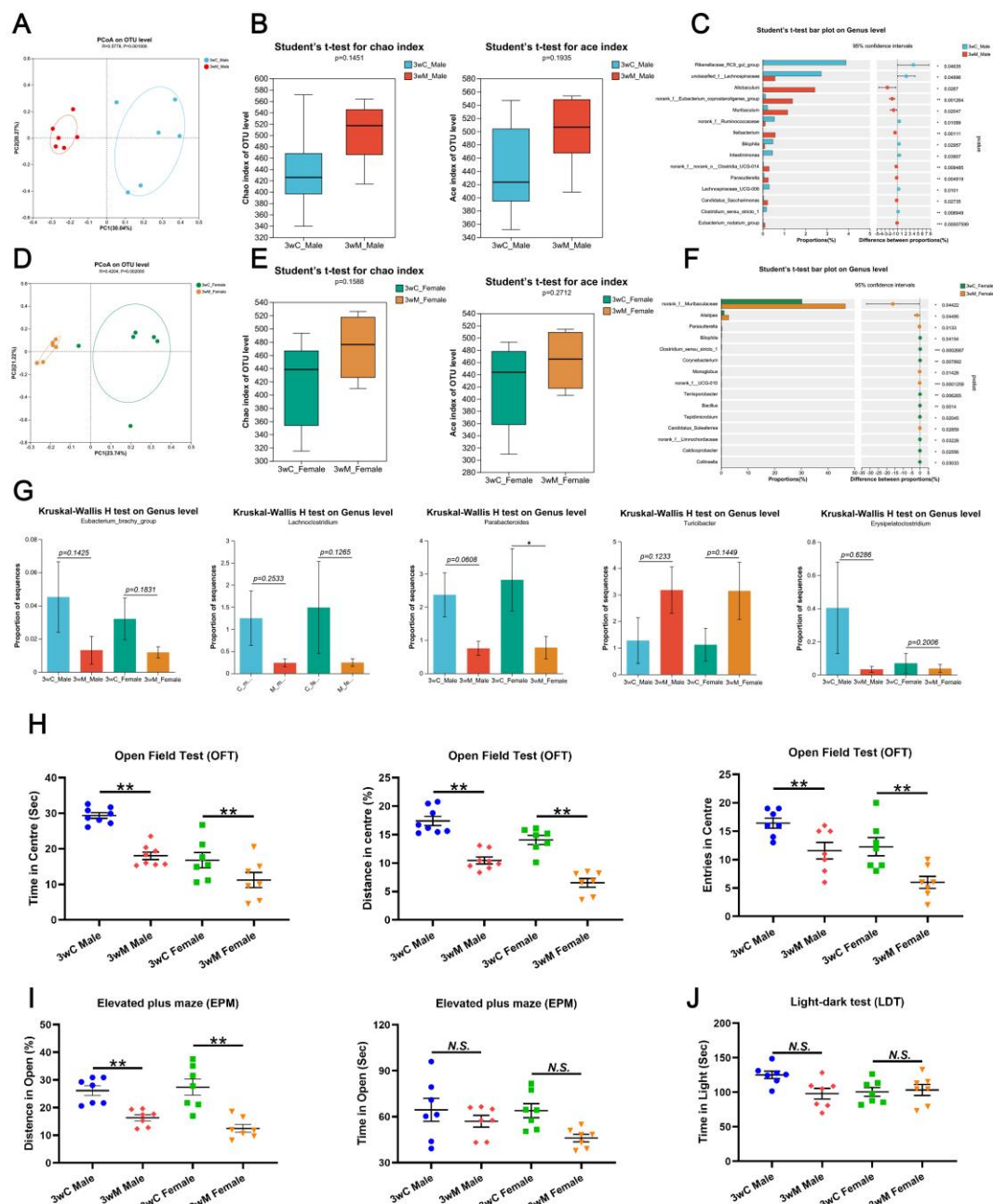


Fig. 4 Gut microbiota mediated anxiety- and depression-like behaviors induced by prenatal METH exposure

(A) PCoA plot analysis at OUT level of 16S rRNA gene sequences in male offspring. n = 6 per group. (B) Chao index and ACE index of the gut microbiota. (C) The top 15 differential microbiota between the 3wC-male and 3wM-male group at the genus level. (D) PCoA plot analysis at OUT level in female offspring. n = 6 per group. (E) Chao index and ACE index of the gut microbiota. (F) The top 15 differential microbiota between the 3wC-female and 3wM-female group at the genus level. (G) The abundance of *Eubacterium_brachy_group*, *Lachnospirillum*, *Parabacteroides*, *Turicibacter* and *Erysipelatoclostridium*. n = 7 per group. * $P < 0.05$. (H) OFT: the residence time and distance in the central zone, and total distance. n = 7 per group. (I) EPM: the residence time and distance in open arm. n = 7 per group. (J) LDT: the time spent in the light chamber. n = 7 per group. Data are expressed as mean $\pm$ SEM, ** $P < 0.05$, N.S.: no significant difference ($P > 0.05$).

We switched newborn pups from PME and Con dams to further examine whether maternal microbiota colonization affects offspring behaviors after birth. The pups in the PME group housed with lactating Con mothers were termed the MOCM group, while the same-day-born pups in the Con group housed with lactating mothers in the PME group were termed the COMM group. In the male offspring, anxiety-like behaviors were observed in the COMM offspring, which specifically manifested as shorter residence time and shorter distance in the central zone in the OFT (Fig. S5A), and a reduced number of entries into the open arms in the EPM (Fig. S5B). Offspring cross-fostered by dams in the PME group exhibited a significantly shortened latency to immobility and a significantly increased immobility time in the TST, as well as a shorter immobility latency in the FST (Fig. S5D, E). However, cross-fostering from Con dams only reversed the depression-like phenotype of PME offspring and did not significantly ameliorate anxiety-like behaviors (Fig. S5A-E). Similarly, in female offspring, cross-fostering by the PME group also caused anxiety- and depression-like phenotypes in the Con offspring (Fig. S5I-J). The difference was that cross-fostering by Con mothers significantly ameliorated the anxiety- and depression-like phenotypes of PME offspring, in addition to the results of the LDT (Fig. S5C, H). Overall, these results suggest that maternal colonization of the microbiota from mothers with PME can induce anxiety- and depression-like behaviors in normal offspring. Additionally, the gut microbiota from Con mothers can partially alleviate PME-induced anxiety- and depression-like behaviors in offspring, suggesting that the gut microbiota may be involved in mediating prenatal METH exposure-induced anxiety- and depression-like behaviors in offspring.

These findings suggest that the vertical transmission of the gut microbiota from mothers has a profound effect on offspring and mediates anxiety- and depression-like behaviors in offspring.

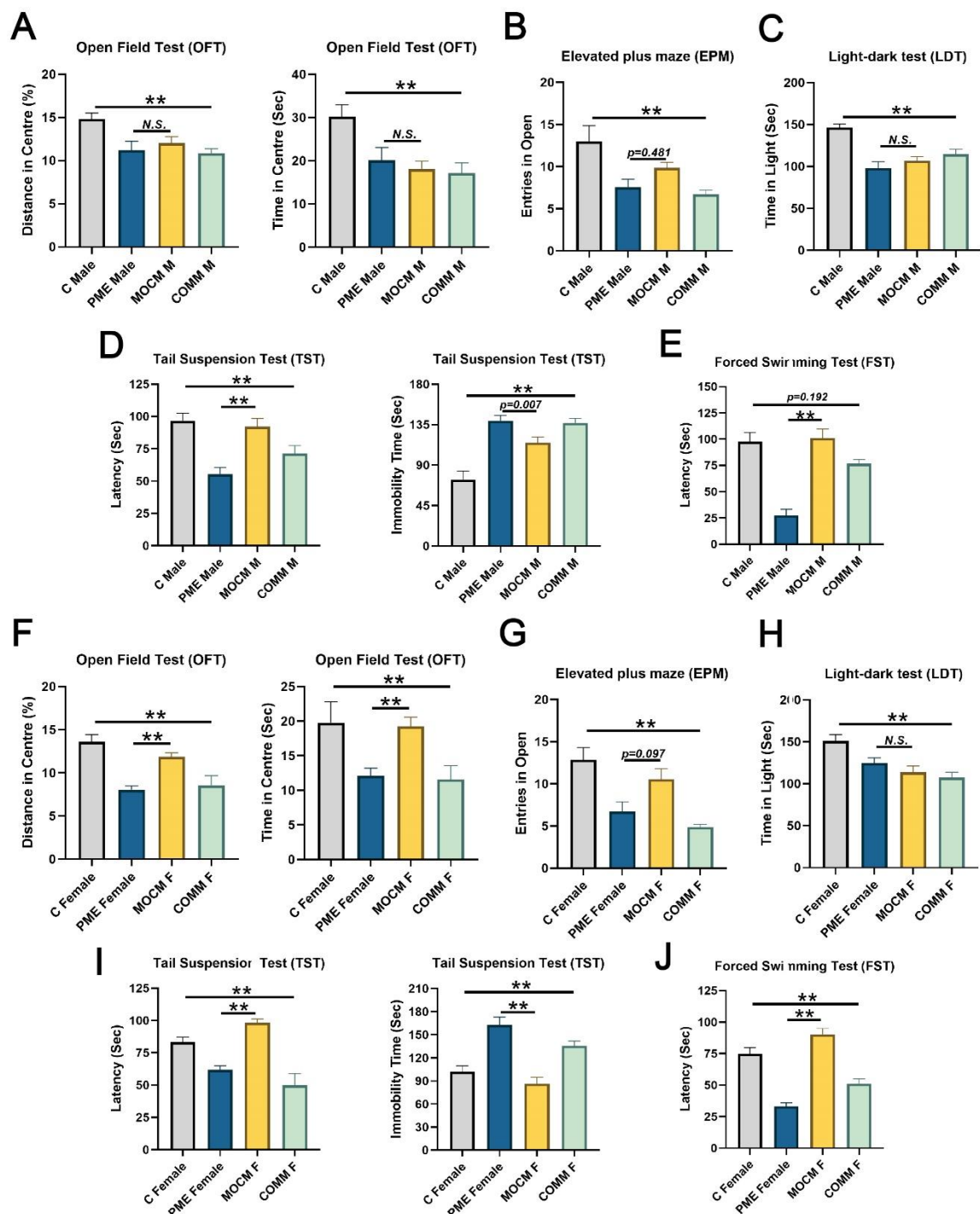


Fig. S5 Cross-fostering affects anxiety- and depression-like behaviors in offspring

Behavioral results of male offspring after cross-fostering. $n = 7-9$ per group. (A) Residence time and distance in the central zone in the OFT. (B) Number of entries into the open arms in the EPM. (C) Time spent in the light chamber in the LDT. (D) Latency to immobility and immobility time in the TST. (E) Latency to immobility in the FST. Behavioral results of female offspring after cross-fostering. $n = 7-9$ per group. (F) Residence time and distance in the central zone in the OFT. (G) Number of entries into open arms in the EPM. (H) Time spent in the light chamber in the LDT. (I) Latency to immobility and immobility time in the TST. (J) Latency to immobility in the FST. Data are expressed as mean $\pm$ SEM, $**P < 0.05$, N.S.: no significant difference ($P > 0.05$).

3.4 Maternal inulin supplementation ameliorated anxiety- and depression-like behaviors in prenatal METH-exposed offspring by reversing gut microbiota

To investigate whether the gut microbiota mediates prenatal METH exposure-induced anxiety- and depression-like behaviors in offspring and to explore inulin supplementation as a promising therapeutic strategy, supplementation with 1% inulin in the drinking water was administered during pregnancy (Fig. 5A). There was no significant difference in water intake between the Con+I and PME+I groups (Fig. 5B), indicating that the total inulin intake was comparable between the two groups. Inulin supplementation mitigated body weight loss induced by METH in maternal mice (Fig. S6A). The body weight change curve of the offspring mice showed that maternal inulin supplementation did not significantly alter the weight of the offspring (Fig. 5C, S6B).

Behavioral tests were conducted on the offspring of inulin-supplemented mothers demonstrated that maternal inulin supplementation alleviated anxiety- and depression-like behaviors in male offspring (Fig. 5D). Similar results were observed in female offspring (Fig. 5K-N).

Maternal inulin supplementation significantly reduced the Chao and ACE indices in the Con+I and PME+I groups compared to those in the Con and PME groups (Fig. 5E), indicating decreased bacterial diversity. The PCoA plot showed that the Con+I and PME+I groups clustered together and were distinct from the Con and PME groups (Fig. 5F), suggesting that maternal inulin supplementation reshaped and alleviated dysbiosis of the gut microbiota in the offspring. Differential analysis at the genus level identified three bacteria, *Turicibacter*, *Eubacterium_brachy_group*, and *norank_f_UCG_10*, whose abundance significantly changed after prenatal METH exposure and recovered following maternal inulin supplementation (Fig. 5G). All the results were also significantly different in female mice (Fig. 5K-N).

Hippocampal neurogenesis was assessed, and IF analysis of DCX revealed that the mean fluorescence intensity of DCX in the PME+I group was significantly increased compared to the PME group, and comparable to that in the Con and Con+I groups (Fig. 5H, I, O, P). There was no significant difference in the gene expression levels of key molecules in the Wnt signaling pathway between the Con+I and PME+I groups (Fig. 5J, Q), suggesting that maternal inulin supplementation alleviated the inhibition of the Wnt signaling pathway and hippocampal neurogenesis induced by prenatal METH exposure.

Intestinal damage was also assessed in the offspring. Maternal inulin supplementation improved the shortening of colorectal length induced by prenatal METH exposure (Fig. S6C).

The serum concentrations of dextran-FITC were significantly lower in the PME+I group than in the PME group (Fig. S6D). Additionally, the decreased protein and gene expression of tight junction proteins in the offspring caused by prenatal METH exposure were reversed after maternal inulin supplementation (Fig. S6E, F). There was no significant difference in intestinal inflammatory factors between the PME+I and Con+I groups (Fig. S6G)), suggesting that intestinal inflammation was alleviated. These patterns were also observed in female offspring (Fig. S6H-L). Serum ELISA showed that serum LPS and inflammatory factors (IL-6, IL-1 β , TNF- α) were significantly reduced in the PME+I group compared to than in the PME group (Fig. S6M-P), indicating that maternal inulin supplementation reversed prenatal METH exposure-induced systemic inflammation.

Hippocampal inflammation was also evaluated. The percentage of IBA1-positive area in the PME+I group was significantly lower than in the PME group, and comparable to the Con and Con+I groups (Fig. S6Q, R), suggesting that maternal inulin supplementation alleviated hippocampal microglial activation induced by prenatal METH exposure in offspring. The recovery of hippocampal inflammatory cytokine gene expression levels further supported this conclusion (Fig. S6S). These results were also significantly observed in female mice (Fig. S6T-V).

These findings suggest that maternal inulin supplementation ameliorated anxiety- and depression-like behaviors in prenatal METH-exposed offspring by reversing gut microbiota dysbiosis, increasing hippocampal neurogenesis, and reducing hippocampal inflammation.

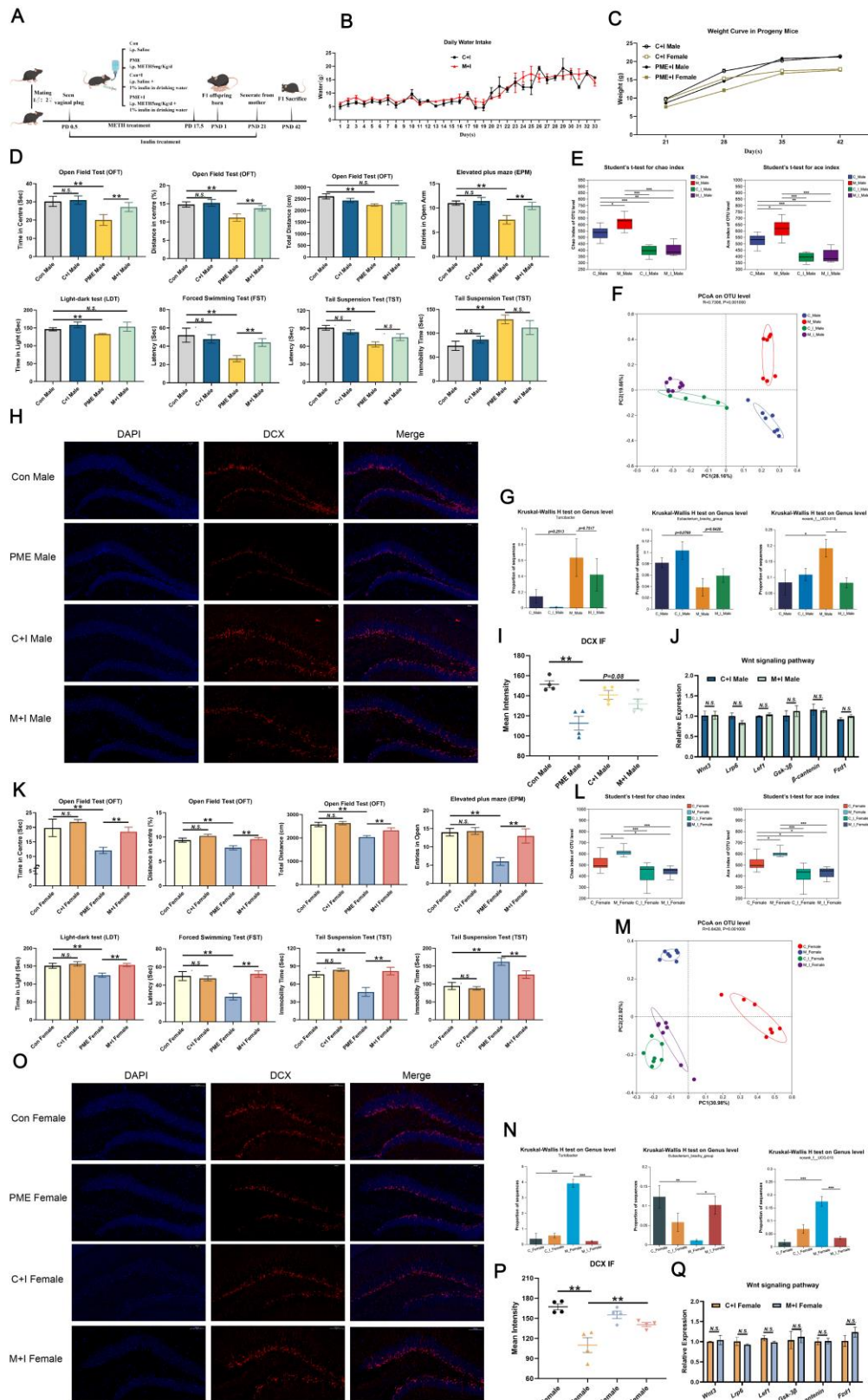


Fig. 5 Maternal inulin supplementation ameliorated anxiety- and depression-like behaviors in prenatal METH exposed-offspring by reversing gut microbiota

(A) Modeling flow chart of maternal inulin supplementation experiment. By Figdraw. (https://www.figdraw.com). (B) Water intake. (C) Weight curve in offspring mice. (D) Behavioral results of male offspring after maternal inulin supplementation. n = 7-8 per group. (E) Chao index and ACE index

of the gut microbiota. (F) PCoA plot analysis at OUT level of 16S rRNA gene sequences in male offspring. n = 7 per group. (G) The abundance of *Turicibacter*, *Eubacterium_brachy_group*, and *norank_f_UCG_010*. n = 7 per group. * $P < 0.05$. (H) Representative images of DCX IF staining (in red) and (I) statistical diagrams of DCX mean fluorescence intensity in the DG of the hippocampus in male offspring. n = 4 per group. (J) The relative mRNA expression levels of key molecule of the Wnt signaling pathway by RT-qPCR in male offspring. n = 3 per group. (K) Behavioral results of female offspring after maternal inulin supplementation. n = 7-8 per group. (L) Chao index and ACE index of the gut microbiota. (M) PCoA plot analysis at OUT level of 16S rRNA gene sequences in female offspring. n = 7 per group. (N) The abundance of *Turicibacter*, *Eubacterium_brachy_group*, and *norank_f_UCG_010*. n = 7 per group. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. (O) Representative images of DCX IF staining (in red) and (P) statistical diagrams of DCX mean fluorescence intensity in the DG of the hippocampus in female offspring. n = 4 per group. (Q) The relative mRNA expression levels of key molecule of the Wnt signaling pathway by RT-qPCR in female offspring. n = 3 per group. Data are expressed as mean $\pm$ SEM, ** $P < 0.05$, N.S.: no significant difference ($P > 0.05$).

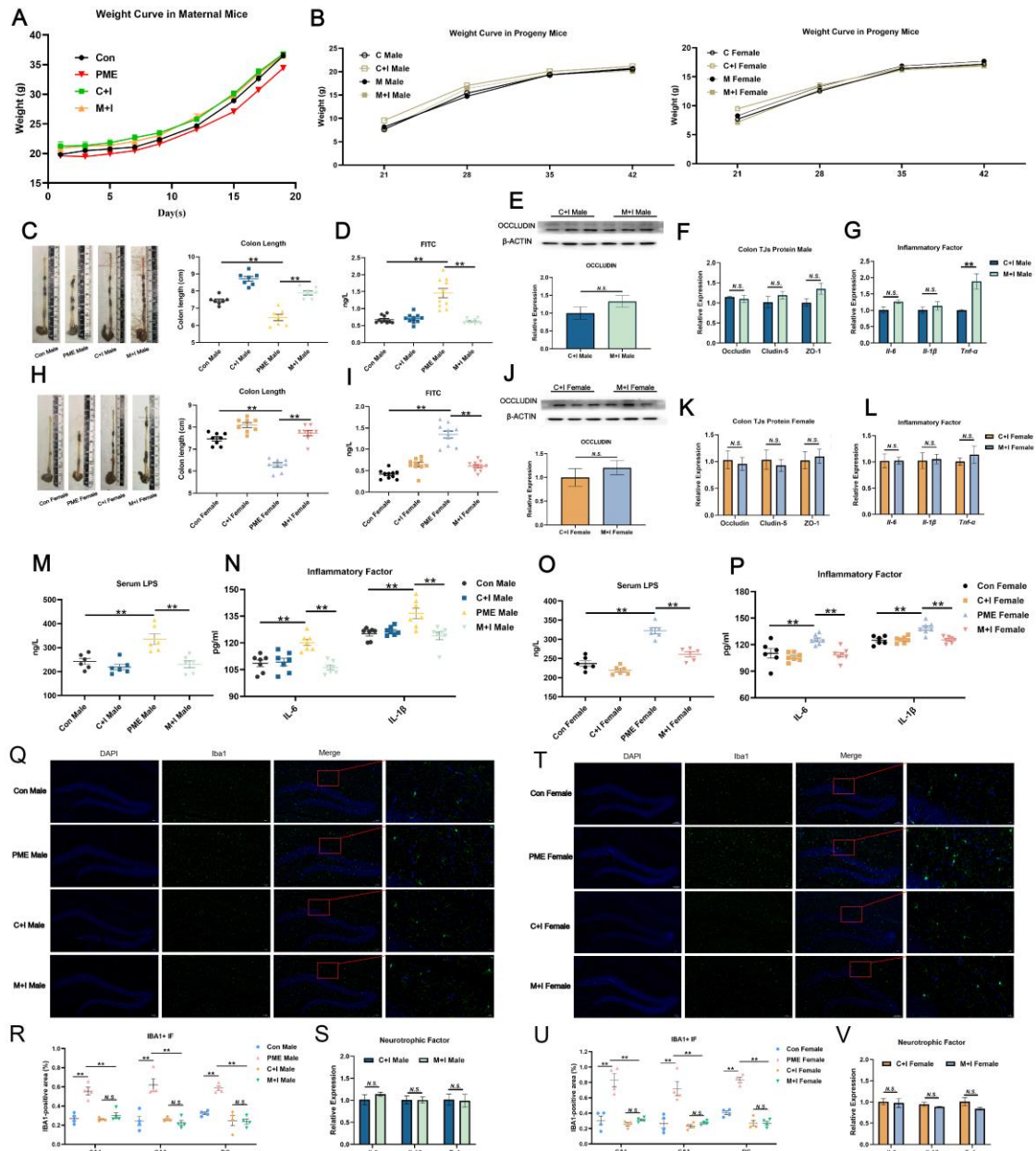


Fig. S6 Maternal inulin supplementation ameliorated intestinal barrier dysfunction, systemic inflammation and hippocampal inflammation in offspring

(A) Weight curve of maternal and (B) offspring mice. (C) Representative images of colon and statistical diagrams of colorectal length in male offspring, $n = 7$ per group. (D) The concentration of FITC-dextran in the serum of male offspring, $n = 10$ per group. (E) The protein expression levels of OCCLUDIN and β -ACTIN in male offspring, $n = 4$ per group. (F) The relative mRNA expression levels of tight junction proteins in male offspring, $n = 3$ per group. (G) The relative mRNA expression levels of inflammatory factors, $n = 3$ per group. (H) Representative images of colon and statistical diagrams of colorectal length in female offspring, $n = 7$ per group. (I) The concentration of FITC-dextran in the serum of female offspring, $n = 10$ per group. (J) The protein expression levels of OCCLUDIN and β -ACTIN in female offspring, $n = 4$ per group. (K) The relative mRNA expression levels of tight junction proteins in female offspring, $n = 3$ per group. (L) The relative mRNA expression levels of inflammatory factors, $n = 3$ per group. (M) The concentrations of LPS ($n = 6$) and (N) serum inflammatory factors ($n = 7$), including IL-6, IL-1 β , and TNF-

α , in the serum of male and (O, P) female offspring. (Q) Representative images of IBA1 IF staining (in green) in male offspring. Nuclei were labeled with DAPI (in blue). Scale bar, 100 μ m. (R) The percentage of IBA1-positive area in the CA1, CA3 and DG regions of the hippocampus in male offspring. n = 4 per group. (S) The relative mRNA expression levels of inflammatory factors in male offspring. n = 3 per group. (T) Representative images of IBA1 IF staining (in green) in female offspring. Nuclei were labeled with DAPI (in blue). Scale bar, 100 μ m. (U) The percentage of IBA1-positive area in the CA1, CA3 and DG regions of the hippocampus in female offspring. n = 4 per group. (V) The relative mRNA expression levels of inflammatory factors in female offspring. n = 3 per group. Data are expressed as mean $\pm$ SEM, ** P < 0.05, N.S.: no significant difference (P > 0.05).

3.5 Inulin supplementation in offspring ameliorated anxiety- and depression-like behaviors in prenatal METH-exposed offspring by reversing gut microbiota

Subsequently, to further confirm the role of inulin, we added 1% inulin to the drinking water of 3-week-old weaned offspring mice exposed to METH during pregnancy for 3 weeks (Fig. 6A). No significant difference in water intake was found between the 3wC+I group and the 3wM+I group (Fig. 6B), and inulin supplementation had no significant effect on the body weight of offspring mice, regardless of gender (Fig. 6C, D). Behavioral tests revealed that the behavioral differences caused by METH exposure during pregnancy disappeared after inulin supplementation in offspring (Fig. 6E), suggesting that inulin supplementation in offspring alleviated anxiety- and depression-like behaviors induced by prenatal METH exposure.

Additionally, the 3wM+I group showed a significant increase in DCX mean fluorescence intensity compared to the PME group, while the 3wC+I group showed a lower level than that in the other groups (Fig. 6F, G). Inhibition of the Wnt signaling pathway was also reversed after inulin supplementation in offspring (Fig. 6H). All the results were significantly different for both male and female mice (Fig. 6I-K).

Regarding intestinal damage, the prenatal METH exposure-induced reduction in colorectal length was reversed by inulin supplementation (Fig. S7A, E). After inulin supplementation, there was no significant difference in the protein or gene expression of tight junction proteins in the colon compared to those in the control group (Fig. S7B, C, F, G), except for *Zo-1*, suggesting that inulin supplementation improved the intestinal barrier damage caused by prenatal METH exposure. Intestinal inflammation (Fig. S7D, H), serum LPS levels (Fig. S7I, K), and serum inflammatory factors levels (Fig. S7J, L) also returned to normal after inulin supplementation in offspring.

Hippocampal inflammation was further examined. Compared to those in the PME group, the percentage of IBA1-positive areas in the CA1, CA3, and DG regions in the 3w+I group

were significantly reduced after inulin supplementation in offspring (Fig. S7M, N, P, Q), and the expression of inflammatory factors was also restored to normal levels (Fig. S7O, R). These results suggest that inulin supplementation in offspring alleviated prenatal METH exposure-induced microglial activation and hippocampal inflammation.

These findings suggest that inulin supplementation in offspring ameliorated anxiety- and depression-like behaviors in prenatal METH-exposed offspring by reversing gut microbiota dysbiosis, increasing hippocampal neurogenesis, and reducing hippocampal inflammation.

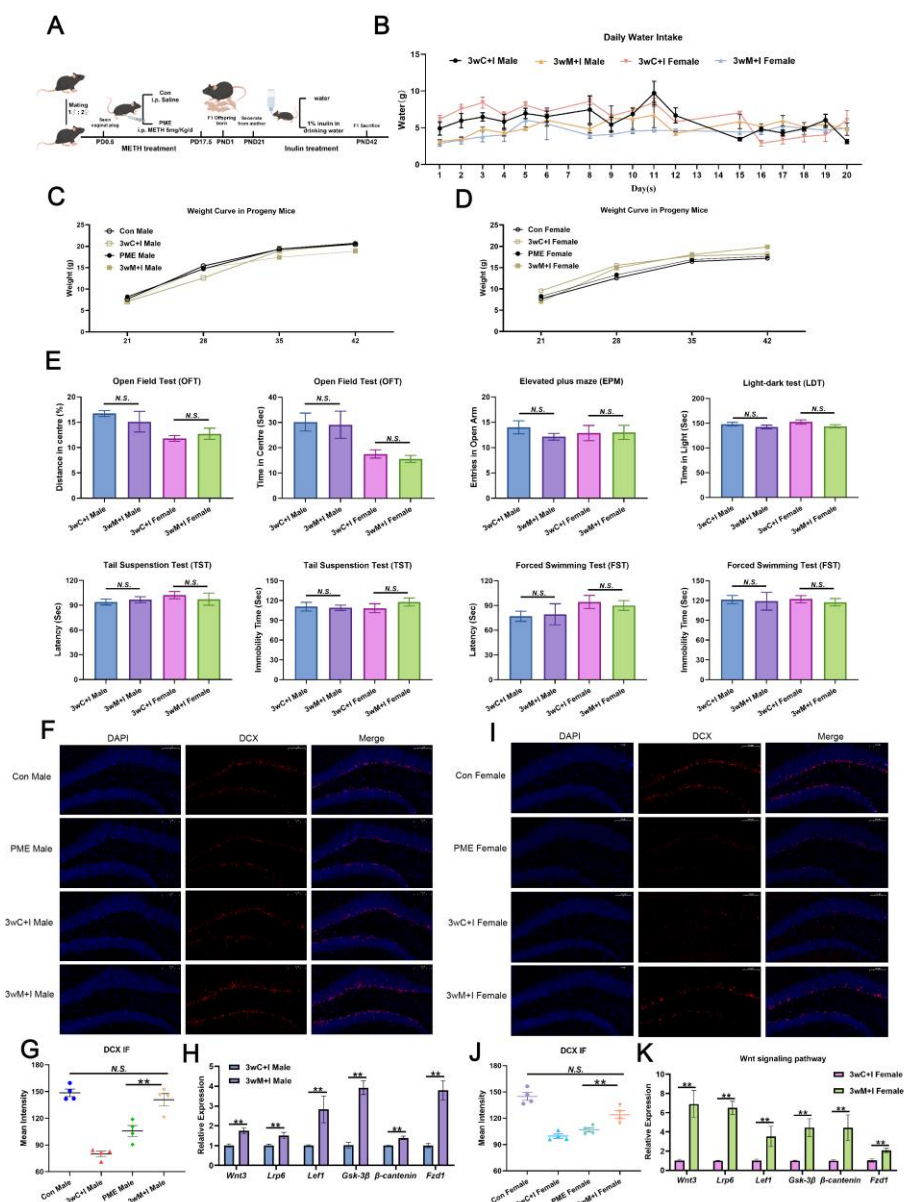


Fig. 6 Inulin supplementation in offspring ameliorated anxiety- and depression-like behaviors in prenatal METH exposed-offspring by reversing gut microbiota

(A) Modeling flow chart of inulin supplementation in offspring experiment. By Figdraw. (https://www.figdraw.com). (B) Water intake. (C) Weight curve in male and (D) female offspring mice. (E) Behavioral results of male and female offspring after inulin supplementation. n = 7-8 per group. (F) Representative images of DCX IF staining (in red) in male and (I) female offspring. (G) Statistical

diagrams of DCX mean fluorescence intensity in the DG of the hippocampus in male and (J) female offspring. n = 4 per group. (H) The relative mRNA expression levels of key molecule of the Wnt signaling pathway by RT-qPCR in male and (K) female offspring. n = 3 per group. Data are expressed as mean $\pm$ SEM, ** P < 0.05, N.S.: no significant difference (P > 0.05).

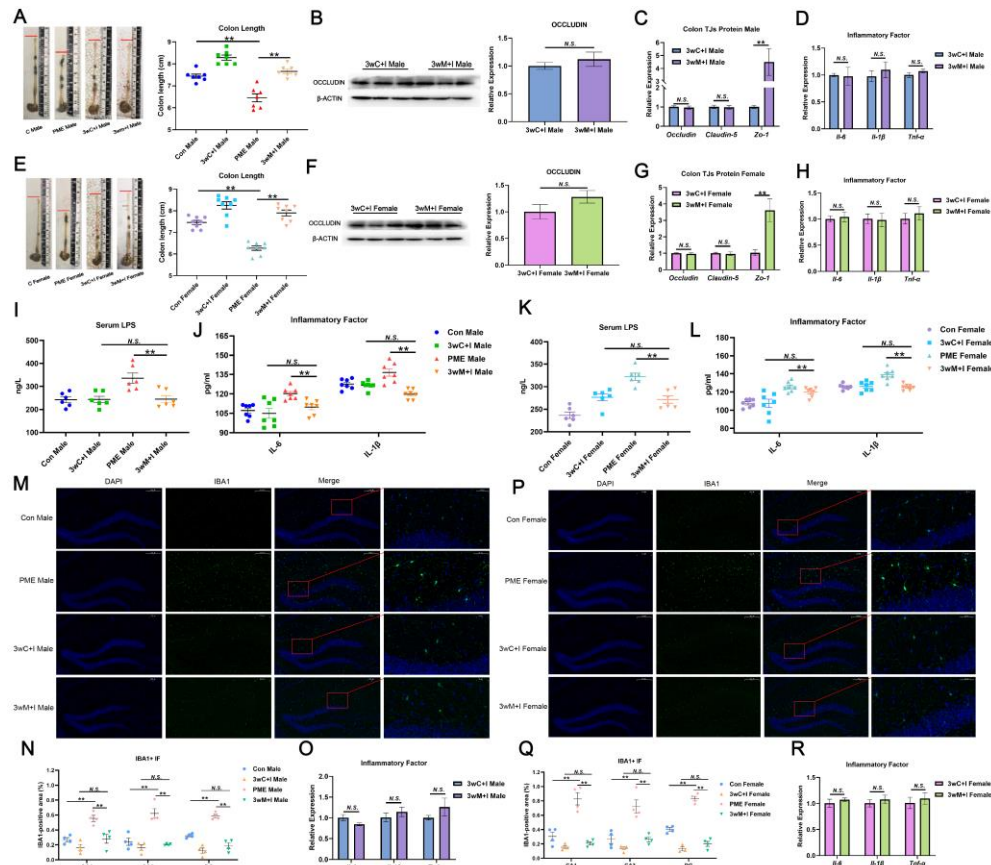


Fig. S7 Inulin supplementation in offspring ameliorated intestinal barrier dysfunction, systemic inflammation and hippocampal inflammation in prenatal METH-exposed offspring

(A) Representative images of colon and statistical diagrams of colorectal length in male and (E) female offspring. n = 7 per group. (B) The protein expression levels of OCCLUDIN and β -ACTIN in male and (F) female offspring. β -ACTIN served as a loading control. n = 4 per group. (C) The relative mRNA expression levels of tight junction proteins (*Occludin*, *Claudin-5*, and *Zo-1*) in male and (G) female offspring. n = 3 per group. (G) The relative mRNA expression levels of inflammatory factors, including *Il-6*, *Il-1 β* , and *Tnf- α* , in male and (H) female offspring. n = 3 per group. (I) The concentrations of LPS (n = 6) and (J) serum inflammatory factors (n = 7), including IL-6, IL-1 β , and TNF- α , in the serum of male and (K, L) female offspring. (M) Representative images of IBA1 IF staining (in green) in male and (P) female offspring. Nuclei were labeled with DAPI (in blue). Scale bar, 100 μ m. (N) The percentage of IBA1-positive area in the CA1, CA3 and DG regions of the hippocampus in male and (Q) female offspring. n = 4 per group. (O) The relative mRNA expression levels of inflammatory factors (*Il-6*, *Il-1 β* , and *Tnf- α*) in male and (R) female offspring. n = 3 per group. Data are expressed as mean $\pm$ SEM, ** P < 0.05, N.S.: no significant difference (P > 0.05).

3.6 Wnt inhibitor hindered the ameliorative effect of inulin on anxiety- and depression-like behaviors in offspring exposed to prenatal METH

To further explore whether the gut microbiota modulates anxiety- and depression-like behaviors in prenatal METH-exposed offspring via the Wnt signaling pathway, SB216763, a Wnt inhibitor, was used along with inulin supplementation in offspring (Fig. 7A). In behavioral tests of the offspring, inulin supplementation ameliorated anxiety- and depression-like behaviors caused by prenatal METH exposure, but the beneficial effects of inulin were reversed after the use of SB216763 (Fig. 7B). IF images of DCX staining of the hippocampus of the offspring were obtained, and the increase in DCX expression induced by inulin was reversed after the administration of SB216763 (Fig. 7C, D). Additionally, the activation of the Wnt signaling pathway was reversed (Fig. 7E), suggesting the suppression of hippocampal neurogenesis. All the results were also significantly different in both sexes (Fig. 7F-I). These results suggest that the Wnt inhibitor reversed the ameliorative effect of inulin supplementation on anxiety- and depression-like behaviors and on the inhibition of the Wnt signaling pathway and hippocampal neurogenesis.

We then performed IBA1 IF staining of the offspring mice's hippocampus. The percentage of IBA1-positive area was significantly lower in the PME+I group than in the PME group, but this decrease was reversed by the use of SB216763 (Fig. S8A, B). The use of SB216763 also reversed the decrease in the expression of inflammatory factors caused by inulin supplementation (Fig. S8C). All the results were also significantly different in both sexes (Fig. S8D-F). These results suggested that the Wnt inhibitor reversed the ameliorative effect of inulin supplementation on hippocampal inflammation.

These results suggest that the use of the Wnt inhibitor hindered the ameliorative effect of inulin on anxiety- and depression-like behaviors in prenatal METH-exposed offspring by inhibiting the Wnt signaling pathway and hippocampal neurogenesis.

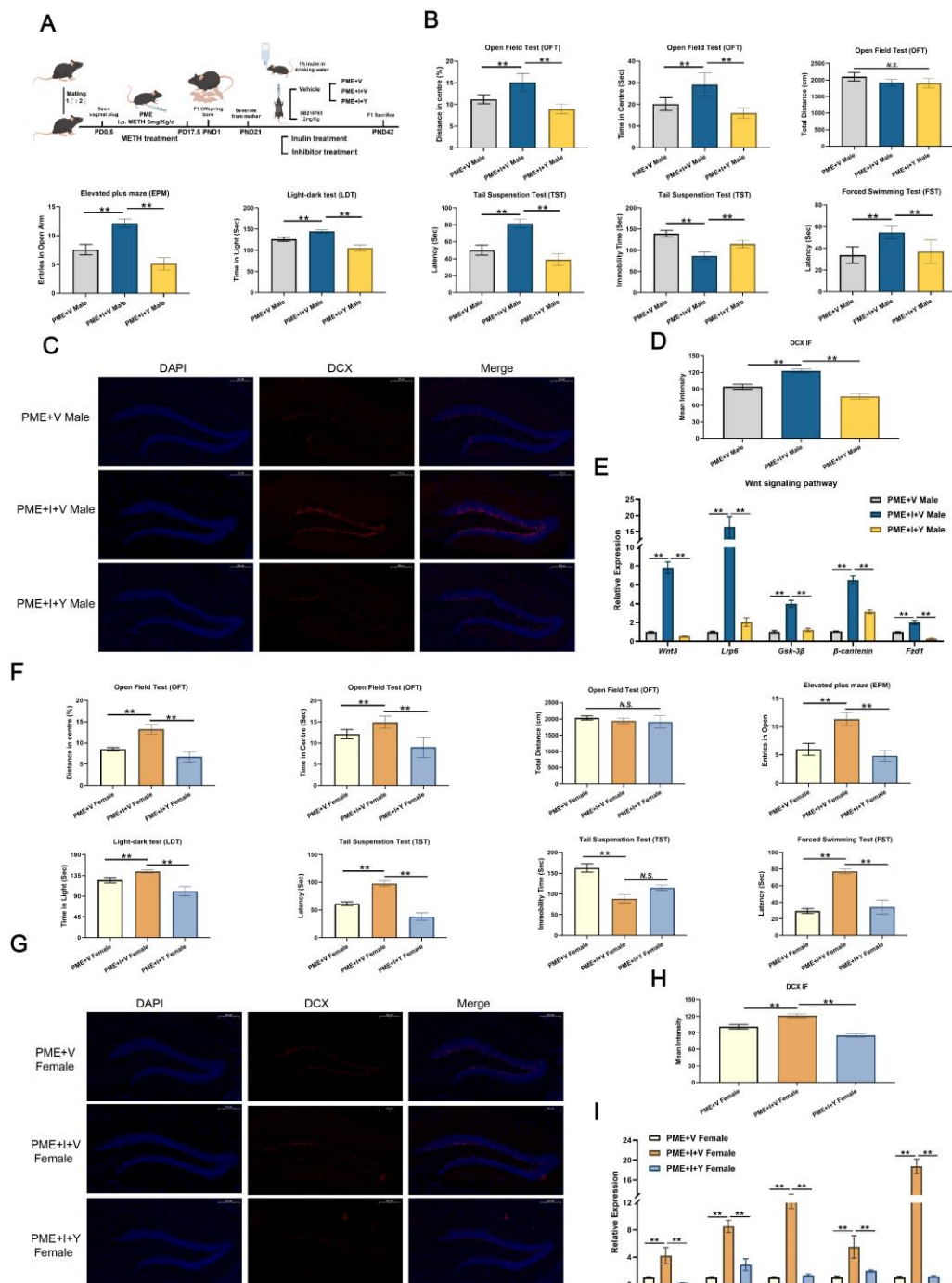


Fig. 7 Wnt inhibitor hindered the ameliorative effect of inulin on anxiety- and depression-like behaviors in offspring exposed to prenatal METH

(A) Modeling flow chart of Wnt inhibitor treatment experiment. By Figdraw. (<https://www.figdraw.com>).
 (B) Behavioral results of male and (F) female offspring. $n = 7-8$ per group. (C) Representative images of DCX IF staining (in red) in male and (G) female offspring. (D) Statistical diagrams of DCX mean fluorescence intensity in the DG of the hippocampus in male and (H) female offspring. $n = 4$ per group. (E) The relative mRNA expression levels of key molecule of the Wnt signaling pathway by RT-qPCR in male and (I) female offspring. $n = 3$ per group. Data are expressed as mean $\pm$ SEM and were analyzed using one-way ANOVA analysis of variance followed by the post-hoc Tukey test, $**P < 0.05$, *N.S.*: no significant difference ($P > 0.05$).

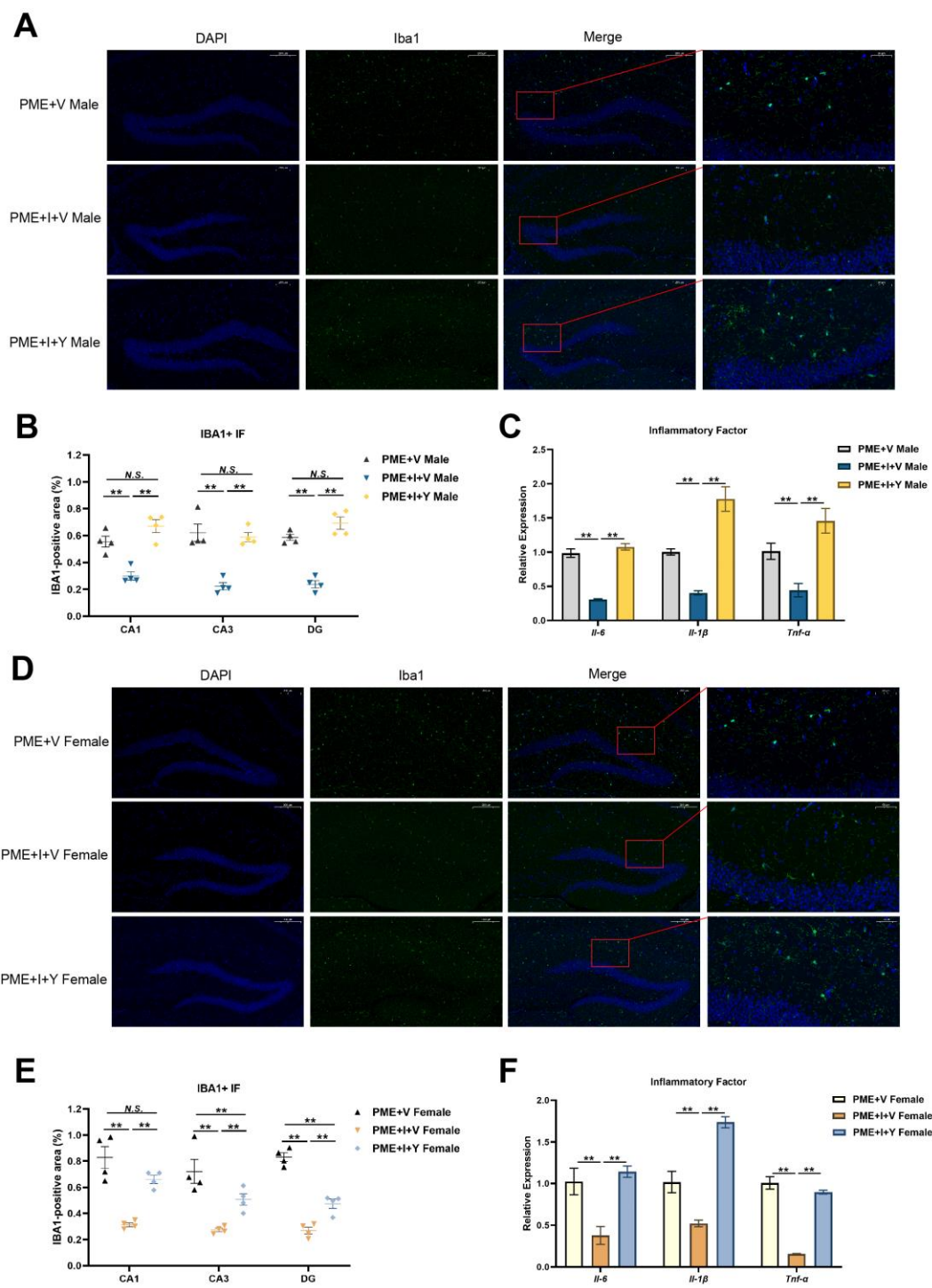


Fig. S8 Wnt inhibitor hindered the ameliorative effect of inulin on hippocampal inflammation in offspring exposed to prenatal METH

(A) Representative images of IBA1 IF staining (in green) in male and (D) female offspring. Nuclei were labeled with DAPI (in blue). Scale bar, 100 μ m. (B) The percentage of IBA1-positive area in the CA1, CA3 and DG regions of the hippocampus in male and (E) female offspring. n = 4 per group. (C) The relative mRNA expression levels of inflammatory factors (*Il-6*, *Il-1 β* , and *Tnf- α*) in male and (F) female offspring. n = 3 per group. Data are expressed as mean $\pm$ SEM and were analyzed using one-way ANOVA analysis of variance followed by the post-hoc Tukey test, ***P* < 0.05, N.S.: no significant difference (*P* > 0.05).

3.7 Wnt agonist ameliorated anxiety- and depression-like behaviors in offspring exposed to prenatal METH

Finally, we attempted to use Ros, which acts as a Wnt agonist, to ameliorate the anxiety- and depression-like behaviors in prenatal METH-exposed offspring (Fig. 8A). In addition to increasing the time spent in the light chamber in the LDT, the use of Ros reversed the anxiety- and depression-like behaviors of the offspring caused by prenatal METH exposure (Fig. 8B). DCX expression in the hippocampus decreased after exposure to METH during pregnancy but increased after the use of Ros (Fig. 8C, D), which was accompanied by activation of the Wnt signaling pathway (Fig. 8E), suggesting the restoration of impaired hippocampal neurogenesis. All the results showed a consistent trend in female offspring (Fig. 8F-I). These results suggested that Wnt agonists reversed the anxiety- and depression-like behaviors and inhibited the Wnt signaling pathway and hippocampal neurogenesis.

Additionally, an IBA1 IF assay was performed on the hippocampus of the offspring mice. Compared with those in the Con group, prenatal METH exposure significantly increased the percentage of IBA1-positive area, which was significantly reduced by the use of Ros (Fig. S9A, B). Ros also reduced the expression of inflammatory factors, which showed a significant increasing trend in offspring with prenatal METH exposure (Fig. S9C). All the results showed a consistent trend in female offspring (Fig. S9D-F). These results suggest that Wnt agonists alleviated the hippocampal inflammation induced by prenatal METH exposure.

These results suggest that the use of Wnt agonists ameliorated anxiety- and depression-like behaviors in prenatal METH-exposed offspring through the activation of Wnt signaling pathways and the restoration of impaired hippocampal neurogenesis.

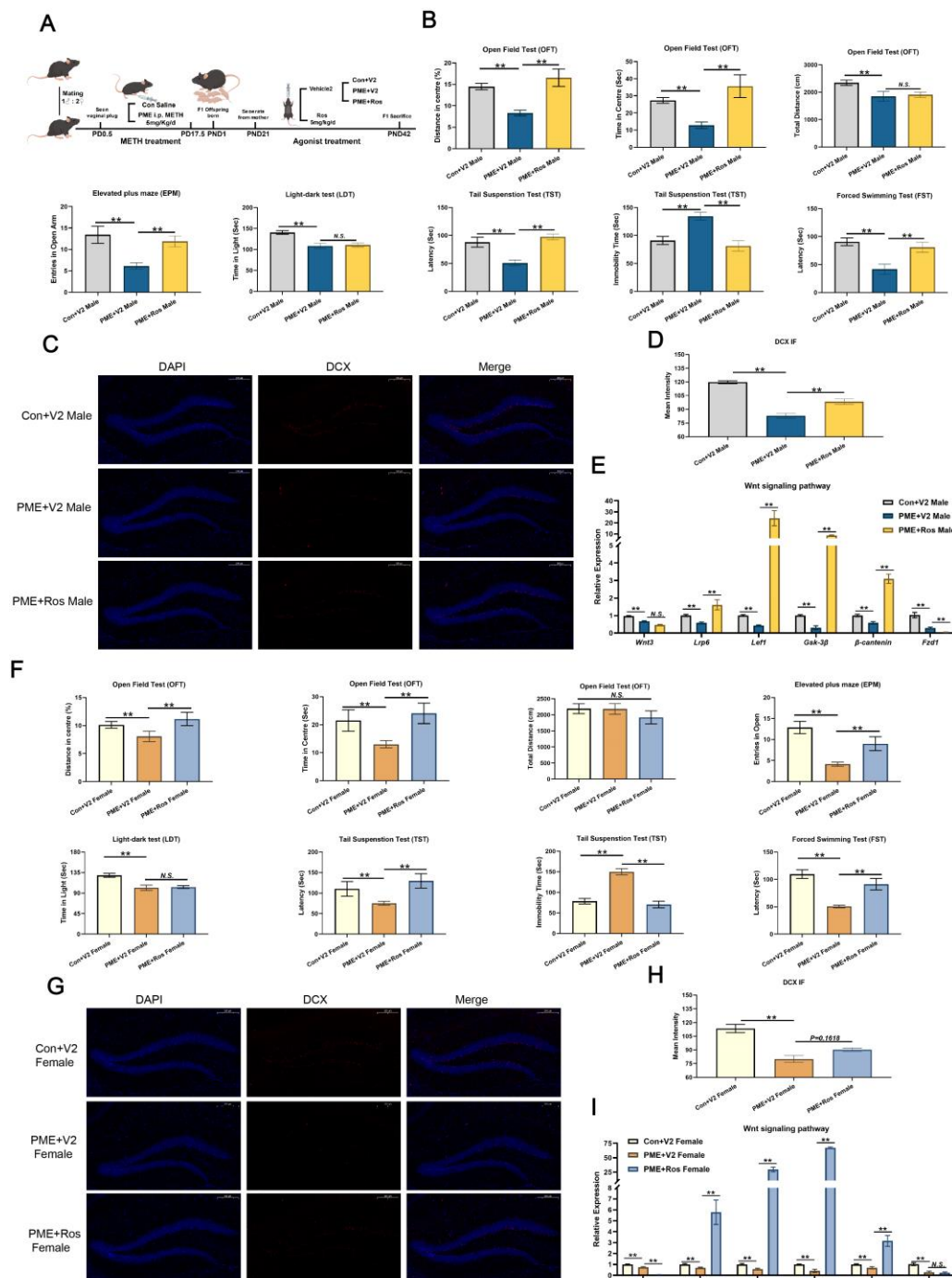


Fig. 8 Wnt agonist ameliorated anxiety- and depression-like behaviors in offspring exposed to prenatal METH

(A) Modeling flow chart of Wnt agonists treatment experiment. By Figdraw. (<https://www.figdraw.com>). (B) Behavioral results of male and (F) female offspring. $n = 7-8$ per group. (C) Representative images of DCX IF staining (in red) in male and (G) female offspring. (D) Statistical diagrams of DCX mean fluorescence intensity in the DG of the hippocampus in male and (H) female offspring. $n = 4$ per group. (E) The relative mRNA expression levels of key molecule of the Wnt signaling pathway by RT-qPCR in male and (I) female offspring. $n = 3$ per group. Data are expressed as mean $\pm$ SEM and were analyzed using one-way ANOVA analysis of variance followed by the post-hoc Tukey test, $**P < 0.05$, *N.S.*: no significant difference ($P > 0.05$).

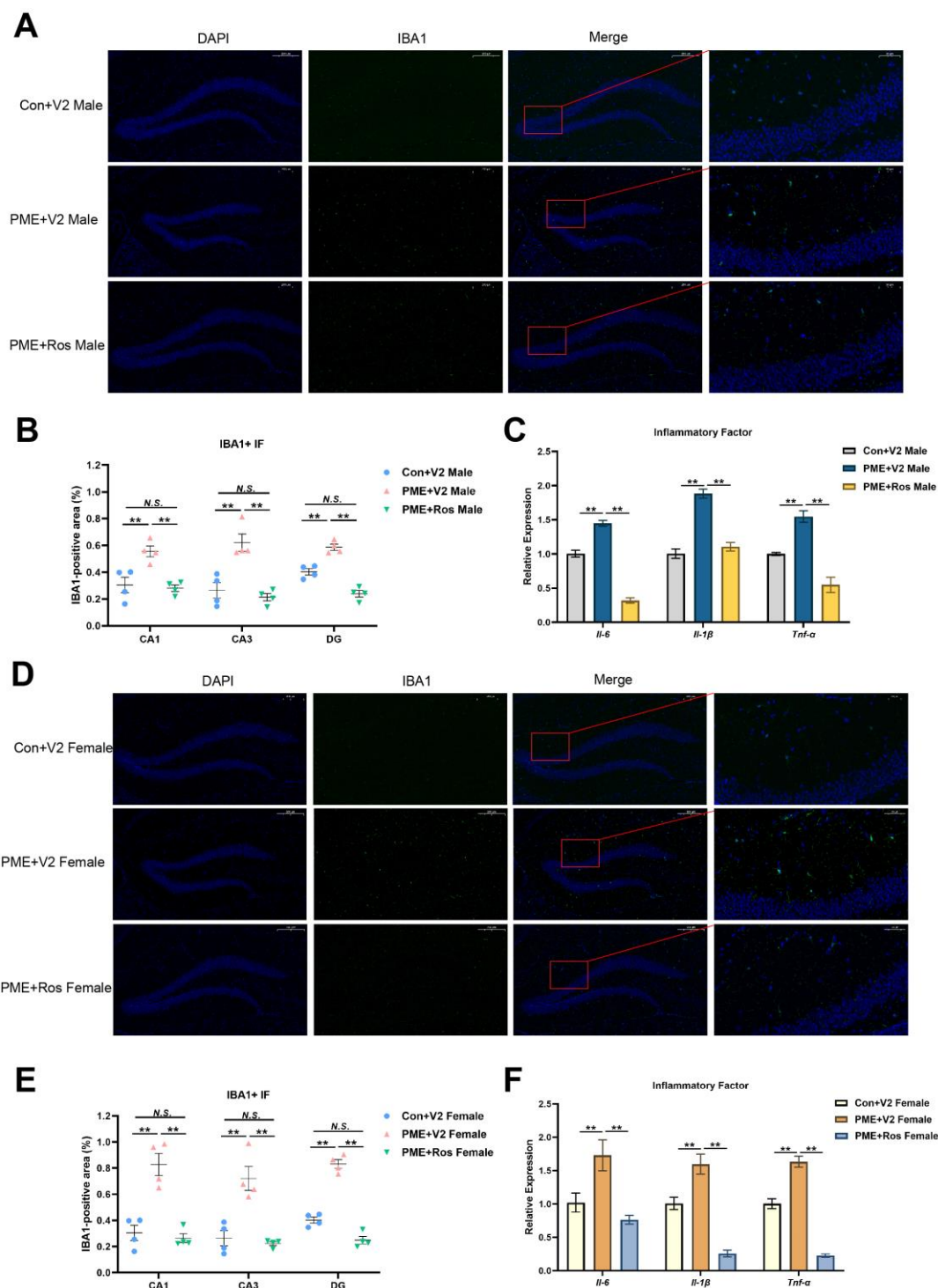


Fig. S9 Wnt agonist ameliorated hippocampal inflammation in offspring exposed to prenatal METH

(A) Representative images of IBA1 IF staining (in green) in male and (D) female offspring. Nuclei were labeled with DAPI (in blue). Scale bar, 100 μ m. (B) The percentage of IBA1-positive area in the CA1, CA3 and DG regions of the hippocampus in male and (E) female offspring. $n = 4$ per group. (C) The relative mRNA expression levels of inflammatory factors (*Il-6*, *Il-1 β* , and *Tnf- α*) in male and (F) female offspring. $n = 3$ per group. Data are expressed as mean $\pm$ SEM and were analyzed using one-way ANOVA analysis of variance followed by the post-hoc Tukey test, $**P < 0.05$, N.S.: no significant difference ($P > 0.05$).

4. Discussion

This study demonstrates that prenatal METH exposure has profound neurotoxic effects on offspring, leading to the development of anxiety- and depression-like behaviors. We found that these behavioral deficits are accompanied by increased hippocampal inflammation and reduced hippocampal neurogenesis in offspring. Furthermore, prenatal METH exposure resulted in intestinal barrier dysfunction and a significant disruption of the gut microbiota composition. To elucidate the role of the gut microbiota in mediating these neurodevelopmental consequences, we conducted cross-fostering and inulin supplementation experiments. Our findings reveal that cross-fostering with control dams partially reversed METH-induced behavioral deficits, suggesting a critical role for maternal microbiota transmission. Furthermore, both maternal and offspring inulin supplementation effectively remodeled the gut microbiota composition, alleviating anxiety- and depression-like behaviors in offspring. This beneficial effect was associated with a reduction in hippocampal inflammation, an increase in hippocampal neurogenesis, and activation of the canonical Wnt signaling pathway. These findings suggest that prenatal METH exposure disrupts gut microbiota homeostasis, leading to a cascade of neurodevelopmental alterations in offspring, which can potentially be mitigated by interventions targeting the gut microbiota and Wnt signaling pathway.

The maternal gut microbiota plays a significant role in shaping the initial gut microbiota of the offspring, which is crucial for immune development and neurodevelopment^[38-39], as evidenced by cohort studies showing correlations between the microbiota in the neonatal gut and the maternal gut at different time points^[40]. The maternal gut microbiota can be vertically transmitted to offspring, profoundly impacting the early colonization of the offspring gut microbiota^[41]. This vertical transmission was evident in our study, as the gut microbiota of 3-week-old offspring clustered according to their mothers. Furthermore, maternal inulin supplementation resulted in a significant remodeling of the maternal gut microbiota, which was mirrored by changes in the offspring's gut microbiota composition. These findings reinforce the notion of vertical transfer of maternal gut microbiota and its impact on the developing offspring gut microbiome.

Maternal microbial dysbiosis during pregnancy can lead to various metabolic and developmental abnormalities in offspring. Maternal obesity or a high-fat diet can mediate innate immune hypo-responsiveness and the progression of metabolic dysfunction associated with steatotic liver disease through alterations in the gut microbiota^[42]. In addition, the maternal

microbiome can affect fetal brain gene expression, potentially leading to abnormal thalamocortical axons. Manipulation of the maternal microbiome during pregnancy has been shown to affect offspring thalamocortical axons and alter somatosensory and tactile sensitivity^[43]. Research using maternal immune activation mouse models has also highlighted the role of the maternal microbiome in offspring development^[44]. Our study adds to this growing body of literature by demonstrating that prenatal METH exposure disrupts the gut microbiota composition in offspring, leading to anxiety- and depression-like behaviors. Notably, these changes in the microbiota, which are significantly related to mental disorders and intestinal dysfunction in 6-week-old offspring, are already present at 3 weeks of age. Specifically, we observed a significant decrease in the abundance of *Roseburia*, *Eubacterium_brachy_group*, and *Parabacteroides*, while the abundance of *Turicibacter* and *Erysipelatoclostridium* increased significantly. *Parabacteroides* has been found to be significantly reduced in children with autism^[45] and may play an anti-anxiety and depression role by producing short-chain fatty acids, especially butyric acid^[46]. *Eubacterium_brachy_group* and *Roseburia* are also significantly reduced in a variety of brain-related diseases^[47], and they have anti-inflammatory and antidepressant effects by producing short-chain fatty acids or affecting tryptophan metabolism^[48-49]. *Turicibacter* is a pro-inflammatory bacterium whose relative abundance is associated with circulating inflammation and abnormal behavioral outcomes such as anxiety-like behavior and sleepiness^[50-51]. *Erysipelatoclostridium* has also been reported to be positively associated with anxiety- and depression-like behaviors caused by Alzheimer's disease (AD) or chronic unpredictable mild stress^[52-53]. These findings suggest that the altered gut microbiota composition induced by prenatal METH exposure may contribute to the development of anxiety- and depression-like behaviors in offspring. This hypothesis was further supported by our cross-fostering experiments and inulin supplementation experiments. Inulin supplementation in both mothers and offspring effectively alleviated anxiety- and depression-like behaviors induced by prenatal METH exposure, accompanied by a recovery in the abundance of the aforementioned microbiota. These results suggest a potential mediating role for the gut microbiota in the development of anxiety- and depression-like behaviors induced by prenatal METH exposure.

Currently, the exact mechanisms by which the gut microbiota influences anxiety and depression remain unclear. The pathogenic mechanism underlying anxiety and depression is complex, while hippocampal neurogenesis has been regarded as one of the important mechanisms^[54-55]. The canonical Wnt signaling pathway has been reported as a regulator of

adult hippocampal neurogenesis^[56], and activation of the Wnt signaling pathway has been found to restore endogenous hippocampal neurogenesis in AD model mice^[57]. Our findings provide further evidence for the involvement of the canonical Wnt signaling pathway in the development of anxiety- and depression-like behaviors in offspring induced by prenatal METH exposure. We observed that anxiety- and depression-like behaviors in the offspring were associated with reduced hippocampal neurogenesis and inhibition of the Wnt signaling pathway. Furthermore, the use of Wnt agonists in offspring effectively relieved anxiety- and depression-like behaviors, suggesting that the canonical Wnt signaling pathway plays a key role in these behavioral changes. A growing body of research suggests that the gut microbiota may influence hippocampal neurogenesis. Studies have shown that mice with disrupted gut microbiota display impaired performance in hippocampal neurogenesis-dependent tasks^[58]. Remodeling of the gut microbiota reverses stress-induced hippocampal neurogenic damage, thereby promoting recovery from stress-induced depression^[59]. Interestingly, we also found that the use of Wnt inhibitors in conjunction with inulin supplementation in offspring hindered the ameliorative effect of inulin on anxiety- and depression-like behaviors in prenatal METH-exposed offspring. These results suggest that the gut microbiota may affect hippocampal neurogenesis by regulating the canonical Wnt signaling pathway and may play an anti-anxiety and anti-depression role. Although our findings provide strong evidence for the involvement of the gut microbiota and the Wnt signaling pathway in mediating prenatal METH exposure-induced anxiety and depression, further research is needed to elucidate the precise mechanisms underlying this interplay.

5. Conclusion

Our study revealed that prenatal METH exposure induced gut microbiota dysbiosis in offspring, leading to suppressed hippocampal neurogenesis and anxiety- and depression-like behaviors. This disruption is associated with the inhibition of the canonical Wnt signaling pathway. Importantly, we demonstrated that both maternal and offspring supplementation with inulin effectively remodeled the gut microbiota composition, promoting hippocampal neurogenesis and reversing anxiety- and depression-like behaviors. Furthermore, we found that a Wnt pathway inhibitor hindered the ameliorative effect of inulin, while a Wnt agonist mitigated the adverse effects of prenatal METH exposure, highlighting the crucial role of the Wnt signaling pathway in mediating these behavioral changes. These findings provide significant evidence for the adverse effects of prenatal METH exposure on offspring, contribute to our understanding of the transmission mechanism of maternal and infant

illnesses, and suggest a potential role for the gut microbiota in the development of mental disorders in offspring. Furthermore, our results highlight the potential of targeting the gut microbiota as a promising therapeutic strategy to prevent the adverse effects of maternal toxicant exposure. However, further prospective research is needed to confirm these findings and elucidate the precise mechanisms underlying the interplay between prenatal METH exposure, the gut microbiota, and Wnt signaling in offspring development.

Declarations

Ethics approval and consent to participate

The Medical Ethics Committee of Southern Medical University approved all animal care and experimental protocols (Approval Code: L2020075).

Consent for participate

Not applicable.

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Availability of data and materials

The original sequence datasets obtained from the 16S rRNA sequencing analysis of fecal bacteria have been submitted to the National Center for Biotechnology Information's Sequence Read Archive (NCBI SRA) and can be accessed using the accession number PRJNA1142237. In addition, the original RNA-seq datasets from the hippocampus have been submitted to the NCBI SRA under accession number PRJNA1141712.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

JHL performed the main part of the experiments and was a major contributor in writing the manuscript. JLL responsible for model construction and performed the IF and ELISA assay. XWL and YL contributed to data collection and analysis. JZY performed the Western blot

and analysis. LJC and KKZ directed the experimental methods and participated in manuscript revision. XLX and QW conceived and designed the study, and provided financial support. All authors read and approved the final manuscript. JHL and JLL contributed equally to this work.

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