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Neurotransmitter disturbances caused by methylmercury exposure: Microbiota-gut-brain interaction

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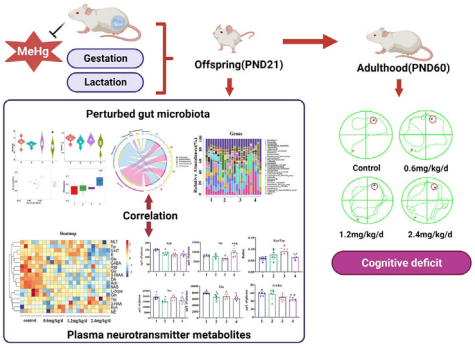
HIGHLIGHTS

- MeHg exposure induced dysregulation of neurotransmitters in rats.
- MeHg significantly changed the diversity of the gut microbiota.
- MeHg reduced tight junctions, which contributed to neurological responses.
- The gut microbiota mediated MeHg exposure and neurotransmitter disturbances.

GRAPHICAL ABSTRACT



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ABSTRACT

The fetal and early postnatal stages are periods of rapid brain development, during which, methylmercury (MeHg) exposure can cause lasting cognitive impairments. MeHg exposure disrupts neurotransmitter metabolites, which increased susceptibility to neurological responses. However, the neurotoxic mechanism underlying the MeHg-induced disruption of neurotransmitter metabolism requires further exploration. To this end, female Sprague-Dawley (SD) rats were administered NaCl (control group) or MeHg (0.6 mg/kg, 1.2 mg/kg and 2.4 mg/ kg body weight (bw), where the body weight refers to the dams) during the perinatal period, and then changes in neurotransmitter profiles and the gut microbiota of offspring were detected. The results showed that tryptophan (Trp) and tyrosine (Tyr) pathway neurotransmitter metabolites, including serotonin (5-HT), 5-hydroxy indole acetic acid (5-HIAA), *N*-acetyl-5-hydroxytryptamin (NAS), Tyr, dopamine (DA) and epinephrine (E), were significantly changed, and the Kynurenine/Tryptophan (Kyn/Trp) ratio was increased in the MeHg-treated groups. Meanwhile, acetylcholine (ACh) and neurotransmitters involved in the amino acid pathway were significantly reduced. Notably, MeHg treatment induced a significant reduction in tight junctions in the colon and hippocampal tissue. Furthermore, fecal

Abbreviations: Methylmercury, MeHg; Acetylcholine, ACh; Alzheimer's disease, (AD); Norepinephrine, NE; Epinephrine, E; γ-aminobutyric acid, GABA; Glutamic acid, Glu; Glutamine, Gln; 5-hydroxytryptamine, 5-HT; Dopamine, DA; Tryptophan, Trp; Kynurenine, Kyn; Levodopa, L-dopa; *N*-acetyl-5-hydroxytryptamine, NAS; Tyrosine, Tyr; 5-hydroxy indole acetic acid, 5-HIAA; Aspartic acid, Asp; 3-hydroxyanthranilic acid, 3-HAA; Melatonin, MLT; Glycine, Gly; Central nervous system, CNS; Blood-brain barrier, BBB; Gestation Day 5, GD 5; Postnatal Day 21, PND21; Morris water maze, MWM; Hematoxylin-eosin staining, H&E; Immunohistochemistry, IHC; Certified reference material, CRM; Electrospray ionization, ESI; Multiple reaction monitoring, MRM; Entrance potential, EP; Collision cell exit potential, CXP; Declustering potential, DP; Collision energy, CE; Principal component analysis, PCA; Orthogonal partial least squares discriminant analysis, OPLS-DA; Total cord blood mercury concentration, THg; Adonis and analysis of similarity, ANOSIM; Operational taxonomic units, OTUs; *Firmicutes/Bacteroidetes*, *F/B*; *Actinobacteria/Proteobacteria*, *A/P*.

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microbiota analysis indicated that the diversity and composition characteristics were significantly altered by MeHg exposure. Mediation analysis showed that the gut microbiota mediated the effect of MeHg treatment on the neurotransmitter expression profiles. The present findings shed light on the regulatory role of the gut microbiota in MeHg-disrupted neurotransmitter metabolic pathways and the potential impact of perinatal MeHg treatment on the "cross-talk" between the gut and brain.

1. Introduction

Methylmercury (MeHg) is a potent neurotoxic chemical substance that accumulates and biomagnifies in marine fish at high trophic levels (Go et al., 2021). Aside from MeHg intake through aquatic products, previous studies have shown that rice can be enriched in MeHg. For example, the average MeHg bioenrichment factor of rice is much higher than that of inorganic mercury in the Wanshan mercury mining area in southwest China, where the total soil mercury (THg) concentration is reported to be 34.2 μg/g, significantly higher than the national background concentration of 0.12 μg/g (Wang et al., 2021; Zhang et al., 2010). MeHg is transported via amino acid transporters in the form of a MeHg–cysteine complex, and readily crosses the placental barrier and blood–brain barrier (BBB) (Li et al., 2021). As a result, the fetal and early postnatal periods have been identified as the most vulnerable developmental stages, and the neurological effects of MeHg have raised concerns (Sakamoto et al., 2020; Tong et al., 2021). Analysis of a Faroese birth cohort revealed that maternal exposure to MeHg resulted in significant neurodevelopmental deficits at birth and during the early school years (Axelrad et al., 2007); however, studies in the Seychelles have observed no such effects (Grandjean et al., 1998; Myers et al., 2003). Additional longitudinal cohort studies of regular fish eaters in Hong Kong (Lam et al., 2013), Canada (Boucher et al., 2016), and Spain (Lozano et al., 2021) have found possible associations between long-term exposure to MeHg and abnormal physiological and behavioral outcomes, including general cognitive function and motor performance. Despite the extensive research and the identification of a number of neurobiological consequences of MeHg exposure, the underlying mechanisms remain largely unexplored. To protect MeHg-vulnerable populations, useful predictive biomarkers associated with future neurological symptoms must be identified to enable early detection, diagnosis, and treatment.

Neurotransmitters are key chemical molecules that transmit information between neurons or between neurons and effector cells (de Vries et al., 2022). Various neurotransmitters, including choline (acetylcholine), monoamines (catecholamines such as norepinephrine, epinephrine, dopamine, 5-HT, histamine, etc.), and amino acids (glutamate, γ-aminobutyric acid, glycine, etc.) (Snyder and Ferris, 2000), are essential to human mental and physical health. Thus, abnormal neurotransmitter concentrations or activities may lead to cognitive deficits and emotional abnormalities. Neurotransmitters are primarily present in the central nervous system (CNS) but are also found in some biological fluids, including plasma, cerebrospinal fluid, saliva, and urine. Research suggests that peripheral blood neurotransmitters are potentially indicative of neurological disorders (Chen et al., 2021). While some reports suggest that MeHg exposure is correlated with some neurotransmitter disturbances (Ben Bacha et al., 2020; Castoldi et al., 2006), systematic studies are still lacking, and detection of neurotransmitter contents remains challenging due to the mixing of neurotransmitters with other substances.

Accumulating evidence suggests that there is a potential regulatory relationship between the gut microbiota and neurotransmitters; this may be involved in neuronal development, CNS physiology, anxiety, cognitive regulation, and social behavior (Kennedy et al., 2017). Moreover, brain-gut "cross-talk" is mainly achieved through neurotransmitters and their precursors. Of note, the complex symbiotic microbes that colonize the mammalian digestive tract have multiple effects on the availability of Trp in the gut and are, therefore, thought to be a driving force affecting Trp metabolism in the gut. One study found that the gut microbiota is disturbed by xenobiotics of heavy metals and pesticides (Jin et al., 2017), and that repeated dietary MeHg intake induces alterations to the gut microbiota and metabolome in fish (Nielsen et al., 2018). In addition, an experimental study found that acute oral MeHg administration in adult rats induced a systemic-level gut microbiota disturbance and metabolic imbalance in rats (Lin et al., 2020). It is acknowledged that the perinatal period is a critical developmental stage for the neonatal microbiome and that maternal exposure to harmful substances is the only variable that shapes the microbial community of neonates and infants up to three weeks of age, an effect that persists even into adulthood (Dai et al., 2020). To date, the effect of perinatal MeHg exposure on regulation of the gut-brain axis in offspring is still unknown.

In the present study, a Sprague-Dawley (SD) rat model was employed to comprehensively evaluate the effects of perinatal MeHg exposure on 18 neuroactive metabolites in the Trp, Tyr and amino acid pathways and on the gut microbiota of offspring. Mediation analysis was also used to evaluate the gut microbiota as a potential mediator of the association between MeHg treatment and disturbances in neurotransmitter profiles. These findings provide mechanistic insights into the neurotoxic effects of MeHg.

2. Materials and methods

2.1. Animals and treatment

Twenty-four female and 12 male SD rats weighing 200 ± 20 g were obtained from Guizhou Medical University. Rats were housed under a constant temperature (22–24 °C) and humidity, with a 12 h light/12 h dark cycle. After acclimatization, the rats were caged together at a 2:1 ratio of males to females. After confirmation of maternal pregnancy, female rats were administered NaCl (4 mg/kg body weight (bw)) or 0.6 mg/kg, 1.2 mg/kg or 2.4 mg/kg bw MeHg (low-, medium-, and high-dose groups, respectively) by oral gavage. These doses were previously used under several exposure paradigms in rats, with the aim of avoiding maternal toxicity and allowing dose-response modeling of a wide range of endpoints. Based on this, our preliminary study showed that relatively few toxic endpoints were affected under the same dosing regime (Wang et al., 2022a; Wang et al., 2022b). The treatments were performed from gestational day 5 (GD5) to postnatal day 21 (PND21), which is the developmental stage that is most susceptible to the neurotoxic effects of MeHg. Each pup was reared with the same number of pups for 21 days to reduce the effect of litter size.

Our previous study on the bioaccumulation and distribution of MeHg in various organs observed sex differences, with higher MeHg concentrations in female rats (Li et al., 2018). In addition, another study of harbor seal pups in central California found that newborn pups acquired mercury from their mothers during fetal development, resulting in higher THg levels in females, and it was hypothesized that the observed sex differences between pups may be related to different rates of transplacental uptake, diffusion, distribution, and/or excretion between the sexes (Lian et al., 2021). Based on our prior findings and the limited sample size of this study, which may limit the biological significance of some of the results of the current study, we focused only on the effects on female offspring in this study.

For 16S ribosomal RNA analysis, at PND21, neurotransmitter levels and Hg measurements were performed on one female pup that was randomly selected from each litter in each treatment group. Moreover, biological samples were collected from these pups (fresh feces, brain, and blood) after sodium pentobarbital anesthesia. Thus, all three samples were taken from the same animal, as appropriate for subsequent correlation analysis and mediated effects analysis. Additionally, one pup was randomly selected from each litter in each group and the brain was obtained at PND21 for

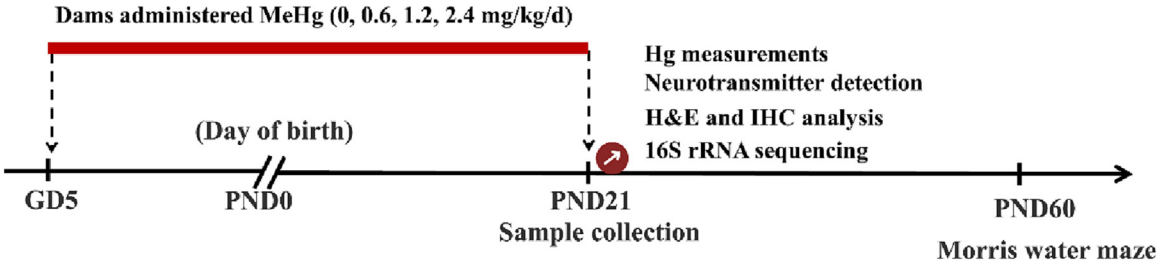

Fig. 1. Schematic workflow of the MeHg treatment in rat.

Nissl and H&E staining, while the small intestine and colon were obtained for H&E staining and immunohistochemistry. The remaining pups were reared to PND60 and a total of 15 animals per group were selected from each group for completion of the Morris Water Maze (MWM). The flow chart of the study design is shown in Fig. 1. All animal handling was approved by the Guizhou Medical University Institutional Animal Care and Use Committee, and a certificate of approval (2000868) is available upon request.

2.2. Morris water maze

To determine whether the cognitive abilities of adult offspring were altered by MeHg treatment during development, offspring completed the MWM, a hippocampus-dependent visuospatial learning memory task, at PND60. As we have described before (Wang et al., 2022a), the test lasted five days and consisted of four days of learning trials and one day of memory trials. For the first four consecutive days, the time taken was recorded for the rat to find the platform as the water escape latency. On the fifth day, the trajectory of the rat, the time and distance of the target quadrant activity, and the number of platform crossings were recorded within 120 s.

2.3. Hg measurements

As mentioned previously (Liu et al., 2021; Wang et al., 2022b), hippocampal tissue samples were enriched in gold trap after nitric acid digestion and reduction by $SnCl_2$, and Hg measurement was performed with cold vapor atomic fluorescence spectrometry. The CRM for Hg determination in the brain was TORT-3 (National Research Council, Canada). THg recovery in the CRM averaged at 96 %. The Relative Standard Devation (RSD) was <10 % when duplicate analyses were performed. Samples, blanks, and CRMs were run once every 10 samples to evaluate the analytical accuracy.

2.4. Nissl staining

Serial brain tissue sections of 4 μm thickness were obtained from 6 pups per group. The tissue sections were stained with toluidine blue for 3 min and then placed in 0.1 % glacial acetic acid for 30 s. The staining was terminated by tap water washing, and the degree of staining was controlled under a microscope. Staining was performed until the Nissl was dark blue and the background was light blue or colorless. Excess water was shaken off the sections, and the sections were oven-dried at 65 °C. The samples were then processed with xylene to transparency for 5 min and were then sealed with neutral resin. The experimental data were analyzed by Image Pro Plus 6.0 software.

2.5. H&E staining

The colon and brain tissues (6 pups per group) were fixed in fixative (10 % formalin) for 48 h to denature and coagulate the tissue and cell proteins. The fixed tissues were embedded in paraffin wax and cut into slices of approximately 4 μm. H&E staining was conducted according to the manufacturer's instructions (Solarbio, China). NanoZoomer Digital Pathology was used for image capture and viewing (Hamamatsu, Japan).

2.6. Immunohistochemistry (IHC)

The colon and brain tissues were sectioned to a thickness of about 4 μm. After dewaxing the tissue sections for transparency processing, they were repaired in citrate buffer (pH = 6.0) at high pressure and sealed with goat serum (Gibco, USA) for 1 h. The sections were then incubated with anti-Occludin (1:100, Abcam, USA), Claudin-1 (1:100, Abcam, USA), and Zonula occludens-1 (ZO-1; 1:500, Abcam, USA) antibodies, followed by secondary antibody (Abcam, UK) at 4 °C. Image Pro Plus 6.0 software was used to analyze the images and the optical density was represented by the mean optical density (MOD) value. Cells positive for Claudin-1, Occludin, and ZO-1 were counted by an investigator blinded to the experimental group assignments. Positive cell counts were performed in at least 3 randomly selected regions in each section, with 6 separate samples analyzed in each group. Cell counts were repeated at least 3 times in each selected region to obtain an average.

2.7. Analysis 16S ribosomal RNA sequencing data

Fecal samples were collected, and DNA extraction procedures were performed using a DNA kit (TIANGEN, China). The primers for 16S were 341F (5′-CCTACGGGNGGCWGCAG-3′) and 805R (5′-GACTACHVGGGTATCTAATCC-3′). The PCR amplification system contained 25 ng of template DNA, 12.5 μL of PCR premix, 2.5 μL of primers, and ultrapure water. PCR conditions were as follows: initial denaturation at 98 °C (30 s); followed by further denaturation at 98 °C (10 s), annealing at 54 °C (30 s), and final extension at 72 °C (10 min). Then, the PCR products were purified and quantified. Amplicon libraries size were assessed using an Agilent 2100 Bioanalyzer (Agilent, USA).The PCR amplification products were sequenced on an Illumina NovaSeq (Kapa Biosciences, USA) platform. Each sample was then normalized according to the SILVA (release 132) classifier. α and β diversity correlation metrics were evaluated with QIIME2 (IBM, USA) and plotted with the "R"package.

2.8. Measurement of neurotransmitter levels

Neurotransmitter concentrations were measured using an AB Triple Quad™ 5500+ tandem mass spectrometer (Foster City, CA, USA). 100 μL plasma was diluted by adding 300 uL of acetonitrile-water and then centrifuged (12,000 r/min, 10 min). Next, 4 μg/mL of 3,4-Dihydroxyphenylaceticacid was added and mixed well. The plasma mixture was then blown dry with nitrogen and re-dissolved with 100 uL of water-methanol (9:1, *v*/v). Finally, 2 uL was obtained for the analysis sample. The chromatographic conditions were as follows: XB-C18 column (100 mm × 2.1 mm, 2.6 μm); mobile phase, acetonitrile (B)-0.1 % formic acid aqueous solution (A); column temperature, 40 °C; flow rate, 0.2 mL/min; injection volume, 2 uL. The gradient elution is shown in Table S1. The mass spectrometry conditions were as follows: AB Triple Quad 5500+ System (SCIEX, USA); electrospray ion source (ESI); positive ion scan mode; multiple reaction monitoring mode (MRM); analysis time, 10 min; ion source spray voltage, 5500 V; temperature setting, 500 °C; Gas1, 45 Psi; Gas2, 50 Psi; dwell time, 50 ms; entrance potential (EP), 15 V; collision cell exit potential (CXP), 6 V. The

instrument parameters, sources of monitored ion pairs, and neurotransmitter standards are shown in Table S2 and Table S3.

2.9. Statistical analysis

Data were analyzed by SPSS statistics 25.0 software (IBM, USA) and the "R" package (v. 2.15.3). Continuous variables and normally distributed data were analyzed using one-way ANOVA followed by the LSD test, while the Kruskal-Wallis H-test was used for non-normally distributed data. The behavioral test results were statistically compared between groups using repeated measures ANOVA and the LSD test for multiple comparisons. Principal component analysis (PCA) was performed to observe group clustering and outliers. Differences between groups were further identified by applying supervised orthogonal projection to latent structure-discriminant analysis (OPLS-DA); R^2 and Q^2 were used to assess the accuracy and reliability of the models. ANOSIM analysis (Analysis of similarities) was performed to test whether the differences between the groups were significantly greater than the differences within the groups, and thus, whether the groups were significantly different.Correlation analysis between two continuous variables was performed using pairwise Pearson analysis. Accordingly, the Benjamini and Hochberg False Discovery method was used to adjust the *P*-value for multiple statistical tests. FDR < 0.1 was considered statistically significant. In this study, MeHg exposure was taken as the independent variable, plasma neurotransmitter levels were the dependent variable, and intestinal microorganisms were the mediator variable. A mediator test model of "MeHg exposure-bacterial taxa-plasma neurotransmitter" was established. The mediation analysis involved division of the total effect of MeHg on the measured neurotransmitter levels into the direct effect of exposure and the mediating effect, the latter of which accounted for mediating factors. The proportion of mediation was the mediating effect of the gut microbiota divided by the total effect of MeHg on the neurotransmitter concentrations; that is, the proportion of the total effect attributable to the mediating factors. This mediating effect analysis enabled quantification of whether and to what extent the effects of MeHg on neurotransmitter metabolism are attributable to the gut microbiota, providing insight into the mechanisms by which MeHg exposure produces neurotransmitter impairments. Before performing the mediation analysis, we identified significant correlations between gut microbes and plasma neurotransmitters, and examined the effect of MeHg on neurotransmitter levels, in order to verify these potential causal links. The mediation effects were analyzed by PROCESS (v3.4.1) software using the Bootstrap method, and the confidence intervals (BootLLCI, BootULCI) were considered significant if they did not contain 0, and insignificant if they did.

3. Results

3.1. Learning and memory impairments in offspring

A dose-dependent MeHg treatment study was performed in rats to explore the causal relationship between maternal MeHg exposure and adverse health outcomes and to investigate the potential mechanisms. The Nissl staining results showed that neurons in the control group were regularly arranged, with abundant neuronal cytosol and Nissl bodies (Fig. 2A). In the low-dose group, there was a small number of loose and missing neurons, the number of surviving neurons was slightly lower, with no significant difference compared to the control group (Fig. 2A, B), while the number of surviving neurons was significantly lower in the offspring of the medium- and high-dose MeHg-treated rats ($F = 21.33$, $P < 0.05$; Fig. 2B), with lighter and blurred Nissl body staining as well as neuronal cell lysis and necrosis. Perinatal treatment with MeHg increased the Hg load in the pups. Fig. 2C shows the THg in hippocampal tissue of offspring at PND21.

To evaluate the effects of maternal MeHg administration on cognitive function in adult offspring rats, the MWM was performed with offspring at PND60 (Fig. 2D, E). After the first four consecutive days of training, rats in the MeHg-treated groups took longer to find the platform ($F = 158.58$, $P < 0.05$; Fig. 2F), and the escape latency of each group gradually decreased as the training time increased ($F = 147.86$, $P < 0.05$; Fig. 2F). MeHg treatment significantly increased the time to the first platform crossing ($H = 38.34$, $P < 0.05$; Fig. 2G) and reduced the number of platform crossings ($H = 19.08$, $P < 0.05$; Fig. 2H) in the offspring. These findings suggest that MeHg exposure produced neurotoxicity with cognitive impairment.

3.2. Disrupted neurotransmitter metabolism in the plasma of offspring

PCA was used to study the extent of neurotransmitter metabolite alterations in the plasma of the rats. A total of 18 neurotransmitter metabolites

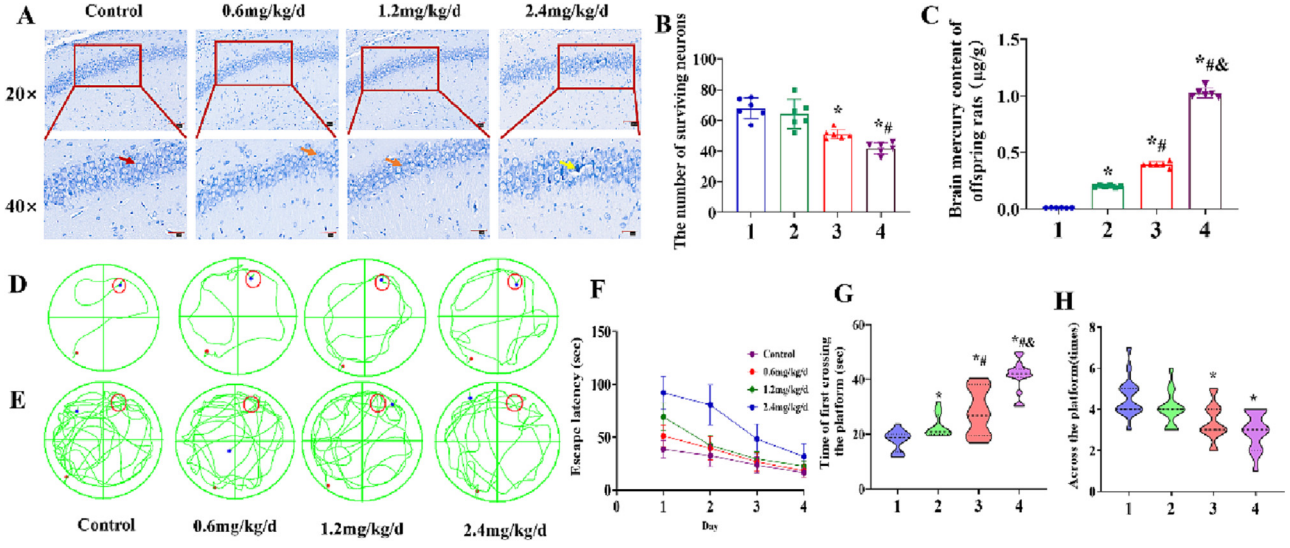

Fig. 2. Effects of maternal MeHg treatment on cognitive deficits and neuronal damage.
(A) Representative diagram of Nissl staining in the hippocampus CA1 region, $n = 6$. (B) The number of surviving neurons statistics, n = 6. The red arrow shows the surviving neurons. The orange arrows show the loose and missing neurons. The yellow arrows show neurons with pyknotic nuclei. The yellow arrows showed the nuclei pyknosis neurons (C) THg contents in the hippocampus of pups, n = 6. (D) and (E) are representative traces of learning trials on day 4 and memory trials on day 5, respectively, $n = 15$. (F) Escape latency statistics, n = 15. (G) Time spent in the probe trial statistics, n = 15. (H) Number of platform crossings statistics, n = 15. Scale bars: 50 μm. 1, 2, 3, and 4 represent the control, 0.6 mg/kg/d, 1.2 mg/kg/d, and 2.4 mg/kg/d MeHg groups, respectively. $^{*}P < 0.05$ vs. Control, $^{\#}P < 0.05$ vs. 0.6 mg/kg/d MeHg group, $^{\&}P < 0.05$ vs. 1.2 mg/kg/d MeHg group.

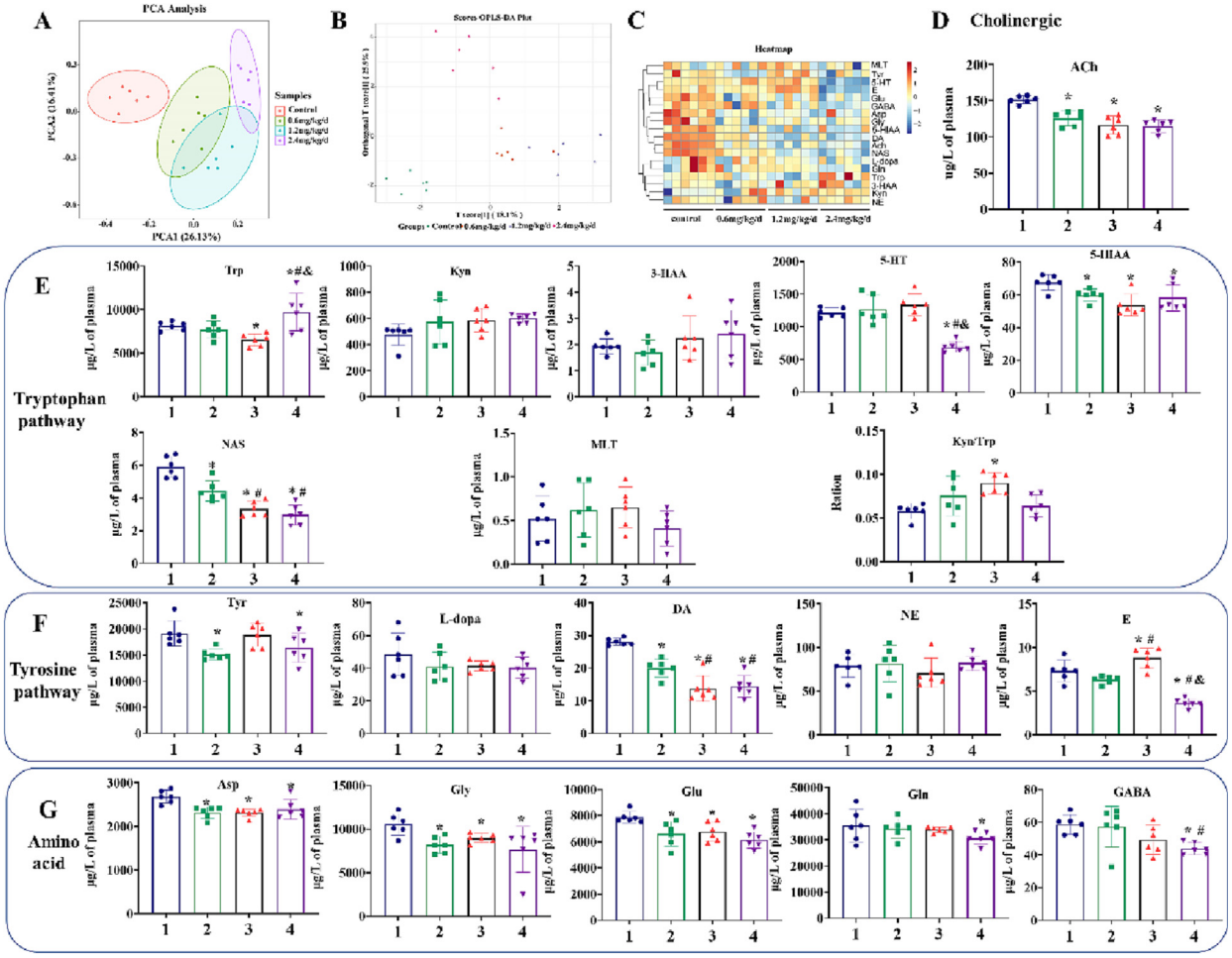

Fig. 3. MeHg treatment disrupted neurotransmitter metabolism in plasma.
(A) PCA plot analysis of neurotransmitters between different groups. (B) OPLS-DA plot analysis of neurotransmitters between different groups. (C) Heatmap of differential neurotransmitter expression in plasma. (D), (E), (F) and (G) are histograms of neurotransmitter contents in the cholinergic, tryptophan, tyrosine, and amino acid pathways in plasma. 1, 2, 3, and 4 represent the control, 0.6 mg/kg/d, 1.2 mg/kg/d, and 2.4 mg/kg/d MeHg groups, respectively. Data are reported as means ± SD, n = 6. $^{*}P < 0.05$ vs. Control, $^{\#}P < 0.05$ vs. 0.6 mg/kg/d MeHg group, $^{\&}P < 0.05$ vs. 1.2 mg/kg/d MeHg group.

were included in the PCA, with two principal components (PC1/PC2) accounting for 42.54 % of the total variance; these components were selected for the construction of the score plot. In the PCA scoring plot of the positive ion mass spectrometry scan, clear separation was observed between the control and MeHg-treated groups (Fig. 3A). OPLS-DA was then performed to enhance the sample separation observed in the PCA analysis. R^2 represents the goodness of fit, and the cross-validation parameter Q^2 indicates the predictive power of the model. In the OPLS-DA model, the R^2Y (0.886) and Q^2 (0.803) were significant, indicating that the model had a good fit and predictive power, and the score plot showed clear separation in the expression levels of neurotransmitters in the offspring of the control group versus the MeHg-treated groups (Fig. S1, Fig. 3B). The above results indicate that maternal MeHg treatment resulted in significant alterations in neurotransmitters in rats (Fig. 3C).

Multiple comparisons of plasma neuroactive metabolite levels in the different groups were then performed. In terms of cholinergic neurotransmitters, ACh was reduced in all MeHg-treated groups (Fig. 3D). In the tryptophan metabolic pathway (Fig. 3E), Trp was decreased in the medium-dose group but showed a significant increase in the high-dose group. 5-HIAA and NAS were decreased in all MeHg-treated groups, and 5-HT was only decreased in the high-dose group. Moreover, the Kyn/Trp ratio was increased in the MeHg-treated groups. In the tyrosine metabolic pathway (Fig. 3F), Tyr and DA were reduced in MeHg-treated groups, and E was only decreased in the high-dose group. For the amino acids (Fig. 3G), Asp, Gly, and Glu were decreased in all MeHg-treated groups, and Gln and GABA were reduced in the high-dose group.

3.3. Gut-brain barrier damage by disrupting tight junctions in offspring

H&E staining was further performed on the colon to evaluate intestinal barrier function, and Fig. 4A shows that the colonic tissue structure of the control group was intact, with neat and tight arrangement of crypt, gland, and cup cells; in contrast, each MeHg-treated group showed damaged intestinal mucosa, disordered arrangement of glands, significantly reduced crypt and cup cells. The Occludin, Claudin-1, and ZO-1 proteins are critical markers of intestinal mechanical barrier function. IHC staining was performed to assess whether MeHg affected the tight junction expression level in the colon (Fig. 4B). Consistent with the H&E staining results, Claudin-1, Occludin, and ZO-1 protein expression levels in the colon were reduced in the MeHg-treated groups (Fig. 4C, D, E). Similarly, in the control group, the hippocampal structure was clear, the neuronal cells were neatly arranged, the cytosol was intact, the cytoplasm was evenly stained, and the nuclei were obvious, while the number of neurons in the hippocampal vertebrae was reduced, the arrangement was disordered, and the nuclei were concentrated in the MeHg-treated groups (Fig. 4F). The Claudin-1, Occludin, and ZO-1 protein expression levels in the hippocampus were also down-regulated to various degrees by MeHg treatment (Fig. 4G, H, I, J). This suggests that MeHg may compromise the integrity of the blood-

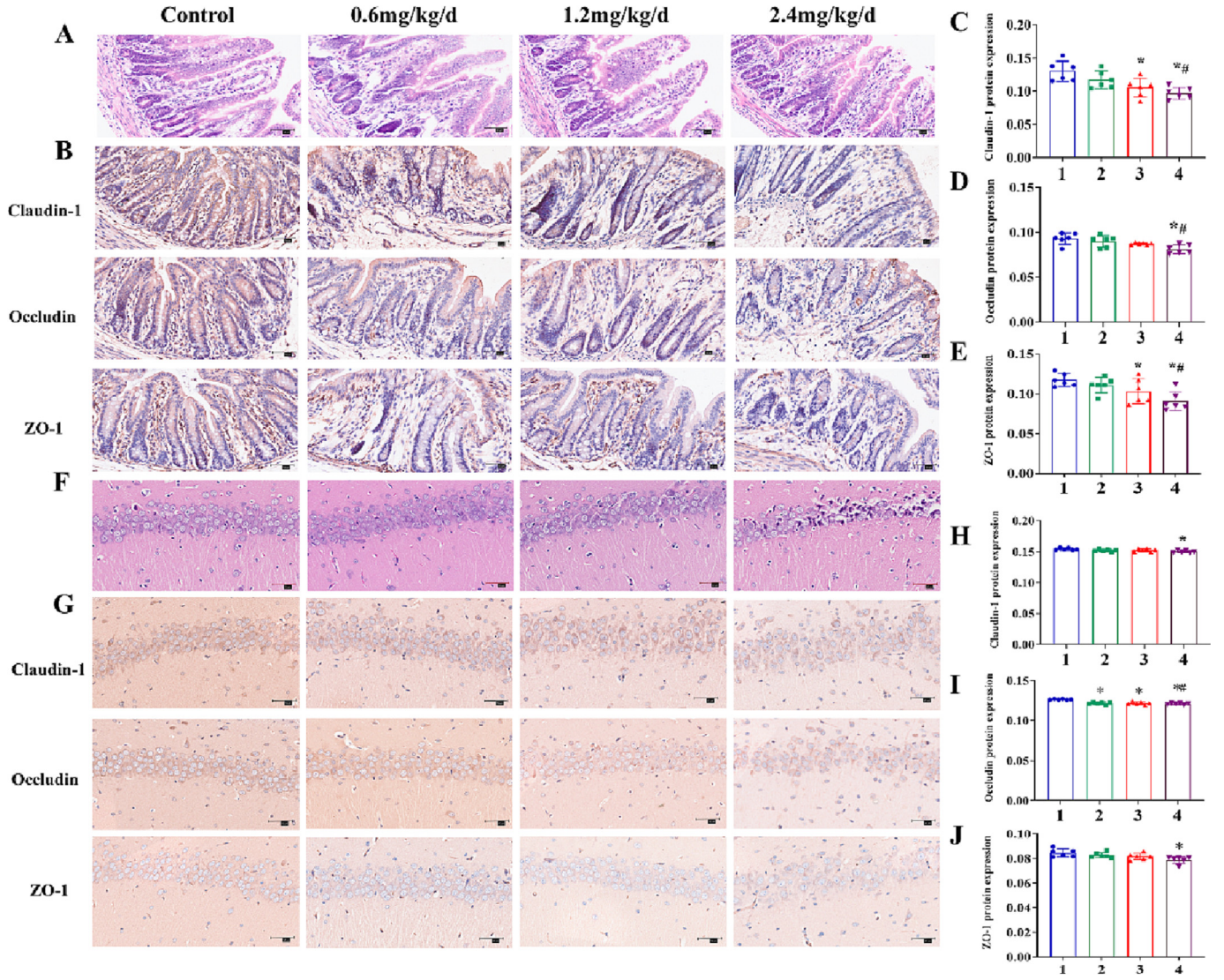

Fig. 4. MeHg treatment reduced tight junctions in the colon and brain in rats.
(A) and (B) are representative images of H&E and IHC of the colon, respectively. Histogram of the protein levels of Claudin-1, Occludin, and ZO-1 in the colon (C, D, E). (F) and (G) are representative images of H&E and IHC of the brain, respectively. Histogram of the protein levels of Claudin-1, Occludin, and ZO-1 in the hippocampus (H, I, J). 1, 2, 3, and 4 represent the control, 0.6 mg/kg/d, 1.2 mg/kg/d, and 2.4 mg/kg/d MeHg groups, respectively. Scale bars: 50 μm. Data are reported as means ±SD, $n = 6$. $^{*}P < 0.05$ vs. Control, $^{\#}P < 0.05$ vs. 0.6 mg/kg/d MeHg group, $^{\&}P < 0.05$ vs. 1.2 mg/kg/d MeHg group.

brain barrier in pups, allowing tight junction proteins to move out of the cerebrovascular system, resulting harmful substances to enter the circulatory system and CNS via the gut-brain axis, thus exacerbating cognitive dysfunction.

3.4. Gut microbiota profile composition in offspring

α diversity analysis was performed based on OTUs sequences. The Shannon index, which reflects the uniformity of the distribution of the gut microbiota, was reduced in the high-dose MeHg-treated group compared with the control group ($P < 0.05$, Fig. 5A), while a decrease in the Simpson index was also observed (though statistically insignificant, Fig. 5B). Mainstream PCA was performed to analyze β diversity. As shown in Fig. 5C, the gut microbiota of the control and MeHg-treated groups were in relatively different regions, and the high-dose group was further isolated from the control group. ANOSIM analysis indicated significant separation of the gut microbiota communities of the control and high-dose groups ($P < 0.05$, Fig. 5D), suggesting that MeHg exposure reduced the diversity of the gut microbiota in rats.

In terms of phylum classification, Fig. 5E shows the composition of the gut microbiome at the phylum level. *Firmicutes* and *Bacteroidetes* were dominant in the feces, followed by *Proteobacteria, Epsilonbacteraeota* and *Chlamydiae*, etc. The *F/B* ratio (*Firmicutes/ Bacteroidetes*) was increased, while the *A/P* ratio (*Actinobacteria/ Proteobacteria*) was reduced after MeHg treatment (Fig. 5F, G). In terms of family classifications, MeHg treatment induced increases in the abundances of *Prevotellaceae, Lactobacillaceae, Veillonellaceae, Tannerellaceae*, and *Desulfovibrionaceae* and decreases in the abundances of *Lactobacillaceae, Bacteroidaceae, Muribaculaceae, Ruminococcaceae, Bacteroidetes_unclassified, Erysipelotrichaceae,* and *Streptococcaceae* (Table S4). At the genus level, a total of 118 taxa were detected, among which, 11 differential taxa were identified (in the MeHg-treated groups compared to the control group). The abundances of *Prevotella_9, Alloprevotella, Parabacteroides, Helicobacter* and *Roseburia* were elevated, while levels of *Lachnospiraceae_UCG-010,* and *Ruminococcaceae_UCG-005* were reduced. Moreover, two unclassified bacteria (*Muribaculaceae_unclassified* and *Bacteroidetes_unclassified*) were significantly reduced in the MeHg-treated groups (Fig. 5H, Fig. S2, S3), while *Chlamydia* was absent in the MeHg-treated groups (data not shown).

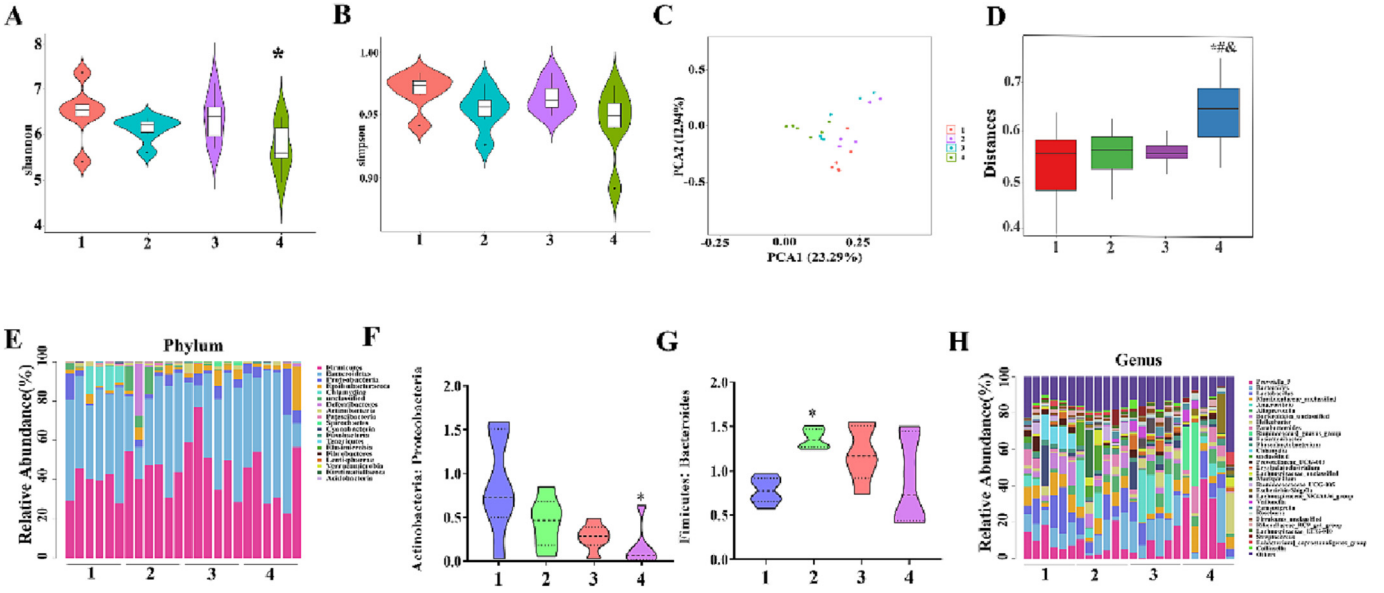

Fig. 5. Maternal MeHg perturbed gut symbiosis in offspring rats.
(A, B) Analysis of the alpha diversity of the gut microbiota by the Shannon and Simpson indices. (C) PCA plots describing microbial beta diversity. (D) Beta diversity based on weighted UniFrac ANOSIM analysis in different groups. (E) Relative abundance of gut microbiota at the phylum level. (F, G) *Firmicutes*: *Bacteroidetes* ratio and *Actinobacteria*: *Proteobacteria* ratio. (H) Relative abundance of gut microbiota at the genus level. n = 6. $^{*}P < 0.05$ vs. Control, $^{\#}P < 0.05$ vs. 0.6 mg/kg/d MeHg group, $^{\&}P < 0.05$ vs. 1.2 mg/kg/d MeHg group.

3.5. Gut dysbiosis contributed to MeHg-induced neurotransmitter disorders in offspring

Next, the potential functional interactions between MeHg treatment, gut microbes, and plasma neurotransmitters were evaluated. The control as well as the low-, medium-, and high-side MeHg-treated offspring rats were included in this analysis. Of the 11 bacterial taxa at the genus level and 18 neurotransmitters associated with MeHg treatment, 9 bacterial taxa at the genus level were found to be significantly correlated with both THg and 9 neurotransmitters according to pairwise Pearson analysis

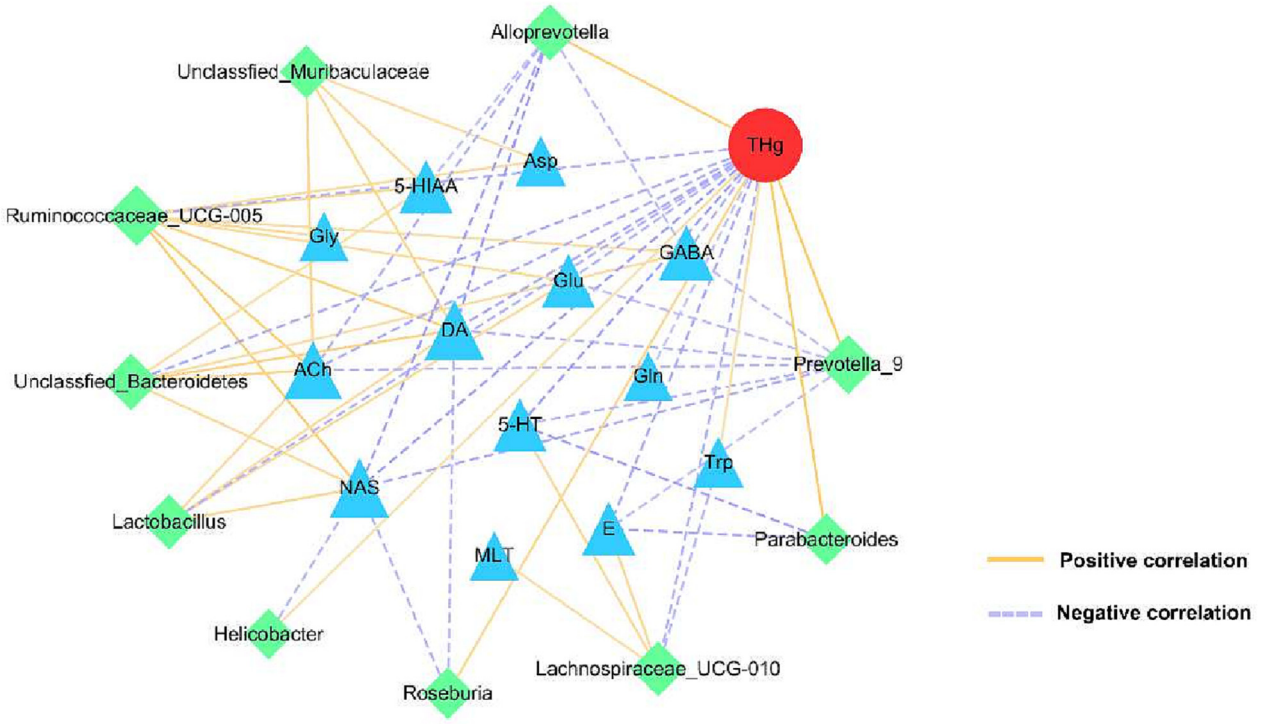

Fig. 6. Correlations between THg, neurotransmitters, and genera.
Pairwise Pearson correlation analysis between the gut microbes that were significantly associated (FDR < 0.1) with THg and neurotransmitter metabolism. Each substance is represented by different colors, as follows: THg (red), neurotransmitter metabolism (blue), and gut microbiota (green). The scale (right legend) represents the degree of positive (yellow) or negative (purple) correlation.

(Fig. 6). We further selected 8 of these bacterial species that were significantly associated with THg and examined their correlations with the altered neurotransmitters, which were not included in this analysis due to the disappearance of *Chlamydia* in the MeHg-treated groups.

Mediation analysis was performed to identify whether bacterial taxa mediated the effect of MeHg treatment on neurotransmitter disturbances. The results indicated that MeHg may contribute to neurotransmitter disturbances by affecting the gut microbiota at the abundance level. Specifically, *Alloprevotella* was found to mediate the impact of MeHg on 5-HT, DA, and E disturbances (mediation proportion = 55.49 %, 73.76 %, and 96.67 %, respectively). *Parabacteroides* mediation was found to account for 87.15 %, 70.11 %, 74.44 % and 61.49 % of the impact of MeHg on ACh, NAS, DA and E disturbances. Helicobate, as a significant mediator, mediates the NAS disturbances caused by MeHg with a mediation ratio of 13.94 %. The mediation of Ruminococcaceae_UCG-005 accounted for 55.96 %, 41.93 %, 26.48 %, 40.07 %, 41.30 %, 24.09 % and 31.01 % of the MeHg-mediated disorders of ACh, Trp, 5-HT, NAS, DA, E and Glu. In addition, inconsistent mediation effects were observed for some gut microbes; that is, mediation effects (indirect effect/total effect) were >100 %. This may be because the direct and indirect effects are in opposite directions such that the total effect is less than the indirect effect (Table S5).

4. Discussion

The host gut microbiota is emerging as an important regulator of brain activity and behavior. MeHg is a notoriously potent neurotoxin that can be transmitted from mother to offspring, and the gut microbiota composition of offspring is thought to be regulated by the mother. Therefore, the present study comprehensively explored the effects of perinatal MeHg exposure on the gut microbiota composition and neurotransmitter expression profiles of offspring. The results revealed that MeHg treatment caused delays in the WMW and altered neurotransmitter metabolic pathways, particularly for the Trp, Tyr, and amino acid metabolic pathways. Mediation analysis and 16S rRNA gene sequencing showed significant microbiota dysregulation that appeared to be partially associated with the levels of neurotransmitters and corresponding metabolites.

Increasing evidence suggests that MeHg exposure induces learning and memory impairments. In this study, MeHg exposure was found to alter neurotransmitter metabolism, and this was correlated with cognitive deficits in the host. The central cholinergic system is key to the normal maintenance of learning and memory functions, and ACh is an important transmitter of the central cholinergic nervous system, which is involved in physiological activities related to cognition. Reduced cholinergic neural activity, decreased synthesis of ACh, and an altered number of choline receptors are the pathological basis for cognitive impairments (Picciotto et al., 2012). Rats studies have shown that MeHg treatment significantly reduces the levels of ACh in the cortex (Hrdina et al., 1976), and that administration of rice contaminated with MeHg significantly decreases ACh levels in the rat brain (Li et al., 2018). In line with this, the current results indicated that MeHg treatment produced a significant reduction in ACh levels in offspring, and it is hypothesized that reductions in ACh are at least partially involved in the neurotoxicity of MeHg.

We further explored the metabolic pathways of the Trp and Tyr neurotransmitters. The levels of the Trp pathway metabolites, including 5-HT and its metabolite 5-HIAA, as well as NAS, exhibited decreasing trends. Additionally, Tyr, DA, and E were significantly changed by MeHg treatment. These findings are partially in line with previous studies reporting significant reductions in some neurotransmitters in the tryptophan metabolic pathway in MeHg-treated rats (Lin et al., 2021). 5-HT is a major metabolite of Trp that affects almost every aspect of brain activity, playing an important role in neuronal differentiation and migration, learning and memory, mood, and energy metabolism (Cryan and Leonard, 2000). Deficient 5-HT in the CNS affects the normal functioning of the brain and may trigger the emergence of emotional behaviors and psychiatric disorders (King et al., 2008). In the current study, a significant decrease in 5-HT was observed. However, very few reports have assessed the association between MeHg exposure and Trp metabolic pathway dysfunction, particularly the Kyn pathway of Trp metabolism. Activation of the Kyn pathway is thought to contribute to the pathogenesis of psychiatric disorders (Zhu et al., 2020). Recently, the adverse effects of Kyn metabolism on the pathogenesis of neurological disorders have attracted attention. For instance, increased Kyn/Trp ratios in plasma were observed in depression and Parkinson's patients (Widner et al., 2002; Zhou et al., 2018). The conversion of Trp to Kyn is mediated by indoleamine 2,3-deoxygenase (IDO) and tryptophan 2,3-deoxygenase (TDO), which play important roles as rate-limiting enzymes (Lim et al., 2017). The current study found that the Kyn/Trp ratio was markedly elevated, presumably related to the activation of the IDO or TDO enzymes.

The tyrosine pathway is involved in the synthesis of monoamine neurotransmitters (e.g., DA, NE and E) and other neuroactive substances (e.g., DOPAC, 3-MT and HVA). DA is an important metabolite in the tyrosine pathway and plays a key physiological role in motor, mood, and neurological sequalae. It has been reported that MeHg causes a decrease in DA synthesis while inorganic mercury does not (Lin et al., 2021). Another study found that although MeHg treatment did not significantly affect DA expression in the striatum, MeHg synergistically promoted PCBs-mediated decreases in DA concentrations (Bemis and Seegal, 1999). In addition to neurotransmitter alterations, in the current study, hippocampal neurons were also significantly damaged, as evidenced by neuronal degeneration, necrosis, and a significant reduction in Nissl-positive neurons. We also examined excitatory amino acid neurotransmitters and inhibitory amino acid neurotransmitters, as well as glutamine, which is the primary source of energy for various cells in the brain and immune system. Glutamine readily crosses the BBB, is the primary source of energy for the brain, and mediates Glu and GABA activity. GABA is produced by Glu catalyzed by glutamate decarboxylase. These two neurotransmitters are key factors influencing social behavior, and disruptions in their balance can lead to psychiatric disorders (Zhang et al., 2022). Earlier studies reported increased GABA contents in fecal samples from MeHg-treated rats (Lin et al., 2020). Both excitatory and inhibitory amino acid neurotransmitters showed different degrees of change in the present study, suggesting that MeHg exposure induced a metabolic imbalance between GABA and Glu.

The current results also indicated that the abundance and diversity of the gut microbiota were reduced in MeHg-treated rats; this may, in turn, affect neurotransmission. Intriguingly, after perinatal exposure to MeHg, the low-dose MeHg-treated group, but not the medium- and high-dose groups, showed an increase in the *F/B* ratio. This increase in the *F/B* ratio may also be related to cognitive function, as an increase in *F/B* is positively associated with aging-related learning and memory deficits (Hoffman et al., 2017). This finding, and the possible mechanism, should be confirmed in future studies with larger sample sizes. Of particular note was the increased abundance of *Desulfovibacteriaceae* in rats treated with all doses of MeHg, which has been reported to be involved in Hg cycling in the environment, demethylation of MeHg, or methylation of Hg (Lin et al., 2021). Notably, significant elevation in *Prevotellaceae*, a conditionally pathogenic bacterium, was observed in this study. *Prevotellaceae* mediate increased intestinal permeability by promoting mucus protein degradation (Yi et al., 2021). Corresponding to the above results, MeHg treatment significantly reduced the expression levels of these protein markers, revealing disruption of tight junctions between intestinal epithelial cells, and reduced levels of these proteins in the hippocampus were observed in this study. This finding was further supported by the H&E staining results, where damaged intestinal mucosa, disorganized arrangement of glands, and reduced mucus secretion from colonic goblet cells were observed. Given that tight junction-related proteins are essential for maintaining the integrity of the epithelial barrier, reduced expression of these proteins may affect gut-brain axis communication.

Based on the above-mentioned analyses, we aimed to screen out key gut microbes that mediate the effect of MeHg exposure on neurotransmitter metabolites with the use of correlations and mediation analysis. *Parabacteria* are common gut microbes and have been observed in humans and animals; changes in the levels of these microbes have been associated

with a variety of psychiatric disorders, and their abundance can be affected by heavy metal exposure (Zhai et al., 2019). In the current study, *Parabacteroides* abundance was increased by MeHg treatment, and it has been suggested that *Parabacteroides* may be involved in the metabolism of DA and NE (Yang et al., 2021). This was further corroborated in the analysis of the mediating effects in the current study. It is speculated that this may be the potential mechanism by which *Parabacteroides* mediated the exposure-response correlations between MeHg and decreases in ACh, DA, E, and NAS. The increase in *Helicobacter* species observed in this study may induce health outcomes such as gastroenteritis, gastric ulcers (Mo et al., 2021), and impairment in the intestinal barrier following exposure to MeHg. In addition, the observed decrease in the number of *lactobacilli* supports this; many strains of the *Lactobacillus* family are probiotics, they have health-promoting effects. *Helicobacter* is involved in the metabolism of NAS, and its increased abundance may be responsible for the increased NAS observed in this study; 13.94 % of the total effect of MeHg exposure on NAS metabolism could be explained by the mediation effect of *Helicobacter*. Increased abundance of *Ruminococcus* has been observed in the feces of cognitive deficit Alzheimer's disease (AD) patients (Zhuang et al., 2018). In a Parkinson's mouse model, *Ruminococcaceae* was negatively associated with DA and positively associated with Trp metabolites (Zhu et al., 2020). *Ruminococcaceae* is a strictly anaerobic, mesophilic cellulolytic bacterium that produces short-chain fatty acids (SFCAs) such as acetate and butyric acid. In a previous study, the increased abundance of *Ruminococcaceae* in mice exposed to BPA mediated decreases in SCFA levels and deficits in cognitive function (Ni et al., 2021). In accordance with previous studies, the current results revealed that *Ruminococcaceae_UCG-005* is involved in the tryptophan and tyrosine metabolic pathways and mediates the MeHg-induced reductions in ACh and Glu. Based on the above findings, it is suspected that dysbiosis of the gut flora remains a key factor in the effects of maternal exposure to MeHg; this may, in turn, affect neurotransmission, especially the tyrosine and tryptophan metabolic pathways (Gonzalez-Arancibia et al., 2019). Thus, we hypothesized that MeHg treatment disrupted the intestinal barrier, allowing harmful substances to enter the circulatory system and CNS, thus exacerbating cognitive dysfunction. Gut microbiota-derived products affect the CNS, and neurotransmitters are considered to be the main mechanism by which the gut microbiota affects neurobehavior.

There are several notable limitations to this study. First, this study observed a correlation between gut dysbiosis and plasma neurotransmitter disorder. Given the difficulty and stringent requirements for obtaining brain neurotransmitter samples in clinical practice, blood samples were obtained in this study to evaluate plasma neurotransmitter contents; blood samples are not only easier to obtain but also enable continuous monitoring. The causal relationship between gut dysbiosis and both brain and intestinal secretory neurotransmitter levels should be further explored in the future. Second, future studies are needed to more fully explore possible MeHg treatment-induced differences in the gut microbiome and neurotransmitter metabolism of males and females. Moreover, the present study focused only on the effects of perinatal MeHg treatment on the gut

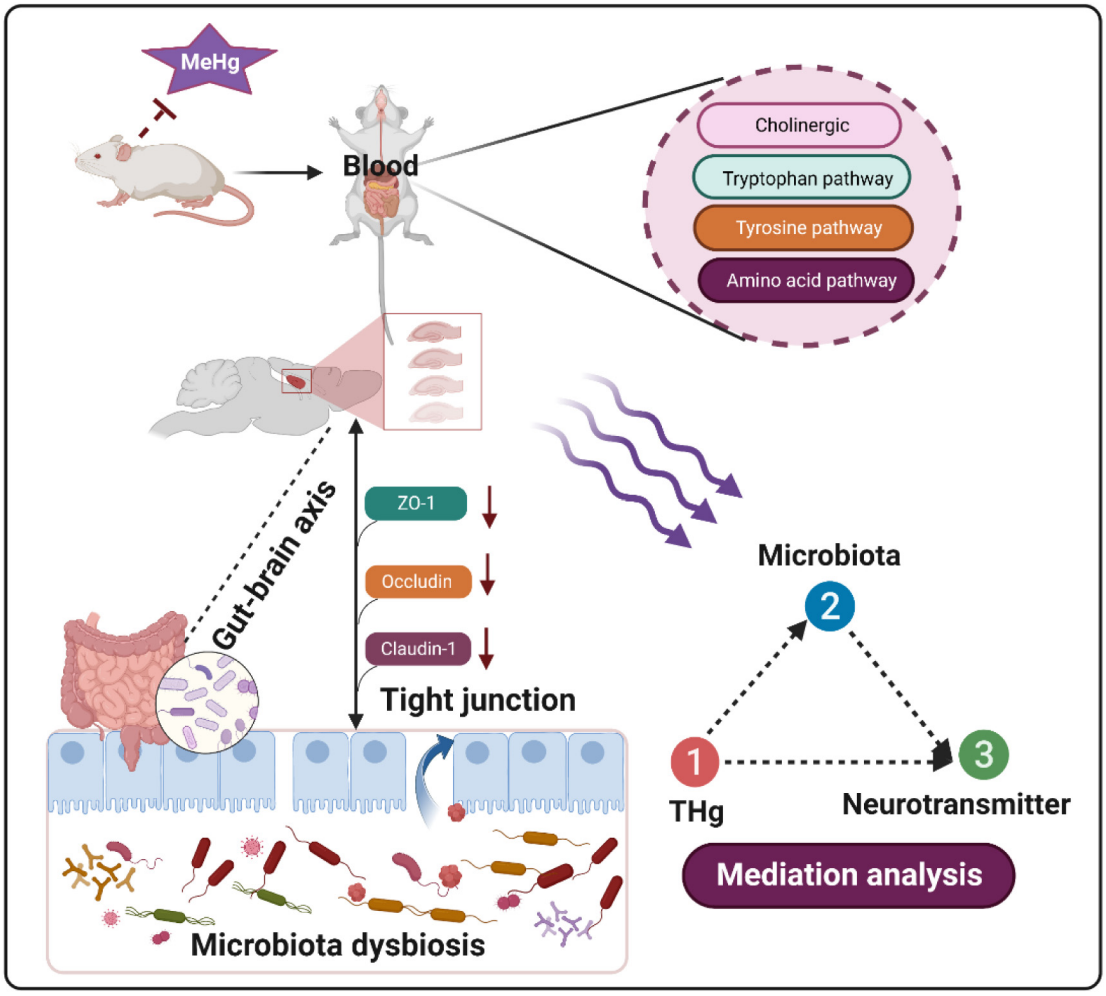

Fig. 7. Schematic diagram showing potential mechanisms of MeHg-induced neurotoxicity.
MeHg disrupts tyrosine, tryptophan, and amino acid neurotransmitter metabolic pathways, impairing the gut barrier, allowing harmful substances to enter the circulatory system and CNS via the gut-brain axis, thus exacerbating cognitive dysfunction. Gut microbiota-derived products affect the CNS, and neurotransmitters are considered to be the main mechanism by which the gut microbiota affects neurobehavior.

microbial and neurotransmitter expression profiles of offspring at PND21. Future studies should extend this to analyze the effects of maternal MeHg exposure on adult offspring in order to explore whether the effects of MeHg exposure persist.

5. Conclusion

In summary, our findings indicate that neurotransmitter disturbances caused by maternal MeHg exposure may be associated with disturbed gut microbiota at the abundance level and alterations in gut-brain-related metabolites at the functional level, thus resulting cognitive dysfunction. These findings strengthen our mechanistic understanding of the effects of maternal MeHg on cognitive function and the role of the microbiota-gut-brain axis in the neurotoxicity associated with environmental contaminants (Fig. 7).

CRediT authorship contribution statement

Wenjuan Wang: conceptualization, performed the research, investigation, writing-review & editing validation, funding acquisition. Fang Chen: methodology, performed the research, data curation, visualization, writing-original draft. Li Zhang: performed experiments. Fuli Wen: investigation. Qing Yu: software. Ping Li: resources, investigation, writing-review & editing, funding acquisition. Aihua Zhang: resources, validation, supervision, funding acquisition.

Data availability

Data will be made available on request.

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.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.scitotenv.2023.162358.

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