Antibiotic cocktail-induced gut microbiota depletion in different stages could cause host cognitive impairment and emotional disorders in adulthood in different manners

Jinxing Li a,1, Fangfang Pu b,1, Chenrui Peng a, Yimei Wang a, Yujie Zhang a, Simou Wu a, Silu Wang a, Xi Shen a, Yun Li a, Ruyue Cheng a,*, Fang He a,2

a Department of Nutrition and Food Hygiene, West China School of Public Health and West China Fourth Hospital, Sichuan University, 610041 Chengdu, Sichuan, PR China
b Department of Clinical Nutrition, West China Hospital, Sichuan University, Chengdu, Sichuan, PR China

ARTICLE INFO

Keywords:
Gut microbiota
Cognitive function
Emotional disorders
Antibiotics
Immune function
Neurochemicals

ABSTRACT

Gut microbiota depletion may result in cognitive impairment and emotional disorder. This study aimed to determine the possible association between host gut microbiota, cognitive function, and emotion in various life stages and its related underlying mechanisms. Seventy-five neonatal mice were randomly divided into five groups (n = 15 per group). Mice in the vehicle group were administered distilled water from birth to death, and those in the last four groups were administered antibiotic cocktail from birth to death, from birth to postnatal day (PND) 21 (infancy), from PND 21 to 56 (adolescence), and from PND 57 to 84 (adulthood), respectively. Antibiotic exposure consistently altered the gut microbiota composition and decreased the diversity of gut microbiota. Proteobacteria were the predominant bacteria instead of Firmicutes and Bacteroidetes after antibiotic exposure in different life stages. Long-term and infant gut microbiota depletion resulted in anxiety- and depression-like behaviors, memory impairments, and increased expression of γ-aminobutyric acid type A receptor mRNA expression of adult mice. Long-term antibiotic exposure also significantly decreased serum interleukin (IL)-1β, IL-10, and corticosterone of adult mice. Gut microbiota depletion in adolescence resulted in anxiety-like behaviors, short-term memory decline, decreased serum interferon-γ (IFN-γ), mRNA expression of 5-hydroxytryptamine receptor 1A, and neuropeptide Y receptor Y2 in the prefrontal cortex of adult mice. Antibiotic exposure in adulthood damaged short-term memory and decreased serum IL-10, IFN-γ, and increased γ-aminobutyric acid type B receptor 1 mRNA expression of adult mice. These results suggest that antibiotic-induced gut microbiota depletion in the long term and infancy resulted in the most severe cognitive and emotional disorders followed by depletion in adolescence and adulthood. These results also suggest that gut microbes could influence host cognitive function and emotion in a life stage-dependent manner by affecting the function of the immune system, hypothalamic-pituitary-adrenal axis, and the expression of neurochemicals in the brain.

1. Introduction

Gut microbiota coexist, interact, and coevolve with the human host for a long time. The gut microbiota has become an important acquired “organ” of the human body and is widely considered an important factor affecting human health (Grice and Segre, 2012; Fan and Pedersen, 2021). Gut microbiota can promote health by inhibiting the growth of pathogens, participating in the absorption and metabolism of nutrients, regulating the immune system, metabolizing some drugs and carcinogens, and affecting the absorption and distribution of fat (Adak and Khan, 2019). Therefore, the influence of gut microbiota on the body is not limited to the gut, and there are also many parenteral effects. More and more studies have shown that gut microbiota is involved in the interaction of the gut-brain axis and plays an indispensable role in the communication between the gut and the brain (Mayer et al., 2015; Cryan et al., 2019).
The development of gut microbiota and brain function is closely related to aging. Changes in the diversity of gut microbiota occur throughout the whole life cycle. After delivery, microorganisms begin to colonize the intestinal tract (Rutayisire et al., 2016; Kapourchali and Cresci, 2020). At this time, the quantity and diversity of gut microbiota are relatively low. With growth, the abundance of gut microbiota increases significantly and becomes adult-like by age ~ 2 years. The gut microbiota composition is relatively stable in adulthood, and then the stability decreases with aging (O’Toole and Jeffery, 2015). Accordingly, the developmental cycle of the brain is largely consistent with that of gut microbiota. If the brain is damaged at infancy, which is a critical period for the growth and physiological development of the brain, it will cause cognitive impairment in adulthood (Als et al., 2004; Kapourchali and Cresci, 2020). Similarly, adolescence and young adulthood are important for brain development and functional perfection (Desbonnet et al., 2015; Dunphy-Doherty et al., 2015; Holliday et al., 2020; Zhao et al., 2020).

Cognitive function is a complex physiological process involving a series of psychological and social behaviors, such as learning, memory, language, thinking, spirit, and emotion. It was initially thought to be mediated entirely by the central nervous system, but later studies have found that other systems are also involved. In recent years, emerging scientific evidence has shown that gut microbiota can affect the brain’s physiological, behavioral, and cognitive functions (Desbonnet et al., 2015; Mayer et al., 2015; Angueci et al., 2019; Cryan et al., 2019; Zhao et al., 2020; Morais and Schreiber, H.L.t., and Mazmanian, S.K., 2021).

Zhao et al. (Zhao et al., 2020) treated mice with antibiotics from adolescence (6–8 weeks) and performed behavioral tests per week. The research found that mice treated with ceftriaxone sodium exhibited depression-like and high aggressive behaviors after gavage for 11 weeks. Lieve et al. (Desbonnet et al., 2015) treated mice with a combination of antibiotics from weaning to adulthood and found that regardless of normal gut microbiota in infant mice, depleted gut microbiota from weaning might contribute to anxiety and cognition dysfunction in adult. Therefore, it can be inferred that gut microbiota depletion may cause abnormal cognitive function and emotional disorder in adulthood. This hypothesis has been supported recently in germ-free (GF) mice. In addition, consistent results were found in mice with gut microbiota disturbances induced by antibiotics (Fröhlich et al., 2016; Zhao et al., 2020). However, whether abnormal gut microbiota in different life periods might have different effects on cognitive function and emotion in adulthood remains unknown. In addition, whether cognitive impairment and emotional disorder will recover with the reestablishment of gut microbiota remains unclear.

Therefore, this study was conducted to confirm how antibiotic-induced gut microbiota depletion in different life cycle stages could affect cognitive function and emotion in adulthood and to elucidate the related underlying mechanisms.

2. Methods

2.1. Animals

Twenty specific pathogen-free (SPF) pregnant Kunming mice were purchased at ~15 days of gestation (Institute of Laboratory Animal Sciences, Sichuan Academy of Medical Sciences and Sichuan Provincial People’s Hospital, Sichuan, China) and raised in the SPF Animal Center of West China School of Public Health of Sichuan University (22 °C ± 1 °C; 50%-60% humidity; 12 h light/dark cycle). After delivery, male pups were randomly divided into five groups (n = 15 per group): vehicle (Veh), antibiotic (Abs), antibiotic infant (Abs infant), antibiotic adolescent (Abs adolescent), and antibiotic adult (Abs adult) groups. Each dam was housed in a cage with its offspring until weaning (PND 21), when male weaned mice were randomly assigned to cages (n = 5 per cage) and fed a normal diet (fat: 51 g/kg, protein: 190 g/kg, fiber: 36 g/kg; Chengdu Dossy Experimental Animals Co., Ltd.).

During the intervention, mice were weighed weekly. On PND 21, 56, and 84, each mouse was placed alone in a transparent plastic case which was wiped with 75% alcohol, and waiting for 10 min during which mice feces were naturally excreted, and we collected feces in sterile tubes and placed the tubes on ice. Feces were frozen at −80 °C until DNA extraction. On PND 107, mice were sacrificed for cervical dislocation after blood collection via extracting the eyeballs after anesthetized with 70% carbon dioxide in a draught box and the hippocampus and prefrontal cortex were collected on ice and immediately frozen at −80 °C. Meanwhile, the thymus, adrenal gland, spleen, and liver were collected and weighed. Organ indexes including adrenal, thymus and spleen index were equal to the organs weight divided by the body weight of mice on PND 107. 1 cm ileum and 1 cm colon tissues were collected and soaked in 10% formalin solution for further measurement.

The animal experiment facility was officially approved by the Experimental Animal Management Committee of Sichuan Government (approval no. SYXK2018-011). All experimental procedures were performed in accordance with the Guidelines for Animal Experiments at West China School of Public Health, Sichuan University (Sichuan, China).

2.2. Antibiotic treatment

The antibiotic cocktail comprised 100 mg/kg ampicillin, 50 mg/kg vancomycin, 100 mg/kg neomycin, 100 mg/kg bacitracin, 50 mg/kg imipenem, and 1 mg/kg amphotericin B (Dalian Meilun Biotechnology, Dalian, China) (Zhang et al., 2020). The volume of the solution was fixed at 10 μL/day from postnatal days (PND) 0–10. Since the 10th day, the volume of the solution was fixed at 100 μL/day. The antibiotics were chosen for two reasons. The first is that these antibiotics are widely effective at killing gram-positive bacteria, gram-negative bacteria and fungi, which could help construct a pseudo-sterile intestinal tract and be beneficial to study the causal relationship between gut microbiota and disease. The second reason is that selected antibiotics are barely absorbed through oral administration and do not cross the blood-brain barrier. Therefore, antibiotics administration could not directly damage the parenteral organs, which could ensure the effects of antibiotics on behavior were limited within the intestines and resulted from its disturbance on gut microbiota.

The protocol of antibiotic treatment is presented in Fig. 1A. Mice in the Veh group were administrated with distilled water by oral gavage from birth to death, whereas mice in the Abs group were administrated with antibiotics by oral gavage from birth to death. Mice in the Abx infant, Abx adolescent, and Abx adult groups were administrated with antibiotics by oral gavage from PND 0 to 21, 22 to 56, and 57 to 84, respectively. The gavages performed at 10:00 am daily.

(A) Neonatal mice were divided into 5 groups (n = 15/group) and given distilled water or antibiotics by gavage. (B) Behavioral tests were carried out as well as treatment.

2.3. Behavioral tests

To measure the cognitive function and emotion of treated mice, we detected depression-like and anxiety-like behavior, learning ability, short-term and spatial memory, which are always accompanied by cognitive impairment and emotional disorder (Liu et al., 2021). Open-field, passive avoidance, morris water maze, and tail suspension tests are four traditional and classic behavioral tests that could detect the above indexes of animals. They were conducted after PND 85 under the following sequence (Fig. 1B): open-field test (OFT), passive avoidance test (PAT), morris water maze test (MWM), and tail suspension test (TST). Thirteen mice from each group were randomly selected for behavioral tests. For all the behavioral tests, one mouse in the Veh group started an experiment, followed by the Abs, the Abx infant, the Abx adolescent, and the Abx adult groups. A total of five mice in all groups completed one round of the experiment, then the next, until all mice
completed a behavioral test.

2.4. OFT

OFT was used to observe the independent behavior, inquiry behavior, and tension of animals in an unfamiliar environment. The OFT equipment consisted of an open-field reaction chamber with parallel light and an automatic data acquisition and processing system. The bulk of the open-field reaction chamber was $625 \times 740 \times 510$ mm, and the bottom surface was divided into 25 small grids on average. Mice were set in the central grid and allowed to act freely for 5 min. The total movement distance, total movement time, central time, and other indicators were collected by a video monitor.

2.5. PAT

PAT is widely used in behavioral and cognitive experiments to test the spatial memory and short-term memory of animals. The PAT chambers contained a bright compartment and a dark compartment connected with a mobile door ($155 \times 140 \times 210$ mm). When the mice first entered the dark compartment and were shocked, the latency was automatically recorded. Meanwhile, mice were electrically stimulated to return to the bright compartment. If mice entered the dark compartment again, it was recorded as an error. In the training period, mice were placed in the bright compartment for 300 s observation after habituating in the dark compartment without electrical stimulation for 15 min, and the relevant parameters were collected. Mice maintained for $>180$ s in the bright compartment were forced to enter the dark compartment or abandoned. After 24 h, the formal test was carried out the same as the training test.

2.6. MWM

The MWM equipment consisted of a slick pool (diameter 800 mm, height 400 mm) and a circular platform (diameter 65 mm, height 150 mm). The pool was filled with warm water within titanium dioxide during training and test, and the platform was fixedly submerged 1 cm underwater. In the training period, mice practiced four times daily in each pool quadrant for 5 days. During training, mice were placed into water and allowed to swim and explore the platform for 60 s. The incubation time was recorded. When mice found the platform, they were left to stand on it for 15 s. If mice failed to find the platform, the
incubation time was recorded as 60 s, and mice were placed on the platform to rest for 15 s. At the end of each practice, mice were dried out and put back into the cage. On the sixth day, the incubation time for mice reaching the original position of the removed platform was recorded. To exclude the effects of sensory, visual, or motor dysfunction, mice were suspended upside down on a bar ~15 cm from the ground. Motionless state, in which the animal gives up active struggle and the body hangs without twisting, meant that mice were depressed. After being hung upside down for 2 min, the camera system was turned on to record the duration of quiescence and activity within 4 min.

2.7. TST

TST was not only used to evaluate the depression state of mice but also to give mice an acute stimulus, which was beneficial to the follow-up observation of the hypothalamic-pituitary-adrenal (HPA) axis alteration on spatial learning and memory, the platform was allowed up to 1 cm away observation of the hypothalamic-pituitary-adrenal (HPA) axis alteration. Other operations were the same as before.

2.8. Intestinal pathology

Ileum and colon tissues were immersed in 10% neutral formalin for 24 h. After being dehydrated with gradient ethanol, the tissues were fixed in paraffin and cooled at ~20°C. Hematoxylin-eosin staining was performed on three μm paraffin sections. Fifteen villi and crypts in 3 microscopic fields (100× magnification) were measured and photographed under a light microscope (Image-Pro Plus 6.0). When taking photographs, the background light of each photo was consistent, and the entire field of view was filled with tissues as possible.

2.9. Serum corticosterone

As serum glucocorticoid concentration varied with the circadian rhythm, it could fluctuate three to five times in 24 h, reaching the highest level before waking up and the lowest level before falling asleep (Bering et al., 2020). Therefore, to avoid the effect of circadian rhythm on serum corticosterone, blood samples were collected uniformly within 30 min after TST. Serum corticosterone was detected by enzyme-linked immunosorbent assay (R&D Systems, Inc., Minneapolis, MN, USA).

2.10. Serum cytokines

Serum interleukin (IL)-1β, IL-6, IL-10, tumor necrosis factor-α (TNF-α), and interferon-γ (IFN-γ) were detected by a Lumex assay (R&D Systems). The experimental protocol was in accordance with the manufacturer’s instructions. A Lumex 200™ multiplexing instrument was used to monitor reactions using a two-color laser.

2.11. Regulatory T (Treg) cells in the spleen

Spleen tissue of mice were collected, and single-cell suspensions were obtained as described previously with some modifications (Atarashi et al., 2013). 100 μL single-cell suspensions were stained for 60 min at 4°C with fluorescent-labeled antibodies (Anti-Mouse CD3-FITC antibody, Anti-Mouse CD4-FITC antibody, and Anti-Mouse CD25-APC antibody, and Anti-Mouse Foxp3-PE antibody) after using an FC receptor-blocking agent. Then anti-Mouse Foxp3-PE antibody was added and incubated for 60 min at room temperature after cells were permeated and fixed. Single-stained and unstained cells served as a control. The operation was carried out in strict accordance with the instruction of the Mouse Regulatory T Cell Staining Kit (eBioscience, USA). Treg cells were detected by flow cytometry using the preceding protocol (Cheng et al., 2017).

2.12. mRNA expression

The hippocampi and prefrontal cortices of mice were collected on ice and frozen at ~80°C. Brain-derived neurotrophic factor (BDNF), γ-aminobutyric acid (GABA) type A receptor α1 (GABA_A1), and type B receptor 1 (GABA_B1), 5-hydroxytryptamine receptor 1A (5-HT1A), nerve growth factor (NGF), glucocorticoid receptor (GR), and mineralocorticoid receptor (MR) mRNA expression levels in the tissue were detected, as described previously (Zhang et al., 2020). Claudin-1, claudin-2, occludin, tight junction protein 1 (ZO-1), serotonin transporter (SERT), neuropeptide Y (NPY), neuropeptide Y receptor Y1 (NPY1R), and neuropeptide Y receptor Y2 (NPY2R) mRNA were extracted and detected using Animal Total RNA Isolation Kit (FOREGENE, Chengdu, China), iScript cDNA synthesis kit (Bio-Rad, Berkeley, USA), and SsoAdvanced Universal SYBR Green (Bio-Rad, Berkeley, USA). β-actin was used as a reference gene. The reaction solution consisted of 0.5 μL forward primer, 0.5 μL reverse primer, 1 μL cDNA, and 3 μL enzyme-free double distilled water. The condition of RT-qPCR was 98°C for 30 s, followed by 40 cycles of 98°C for 15 s and 60°C for 30 s. The primer sequences are enumerated in Table 1.

2.13. 16S rRNA encoding gene sequencing and bioinformatics analysis

On PND 21, 56, and 84, stool samples were collected, and a TIANamp Stool DNA Kit (Tiangen Biotech Co., Ltd., Beijing, China) was used to extract DNA. The details of 16S rRNA sequencing were elucidated in a

| Target | Sequences | amplicon (bp) | Tm (°C) |
|--------|-----------|---------------|---------|
| β-actin | F: 5′- GTGGCCGCGCTTACGAGCCAACAA -3′ | 174 | 60 |
| BDNF | F: 5′- TGGACTGACCCTCGCTCTAT -3′ | 88 | 58 |
| NGF | F: 5′- GCCAGAAGGTGACACAGTTTC -3′ | 84 | 60 |
| GABA_A1 | F: 5′- AAAAATCGGGGGCTATCCTGA -3′ | 138 | 58 |
| GABA_B1 | F: 5′- CAGTGGTTCGAAACTTGTAG -3′ | 107 | 57 |
| 5-HT1A | F: 5′- TGGCAAATCAGCCACCTAT -3′ | 179 | 55.5 |
| MR | F: 5′- GAAAGGGCGTCTGGACATAG -3′ | 127 | 58 |
| GR | F: 5′- GTGGTTGACTTACGCTTTCT -3′ | 110 | 64 |
| Claudin-1 | F: 5′- CCTGGAGGAGCGAATGGTTC -3′ | 150 | 64 |
| Claudin-2 | F: 5′- TCAGGAGGACCCGGGGGTTAG -3′ | 132 | 64 |
| Occludin | F: 5′- GGAAGGACCTGAGGAGAC -3′ | 112 | 64 |
| ZO-1 | F: 5′- GGAACAGAAGAGGAAAGGAG -3′ | 123 | 58 |
| SERT | F: 5′- GCTTTTATATCGCCTTCCTAC -3′ | 314 | 60 |
| NPY | F: 5′- GCTTCTGACTTCTGCTGCTGCTG -3′ | 325 | 70 |
| NPY2R | F: 5′- CTCCTGATTCCTCAGCTGGATGGAAG -3′ | 518 | 58 |

BDNF, brain-derived neurotrophic factor; GABA_A1,  γ-aminobutyric acid type A receptor α1; GABA_B1, γ-aminobutyric acid type B receptor 1; 5-HT1A, 5-hydroxytryptamine receptor 1A; NGF, nerve growth factor; GR, glucocorticoid receptor; MR, mineralocorticoid receptor; ZO-1, tight junction protein 1; SERT, solute carrier family 6 (neurotransmitter transporter, serotonin), member 4; NPY, neuropeptide Y; NPY1R, neuropeptide Y receptor Y1; NPY2R, neuropeptide Y receptor Y2.
previous study (Cheng et al., 2019). Briefly, polymerase chain reaction (PCR) was performed to amplify DNA (primer: 338F ACTCCTAGGG-GAGGCGAGCAG and 806R GGACTACNNNGGGTATCTAAT). PCR reaction volume was 25 μL, consisting of 12.5 μL of Phusion Hot Start Flex 2× Master Mix, 1 μmol/L of forward and reverse primers, approximate 50 ng template DNA, and double-distilled water. The condition of PCR was 98 °C for 30 s, followed by 35 cycles of 98 °C for 10 s, 54 °C for 30 s, and 72 °C for 45 s. 2% agarose gel electrophoresis was used to detect PCR products (mixed with the same volume of 1 × loading buffer contained SYBR green). After purification and quantification, the amplicon library was sequenced on an Illumina MiSeq instrument (Illumina, Inc., Foster City, CA, USA).

The de novo Uchime algorithm and Qiime script (v1.9.1) was used to screen and collect high-quality reads. After clustering according to 97% similarity and comparison of the Greengene database, an operational taxonomic unit (OTU) table was generated. The representative sequences were aligned using PyNAST (v0.1) and a taxonomic tree was constructed using FastTree (v2.1.7). OTU with relative abundance less than 0.001% was removed from the OTU table to obtain a simplified OTU table. The alpha diversity of gut microbiota, including ACE, Chao 1, Shannon, and Simpson indexes was calculated based on the simplified OTU table and taxonomic information. The principal co-ordinates analysis (PCoA) was used to analyze microbial community composition among groups based on weighted UniFrac distance. Welch analysis of variance and Kruskal–Wallis H-test were used to detect differences in alpha diversity and relative abundance at phylum and genus levels between groups, and the Benjamini-Hochberg method was used to adjust P value for post hoc comparisons. Adjust P value less than 0.05 was considered statistically significant.

In this study, data from 16S rRNA sequencing can be found in the National Center for Biotechnology Information BioProject database (accession no. PRJNA546058).

2.14. Statistical analysis

Data were analyzed using IBM SPSS version 22.0 (SPSS, Inc., Chicago, IL, USA) and charted using GraphPad Prism version 8.0.2 (GraphPad Software, San Diego, California USA). Quantitative data were uniformly expressed as mean ± standard deviation (X ± SD). The body weight data measured repeatedly were analyzed by repeated measures analysis of variance. One-way analysis of variance at each time point was performed if there was an interaction between treatment and time. One-way analysis of variance or the Kruskal–Wallis H-test was used for multiple comparisons, and Benjamini-Hochberg method was used to adjust P value for pairwise comparisons. Adjusted P < 0.05 was considered statistically significant (two-tailed).

3. Results

3.1. Growth rate and organ indices of mice

Body weight was measured per week after birth. On PND 14, the growth rate of the Veh group was significantly lower than the Abx adult group (Supplementary Fig. 1A). On PND 21, the growth rate of test mice was lower in the Abx infant group than in the Veh group (Supplementary Fig. 1A). Since PND 49, fold changes of body weight in the Abx adult group were lower than the Veh group, and the reverse was found in the Abx adolescent group (Supplementary Fig. 1B).

Compared to the Veh group, the adrenal index of the Abx, Abx adolescent, and Abx adult groups was significantly decreased (Supplementary Fig. 1C). The spleen index of the Abx group was significantly lower than the Veh group and the Abx infant group (Supplementary Fig. 1D). There was no significant difference in the thymus index of mice among the groups during the intervention (Supplementary Fig. 1E).

3.2. Detection of intestinal pathology

The villi height in the ileum was significantly lower in the Abx, Abx adolescent, and Abx adult groups than in the Veh group (Supplementary Fig. 2A). The crypt depth in the ileum and colon of the Abx group was significantly lower than the Veh group (Supplementary Fig. 2B and C).

3.3. Behavioral tests

3.3.1. OFT

OFT was the first behavioral test that involved a low stress level. It revealed the anxious degree of mice by observing how they moved in an open field for 5 min. The total movement time of the Abx and Abx infant groups was significantly lower than the Veh group (Fig. 2A). No significant differences were found in the total movement distance among groups (Fig. 2B). Compared to the Veh group, the movement time in the center of mice in the Abx, Abx infant, and Abx adolescent groups was significantly decreased (Fig. 2C). The level and vertical scores in the Abx, Abx infant, and Abx adolescent groups were lower than the Veh and Abx adult groups, although no statistical difference was found (Fig. 2D and E).

3.3.2. PAT

All animals passed the training test. Antibiotic treatment significantly decreased the difference in the latency regardless of the treatment period (Fig. 2F). Compared to day 1, the latency of mice increased in all groups on day 2, but the latency in the Abx and Abx adult groups remained lower than the Veh group on day 2 (Fig. 2G). Although there was no statistical significance between the difference of error times in the Veh group and other treatment groups, it was the lowest in the Abx group (Fig. 2H).

3.3.3. MWM

During the 5-day orientation training, the incubation time decreased as training time went on. Compared to the first day of MWM, the incubation time of the Veh and Abx adolescent groups on the fifth day was significantly decreased (47.44 ± 7.52 s vs. 34.55 ± 11.15 s and 41.35 ± 10.97 s vs. 30.50 ± 14.52 s, P < 0.05, respectively). The average incubation time of the Abx group for 5 days was significantly higher than the Veh group (47.43 s ± 2.44 s vs. 39.65 s ± 2.44 s; P < 0.05).

The incubation time of both Abx and Abx infant groups was significantly higher than the Veh group on the formal test, which was not statistically different in the incubation time of the Abx adolescent and Abx adult groups compared to the Veh group (Fig. 2I).

3.3.4. TST

Compared to the Veh group, the duration of quiescence in the Abx and Abx infant groups was significantly increased. The quiescence duration in the Abx was statistically lower than that in the Abx, Abx infant and Abx adult groups (Fig. 2J).

(A-E) To figure out the effects of antibiotics on anxiety-like behavior in mice, some parameters in the open field test including total movement time, total movement distance, central time, level score which mean the number of grids crossed by a limb of mice and vertical score which mean the number of times mice stood on its hind legs were recorded. (F, G) To figure out the effects of antibiotics on short-term memory in mice, the difference of latency which mean the latency at formal tests (Day 2) minus the latency at training tests (Day 1) and difference of error times which mean error times at Day 1 minus error times at Day 2 in the passive avoidance test were calculated. (H) Data of the latency at Day 1 and Day 2 in the passive avoidance test were also exhibited. (I) To figure out the effects of antibiotics on spatial learning ability in mice, the incubation time that was regarded as duration of mice swim at each test in the Morris water maze test among 6 days was recorded. (J) To figure out the effects of antibiotics on depression in mice, the quiescence in the tail-suspension test that was regarded as
duration of motionless state of mice was recorded. * adjusted $P < 0.05$, ** adjusted $P < 0.01$, *** adjusted $P < 0.001$ for pairwise comparisons.

3.4. Serum cytokines and corticosterone

Serum IL-1β and IL-10 concentrations in the Abx group and IL-10 in the Abx adult group were significantly lower than the Veh group (Fig. 3A and C). IFN-γ was significantly decreased in the Abx adolescent and Abx adult groups compared to the Veh group (Fig. 3D). There were no statistical differences in the IL-6 and TNF-α levels between the Veh and other groups (Fig. 3B and E). Serum corticosterone in the Abx group was significantly lower than the Veh, Abx adolescent and Abx adult groups (Fig. 3F).

(A) IL-1β, (B) IL-6, (C) IL-10, (D) IFN-γ and (E) TNF-α were detected to measure the impact of antibiotics on immune system in mice. (F) Serum corticosterone was detected to measure the impact of antibiotics on hypothalamus pituitary adrenal axis in mice. (G) CD3+CD4+CD25+Foxp3+ T cell was regarded as Foxp3+ Treg cell that was a kind of regulatory T cell which played an important role in immune homeostasis. The percentage of Foxp3+ Treg cell in spleen was exhibited. * adjusted $P < 0.05$, ** adjusted $P < 0.01$, *** adjusted $P < 0.001$ for pairwise comparisons.

3.5. Foxp3+ Treg cells

Foxp3+ Treg cells in the spleen counted by flow cytometry in the Abx group was significantly lower than the Abx infant group. However, no statistical differences were found between the Veh and antibiotic
3.6. Expression of neural signaling molecules

In the prefrontal cortex, compared to the Veh group, BDNF and GABA<sub>A</sub><sub>α</sub><sub>1</sub> mRNA expression in the Abx, Abx infant, and Abx adolescent groups significantly increased. GABA<sub>B1</sub> expression in the Abx adolescent and Abx adult groups was significantly higher than the Veh group, and the reverse was found for 5-HT<sub>1A</sub>. NPY2R expression in the Abx adolescent group and MR expression in the Abx adult group were significantly lower than in the Veh. The differences in NGF, GR, claudin-1, claudin-2, occludin, SERT, NPY, and NPY1R expression between five groups were not significant (Table 2).

In the hippocampus, GABA<sub>A</sub><sub>α</sub><sub>1</sub> expression in the Abx, Abx infant, and Abx adult groups and GABA<sub>B1</sub> expression in the Abx and Abx adult groups significantly increased compared to the Veh group. In contrast, GR mRNA expression in the Abx infant group and NPY2R expression in the Abx adult group were significantly lower than in the Veh group.
The mRNA relative expression of neural signaling molecules in the prefrontal cortex and hippocampus.

### Table 2

| Part       | index | Veh | Abx infant | Abx adolescent | Abx adult |
|------------|-------|-----|------------|----------------|-----------|
| prefrontal cortex | BDNF | 1.11 | 2.65 | 2.55 | 3.16 $\pm$ 0.45 | 2.47 $\pm$ 0.52 |
|            | $\pm$ | $\pm$ | $\pm$ | $\pm$ | $\pm$ | $\pm$ |
|            | NGF  | 1.93 | 1.36 | 1.37 | 2.19 $\pm$ 0.67 | 1.78 $\pm$ 0.26 |
|            | $\pm$ | $\pm$ | $\pm$ | $\pm$ | 50.50 | 13.35 |
|            | GABA<sub>A</sub>A | 1.01 | 2.62 | 2.00 | 2.40 $\pm$ 1.69 | 1.15 |
|            | $\pm$ | $\pm$ | $\pm$ | $\pm$ | $\pm$ | $\pm$ |
|            | GABA<sub>B</sub>B | 1.09 | 1.62 | 1.52 | 2.47 $\pm$ 2.16 | 0.46 |
|            | $\pm$ | $\pm$ | $\pm$ | $\pm$ | $\pm$ | $\pm$ |
|            | GR   | 1.02 | 0.81 | 0.96 | 0.77 $\pm$ 0.96 | 0.24 |
|            | $\pm$ | $\pm$ | $\pm$ | $\pm$ | $\pm$ | $\pm$ |
|            | MR   | 1.02 | 0.80 | 0.88 | 0.71 $\pm$ 0.48 | 0.24 |
|            | $\pm$ | $\pm$ | $\pm$ | $\pm$ | $\pm$ | $\pm$ |
|            | 5-HT<sub>1</sub>A | 1.07 | 0.67 | 0.56 | 0.55 $\pm$ 0.41 | 0.43 |
|            | $\pm$ | $\pm$ | $\pm$ | $\pm$ | $\pm$ | $\pm$ |
|            | Claudin-1 | 1.15 | 0.57 | 0.66 | 0.78 $\pm$ 0.59 | 0.63 |
|            | $\pm$ | $\pm$ | $\pm$ | $\pm$ | $\pm$ | $\pm$ |
|            | Claudin-2 | 1.38 | 0.65 | 0.60 | 0.88 $\pm$ 0.74 | 1.35 |
|            | $\pm$ | $\pm$ | $\pm$ | $\pm$ | $\pm$ | $\pm$ |
|            | Occludin | 1.04 | 0.74 | 0.92 | 0.78 $\pm$ 0.90 | 0.28 |
|            | $\pm$ | $\pm$ | $\pm$ | $\pm$ | $\pm$ | $\pm$ |
|            | ZO-1  | 1.05 | 0.71 | 0.87 | 0.92 $\pm$ 0.92 | 0.20 |
|            | $\pm$ | $\pm$ | $\pm$ | $\pm$ | $\pm$ | $\pm$ |
|            | SERT  | 1.03 | 0.70 | 0.92 | 0.95 $\pm$ 0.91 | 0.33 |
|            | $\pm$ | $\pm$ | $\pm$ | $\pm$ | $\pm$ | $\pm$ |
|            | NPY   | 1.03 | 1.69 | 2.13 | 1.90 $\pm$ 1.63 | 0.78 |
|            | $\pm$ | $\pm$ | $\pm$ | $\pm$ | $\pm$ | $\pm$ |
|            | NPY1R | 1.03 | 1.69 | 2.13 | 1.90 $\pm$ 1.63 | 0.78 |
|            | $\pm$ | $\pm$ | $\pm$ | $\pm$ | $\pm$ | $\pm$ |
|            | NPY2R | 1.03 | 1.69 | 2.13 | 1.90 $\pm$ 1.63 | 0.78 |
|            | $\pm$ | $\pm$ | $\pm$ | $\pm$ | $\pm$ | $\pm$ |

### Table 2 (continued)

| Part       | index | Veh | Abx infant | Abx adolescent | Abx adult |
|------------|-------|-----|------------|----------------|-----------|
| hippocampus | BDNF | 1.05 | 1.24 | 2.01 | 1.57 $\pm$ 1.21 |
|           | $\pm$ | $\pm$ | $\pm$ | $\pm$ | $\pm$ |
|           | NGF  | 1.04 | 1.16 | 1.49 | 1.36 $\pm$ 1.26 |
|           | $\pm$ | $\pm$ | $\pm$ | $\pm$ | $\pm$ |
|           | GABA<sub>A</sub>A | 1.06 | 1.45 | 1.66 | 1.41 $\pm$ 1.68 |
|           | $\pm$ | $\pm$ | $\pm$ | $\pm$ | $\pm$ |
|           | GABA<sub>B</sub>B | 0.35 | 0.92 | 0.35 | 0.76 $\pm$ 0.96 |
|           | $\pm$ | $\pm$ | $\pm$ | $\pm$ | $\pm$ |
|           | GR   | 0.31 | 0.54 | 0.31 | 0.76 $\pm$ 0.96 |
|           | $\pm$ | $\pm$ | $\pm$ | $\pm$ | $\pm$ |
|           | SERT  | 1.02 | 1.04 | 1.11 | 1.13 $\pm$ 0.83 |
|           | $\pm$ | $\pm$ | $\pm$ | $\pm$ | $\pm$ |
|           | NPY   | 0.41 | 0.88 | 0.41 | 0.76 $\pm$ 0.96 |
|           | $\pm$ | $\pm$ | $\pm$ | $\pm$ | $\pm$ |
|           | NPY1R  | 0.41 | 0.88 | 0.41 | 0.76 $\pm$ 0.96 |
|           | $\pm$ | $\pm$ | $\pm$ | $\pm$ | $\pm$ |
|           | NPY2R | 0.41 | 0.88 | 0.41 | 0.76 $\pm$ 0.96 |

There was no statistical difference between the Veh and other treatment groups in BDNF, NGF, MR, 5-HT<sub>1</sub>A, claudin-1, claudin-2, occludin, ZO-1, SERT, solute carrier family 6 (neurotransmitter transporter, serotonin), member 4; NPY, neuropeptide Y; NPY1R, neuropeptide Y receptor Y1; NPY2R, neuropeptide Y receptor Y2.

### 3.7. Gut microbiota diversity

On PND 21, in terms of microbiota richness, the ACE and Chao1 indices in the Abx adult group were higher than the Veh group. The Shannon and Simpson indices, which showed the evenness and diversity of microbiota, respectively, significantly decreased in the Abx group compared to the Veh group (Fig. 4A–D).

On PND 56, the Shannon and Simpson index of the Abx and Abx adolescent group was significantly lower than the non-intervention group. No statistical differences were observed in the ACE and Chao1 indices among groups (Fig. 4A–D).

On PND 84, the Shannon and Simpson indexes of the Abx and Abx adult groups significantly decreased compared to the non-intervention group. However, no statistical differences remained in the ACE and Chao1 indices (Fig. 4A–D).

β-Diversity analysis revealed that using antibiotics could seriously damage the normal gut microbiota structure. The principal coordinate analysis plot showed that the microbiota of one group clustered separately from the other groups at a time point. PC1 axis captured 69.02%, 66.95%, and 67.61% of the variability, indicating that antibiotic treatment had an important effect on community composition. However, the effects of antibiotics wore off over time. The recent antibiotic exposure made the main difference. In short, the use of antibiotics had a significant impact on the gut microbiota structure, but once antibiotics were terminated, gut microbiota would recover over time (Fig. 4E–G).

(A-D) ACE index, Chao 1 index, Shannon index and Simpson index of gut microbiota at three time point (PND 21, PND 56 and PND 84) were calculated and shown respectively. (E-G) The principal coordinate analysis (PCoA) based on weighted Unifrac distance was performed to measure the effect of antibiotics on beta-diversity of gut microbiota on PND 21, PND 56 and PND 84. (H, I) Community composition at phylum level and genus level was also exhibited. * adjusted P < 0.05, ** adjusted P < 0.01, *** adjusted P < 0.001 for pairwise comparisons.
3.8. Alterations of gut microbiota composition

The mean relative abundance of gut microbiota is shown in Tables 3 and 4. On PND 21, at the phylum level, the relative abundance of Proteobacteria was significantly higher in the Abx and Abx infant groups than the Veh group. In contrast, Firmicutes, Bacteroidetes, and Verrucomicrobia significantly decreased in the Abx and Abx infant groups (Fig. 4H; Table 3). At the genus level, Bacteroides, Parabacteroides, and Akkermansia accounted for >85% of the relative abundance in the Veh group. In the Abx group, the three predominant bacteria were
Table 3
Mean relative abundance of different taxa at phylum level.

| Stage    | Phylum       | Veh (%) | Abx (%) | Abx infant (%) | Abx adolescent (%) | Abx adult (%) |
|----------|--------------|---------|---------|----------------|-------------------|---------------|
| PND21    | Bacteroides  | 60.38   | 0.35^a | 2.14^a         | 63.83^bc         | 58.15^bc      |
|          | Firmicutes   | 20.11   | <0.01^b| 0.06^b         | 20.48^bc         | 30.42         |
|          | Proteobacteria | 7.15   | 98.74^a| 95.62^a        | 9.85             | 9.44^bc       |
|          | Verrucomicrobia | 11.87   | 0.04^a | 2.18           | 5.81             | 1.78          |
| PND56    | Bacteroides  | 52.85   | 29.11   | 80.05^b        | 32.51^c          | 62.72         |
|          | Firmicutes   | 19.06   | 0.27^a  | 1.37^a         | 0.72             | 28.68^ad      |
|          | Proteobacteria | 21.58 | 66.75   | 3.33^b         | 58.83^f          | 7.99          |
|          | Verrucomicrobia | 5.93   | 3.66    | 2.47           | 7.89             | 0.07^c        |
| PND84    | Bacteroides  | 34.98   | 2.71    | 43.39^b        | 43.43            | 19.14         |
|          | Firmicutes   | 61.69   | 0.67^a  | 42.05          | 46.13            | 0.29^ad       |
|          | Proteobacteria | 2.55 | 93.22^A | 4.55^b       | 5.61             | 79.11^e       |
|          | Verrucomicrobia | 0.26 | 3.37    | 3.83           | 4.71             | 1.45          |

Note: [A] indicate that the corresponding sequence was involved in the Greengene database, but was not involved in NCBI.

Table 4
Mean relative abundance of different taxa at genus level.

| Stage    | Genus         | Veh (%) | Abx (%) | Abx infant (%) | Abx adolescent (%) | Abx adult (%) |
|----------|---------------|---------|---------|----------------|-------------------|---------------|
| PND21    | [Prevotella]  | 2.29    | 0.05    | 0.27           | 1.19              | 2.31          |
|          | Akkermansia   | 15.95   | 0.17^b  | 8.17           | 7.81              | 3.14          |
|          | Bacteroides   | 47.67   | 0.42^a  | 14.97          | 68.33^bc         | 50.12^b       |
|          | Blautia       | 1.75    | 0.01^b  | <0.01^b       | 1.06              | 1.01          |
|          | Clostridium   | 3.33    | 0.01    | 81.73          | 2.74              | <0.01^b       |
|          | Enterobacter  | <0.01   | 1.58^b  | 0.66           | <0.01^b          | <0.01^b       |
|          | Klebsiella    | 0.28    | 13.14   | 24.46          | 0.06^bc          | <0.01^c       |
|          | Lactobacillus | 1.52    | 0.20    | 1.07           | 2.27              | 4.38          |
|          | Oscillospira  | 0.49    | 0.10    | <0.01          | 0.35              | 5.21          |
|          | Parabacteroides | 23.69 | 0.06^a  | 0.56^a         | 11.92             | 17.76         |
|          | Proteus       | <0.01   | <0.01   | 12.59          | <0.01             | <0.01         |
|          | Sutterella    | 2.74    | <0.01   | 28.07^a        | 3.25              | 1.55          |
| PND56    | [Prevotella]  | 22.15   | 0.01    | 15.76          | 0.03              | 12.13         |
|          | Akkermansia   | 15.07   | 6.75    | 7.66           | 12.77             | <0.01         |
|          | Bacteroides   | 25.07   | 53.77   | 38.76          | 55.58             | 25.12         |
|          | Clostridium   | <0.01   | 1.01    | <0.01          | 13.24             | <0.01         |
|          | Coprococcus   | <0.01   | <0.01   | 0.54           | <0.01             | 2.73^b        |
|          | Lactobacillus | 9.06    | <0.01   | 3.72           | 0.20              | 5.74^b        |
|          | Morganella    | 1.86    | 6.39    | <0.01          | 3.81              | <0.01         |
|          | Oscillospira  | 5.84    | <0.01   | 7.26           | <0.01             | <0.01         |
|          | Parabacteroides | 0.43 | <0.01   | 14.55^b        | <0.01^c          | 2.55          |
|          | Ruminococcus  | 0.57    | <0.01   | 1.39           | <0.01             | 2.41^b        |
|          | Sutterella    | 11.11   | 31.10   | 4.03^b         | 13.22             | 16.78         |
| PND84    | [Prevotella]  | 7.10    | 0.03    | 7.53           | 11.91             | 0.02          |
|          | [Ruminococcus] | 3.22 | <0.01^a | 2.00^a         | 2.83^ad          | 10.78^ad      |
|          | Akkermansia   | 1.39    | 13.49   | 10.05          | 11.15             | 3.59          |
|          | Bacteroides   | 10.63   | 7.08    | 40.36          | 27.73             | 58.31         |
|          | Clostridium   | <0.01   | 30.7^a  | <0.01          | <0.01             | 0.09          |
|          | Coprococcus   | 2.33    | <0.01^a | 1.81^a         | 0.74              | <0.01^c       |
|          | Dehalobacterium | 3.20 | <0.01^a | 2.65           | 0.03              | <0.01^a       |
|          | Desulfovibrio | 2.36    | <0.01^a | 0.49           | 0.11              | <0.01^ad      |
|          | Lactobacillus | 31.65   | 0.04^a  | 5.02           | 7.11              | 0.06^a        |
|          | Morganella    | 0.04    | 6.20^a  | <0.01          | <0.01             | 6.31^a        |
|          | Oscillospira  | 23.93   | 0.04^a  | 15.88^b        | 11.19             | 0.02^d        |
|          | Ruminococcus  | 5.71    | <0.01^a | 3.89           | 4.95              | <0.01^ad      |
|          | Sutterella    | 1.85    | 38.06^A | 4.31           | 11.76             | 25.31^a       |

Note: [A] indicate that the corresponding sequence was involved in the Greengene database, but was not involved in NCBI.

\[ \text{Citrobacter}, \text{Klebsiella}, \text{and Enterobacter}. \text{Bacteroides, Parabacteroides, Akkermansia, and Blautia} \text{significantly decreased in the Abx group compared to the Veh group. In the Abx infant group, Sutterella, Klebsiella, and Bacteroides were the three predominant bacteria, but the relative abundance of Bacteroides in the Abx infant group was still lower than the Veh group. Meanwhile, Blautia and Parabacteroides in the Abx infant were significantly decreased (Fig. 4; Table 4).} \]

On PND 56, at the phylum level, Proteobacteria increased in the Abx and Abx adolescent groups compared to the Veh group, whereas Firmicutes showed a decreasing relative abundance in the Abx and Abx adolescent groups (Fig. 4H; Table 3). At the genus level, Bacteroides, Prevotella, and Akkermansia were the three predominant bacteria in the Veh group. Sutterella in the Abx group increased and became one of the three predominant bacteria. Lactobacillus and Oscillospira in the Abx group significantly decreased. Parabacteroides in the Abx infant group significantly increased compared to the Abx group. Citrobacter and Oscillospira increased and decreased in the Abx adolescent group, respectively. Coprococcus and Ruminococcus in the Abx group were
significantly higher than the Abx adult group (Fig. 4i; Table 4).

On PND 84, at the phylum level, Proteobacteria were significantly higher in the Abx and Abx adult groups than the Veh group. Conversely, Firmicutes significantly decreased in the Abx and Abx adult groups; Bacteroidetes significantly decreased in the Abx groups (Fig. 4h; Table 3). At the genus level, Lactobacillus, Oscillospira, and Bacteroides were the predominant bacteria in the Veh group. The relative abundance of Citrobacter, Morganella, and Sutterella in the Abx group significantly increased, and the reverse was found in Coprococcus, Dehalobacterium, Desulfovibrio, Lactobacillus, and Oscillospira, which significantly decreased in the Abx adult group as well. Morganella increased significantly in the Abx and Abx adult group. Accordingly, bacteria in the Abx infant and Abx adolescent groups were altered mildly. Bacteroides in the Abx infant group increased and Lactobacillus decreased compared to the Veh group. Sutterella in the Abx adolescent group were higher than the Veh group. However, Coprococcus, Dehalobacterium, Desulfovibrio, Lactobacillus, and Oscillospira in the Abx adolescent group decreased (Fig. 4f; Table 4).

4. Discussion

Results of animal experiments and human research showed that gut microbiota play an important role in developing cognitive function and emotion via endocrine, neurochemical, and immune pathways, and changes in the gut microbiota structure can be improved or lead to brain disease (McCormick et al., 2020; Tengeler et al., 2020). The development of the brain and gut microbiota was time-consistent. In this study, gut microbiota depletion in mice induced by the antibiotic cocktail was to investigate whether gut microbiota depletion that occurs in different life cycle stages could affect host cognitive function and emotion differently and how gut microbiota could communicate with the brain as possible underlying mechanisms.

In this study, although the growth rate of mice was significantly different among five groups during the intervention, the differences were probably not due to antibiotic exposure. Because antibiotic exposure did not consistently increase or decrease the growth rate. For instance, there was no statistical difference between the growth rate of the mice treated with antibiotics in infancy and throughout their whole life and that of the control group. In contrast, the growth rate of the Abx adolescent and Abx adult groups was significantly higher and lower than that of the control group since PND 49, respectively. Studies found that GF mice are often accompanied by multiple organ abnormalities and abnormal intestinal physiological structure and function (Huo et al., 2017; Li et al., 2020a). Similar results also occurred after extensive use of antibiotics to destroy intestinal microorganisms (Cheng et al., 2019; Zhang et al., 2020). In this study, the organ index and the villus and crypt structure were the most damaged by long-term exposure to antibiotics, whereas antibiotics used in adolescence and adulthood but not infancy also decreased intestinal villus height in mice, revealing that the damage of antibiotics on infant intestinal morphology was recoverable. Notably, mice exposed to antibiotics during infancy had been discontinuing antibiotics for the longest time at the point of tissue collection. Thus, perhaps intestinal morphology could be recovered in the other treatment groups after withdrawal antibiotics for a more extended period.

This study found that antibiotic exposure significantly altered gut microbiota structure and diversity regardless of long- or short-term intervention. Antibiotics used from birth to adulthood can dramatically decrease the evenness of gut microbiota in mice. Firmicutes and Bacteroidetes were the two predominant bacteria of mice, representing 90% of gut microbiota (Riminella et al., 2019) in a normal intestinal environment, and the relative abundance of Firmicutes increased as mice grew up. However, predominant bacteria of mice treated with antibiotics transformed into Proteobacteria dramatically. There were no significant changes in Verrucomicrobia and Firmicutes in adulthood after antibiotic intervention in infancy or adolescence. Citrobacter, Morganella, and Klebsiella increased due to antibiotic treatment as opportunistic pathogens and then rapidly returned to control levels when antibiotics were discontinued. Similarly, beneficial bacteria at the genus level, such as Lactobacillus, decreased on account of antibiotic exposure but increased slightly as antibiotics were discontinued. These results also indicated that gut microbiota could gradually recover after the disuse of antibiotics, consistent with a previous study (Cheng et al., 2020). However, some studies showed that there was a limit to the resilience of gut microbiota (Riminella et al., 2019), which was affected by individual differences and environmental factors, and the repeated use of antibiotics may still result in gut microbiota disturbance.

Abnormal gut microbiota came with cognitive impairment and emotional disorders including anxiety, depression, memory, and learning ability decline. In this study, although gut microbiota disturbance induced by antibiotics in infancy would recover over time, it still resulted in anxiety- and depression-like behaviors, decline of spatial learning ability and short-term memory of mice in adulthood, as found in mice treated with antibiotics from birth to adulthood. However, antibiotic exposure in adolescence led to anxiety and weakened short-term memory, while antibiotic exposure in adulthood damaged only short-term memory in adult mice. These results indicated that cognitive dysfunction and emotional disorders in adulthood induced by only gut microbiota depletion in infancy was similar to that induced by the long-term gut microbiota depletion throughout the host’s life, again emphasizing the importance of infancy in the development of gut microbiota and the brain.

In early life, bacteria will gradually colonize the neonatal gut and develop during childhood and adolescence. Similarly, this period is also absolutely indispensable for the development of brain function. Previous studies demonstrated that gut microbiota depletion in childhood and adolescence would increase anxiety- and depression-like and aggressive behaviors and injure spatial working memory and ability, especially in mice with underlying diseases (Ceylan et al., 2018; Zeraati et al., 2019; Kwon et al., 2020; Lee et al., 2020; Zhao et al., 2020). In adulthood, gut microbiota were relatively stable, and cognitive functions were close to mature, but adult neurogenesis kept continuing, especially in the hippocampus and prefrontal cortex, which are necessary for advanced neural activity in addition to learning and memory (Cameron and Glover, 2015). In animal studies, alteration of gut microbiota in adult mice may cause attention deficit (Tengeler et al., 2020), novel object recognition disorder (Frohlich et al., 2016), and working memory deficit (Li et al., 2020b). In human studies, patients with mild cognitive impairment who underwent mindful awareness practice would have improved cognitive functions and gut microbiota profile (Kchine et al., 2020). Therefore, gut microbiota depletion in early life, adolescence, and adulthood can lead to different cognitive dysfunction and emotional disorders. More studies are needed to determine the effects of changes in gut microbiota on advanced neural activity, such as emotion.

Gut microbiota are connected with the brain via immune, endocrine, and neurochemical pathways (Martin et al., 2018). The HPA axis, one of the major neuroendocrine systems, controls stress and regulates a variety of physical responses (Frankiensztajn et al., 2020). GR and MR are transcriptional regulatory factors that exist in almost all vertebrate cells. By binding to different ligands, GR and MR specifically regulate the transcription of thousands of genes (Luo et al., 2018). Animal studies suggested that expression deletion of MR in the hippocampus and prefrontal cortex would damage memory performance, emotion regulation, and executive function (Glover and Treit, 2011). Consistently, in this study, GR expression in the hippocampus and MR expression in the prefrontal cortex were significantly downregulated after treatment with antibiotics during infancy and adult, respectively. However, it appears insufficient to detect GR and MR only in the hippocampus and prefrontal cortex. Because it was shown that, in functional magnetic resonance imaging studies, the amygdala became highly activated during the initial phase of stress response, whereas the activity of the hippocampus and prefrontal cortex was decreased, leading to a redistribution of MR
Corticosterone, a ligand of GR and MR, could increase attention and vigilance after stress. The effect of corticosterone depends on its concentration and distribution of receptors (Joëls, 2018). High serum corticosterone in GF mice has been supported, whereas the effects of antibiotic-induced depletion on corticosterone have inconsistent results. It was reported that acute stress would increase corticosterone levels in both antibiotic-treated and non-treated animals (Desbonnet et al., 2015; Hoban et al., 2016). Nevertheless, Afia et al. have observed that antibiotics treatment could effectively prevent stress-induced high serum corticosterone concentration (Ait-Belgnaoui et al., 2012). In addition, Garate et al. found that antibiotic-induced gut microbiota depletion would inhibit the increase of corticosterone induced by chronic stress (Garate et al., 2011). The inconsistent effects might be related to differences in animals’ species, the types of antibiotics, and the treatment period in this study, especially. We believed that antibiotic exposure from infancy would cause insensitivity to HPA activation. Low corticosterone concentrations, such as in stress-free conditions, are more likely to activate MR instead of GR (de Kloet et al., 2016). In this study, long-term antibiotic treatment from birth to death significantly decreased corticosterone, leading to cognitive impairment and emotional disorders in mice.

Also, antibiotic treatment during different periods could significantly increase GABA<sub>A₁</sub> and GABA<sub>B₁</sub> mRNA expression in the hippocampus and prefrontal cortex and BDNF in the prefrontal cortex and decrease NPY2R mRNA expression in the hippocampus and prefrontal cortex and 5-HT<sub>1A</sub> mRNA expression in the prefrontal cortex. GABA and 5-HT were the main inhibitory neurochemicals that induce negative mental activities by binging with various receptors. 5-HT<sub>1A</sub> receptors, which regulate spatial learning and memory, are innovatively applied to treat neurodegenerative diseases (Ye et al., 2014; Glikmann-Johnston et al., 2015). GABA<sub>A₁</sub> connecting with ligands is more likely to mediate hypnotic function (Rudolph and Möhler, 2006). Mice lacking functional GABA<sub>B₁</sub> receptors exhibited more activity (Vacher et al., 2006). Thus, it was inferred that GABA<sub>B₁</sub> is more likely to mediate exploration behaviors. BDNF, one of the neural-signaling-related molecules associated with learning and memory, has been reported to decline or remain after antibiotic treatment (Desbonnet et al., 2015; Mosafari et al., 2021).

However, some researchers found that antibiotic exposure or anxiety induced by withdrawal reaction also increased BDNF expression (Bercik et al., 2011; Ait-Belgnaoui et al., 2012; Hammad et al., 2021). Thus, similar to low expression, we assumed that overexpression of BDNF might lead to cognitive impairment and emotional disorders. NPY2R, known for regulating emotion and behavior, has been gradually recognized as essential for learning and memory processing, especially memory retaining (Holzer et al., 2012; Gotsche and Woldbye, 2016). It was also thought that NPY2R depletion would impair the learning process under stress (Redrobe et al., 2004; Hörmer et al., 2018). These results demonstrated that decreased 5-HT<sub>1A</sub> receptors and NPY2R and increased GABA<sub>A₁</sub> and GABA<sub>B₁</sub> receptors might result in abnormal behaviors. Although selected antibiotics did not appear to cross the blood-brain barrier or directly damaged the tight junction proteins of the blood-brain barrier, it is possible that the changes in the microbiota itself led to alterations in the blood-brain barrier. More researches are needed to figure out this issue.

IL-10 is a recognized anti-inflammatory cytokine that exerts multiple immunomodulatory effects. Studies suggested that IL-10 receptor would attenuate the inflammatory response to an acute psychological stress in rats (Mol. Life Sci. 76 (3), 473–493. https://doi.org/10.1007/s00126-018-1494-4). These re


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