Original Article

Changes in short-chain fatty acids affect brain development in mice with early life antibiotic-induced dysbacteriosis

Qixing Zhang[1#], Han Li[1#], Shuang Yin[1], Feng Xiao[2], Chen Gong[3], Jie Zhou[1], Kangkang Liu[1], Yan Cheng[1]

[1]Department of Pediatrics, The Second Affiliated Hospital of Anhui Medical University, Hefei, China; [2]Institute of Clinical Pharmacology, Anhui Medical University, Hefei, China; [3]Department of Pediatrics, The First Affiliated Hospital of Anhui Medical University, Hefei, China

Contributions: (I) Conception and design: Y Cheng, Q Zhang; (II) Administrative support: Y Cheng, F Xiao; (III) Provision of study materials or patients: Q Zhang, H Li, C Gong; (IV) Collection and assembly of data: Q Zhang, H Li, S Yin; (V) Data analysis and interpretation: Q Zhang, K Liu, J Zhou; (VI) Manuscript writing: All authors; (VII) Final approval of manuscript: All authors.

[#]These authors contributed equally to this work.

Correspondence to: Yan Cheng, PhD. Department of Pediatrics, The Second Affiliated Hospital of Anhui Medical University, No. 678 Furong Road, Shushan District, Hefei 230601, China. Email: chengyan1971@163.com.

Background: Early enteral nutrition and the gut microbiota profoundly influence neonatal brain development, with short-chain fatty acids (SCFAs) from the microbiota playing a pivotal role. Understanding the relationship between dysbiosis, SCFAs, and brain development is crucial. In this study, we investigated the impact of antibiotics on the concentration of SCFAs in neonatal feces. Additionally, we developed a model of gut dysbiosis in neonatal mice to examine the potential relationship between this imbalance, SCFAs production, and brain function development.

Methods: We measured the SCFAs content in the feces of two groups of neonates, categorized based on whether antibiotics were used, and conducted the Neonatal Behavioral Neurological Assessment (NBNA) test on all neonates. Then we evaluated fecal SCFAs levels in neonates and neonatal mice post-antibiotic treatment using liquid chromatography-mass spectrometry (LC-MS) analysis. Morris water maze (MWM) tests assessed behavioral performance, and western blot analysis examined brain tissue-related proteins—neuron-specific enolase (NSE), ionized calcium binding adaptor molecule-1 (IBA1), and myelin basic proteins (MBP).

Results: The use of antibiotics did not affect the NBNA scores of the two groups of neonates, but it did reduce the SCFAs content in their feces. Antibiotic administration induced gut dysbiosis in mice, resulting in decreased IBA1 and MBP expression. Interventions to restore gut microbiota ameliorated these effects. Mice with dysbiosis displayed cognitive deficits in the MWM test. SCFAs levels decreased during dysbiosis, and increased upon microbiota recovery.

Conclusions: Neonatal dysbiosis affects the microbiota-gut-brain axis, impairing cognitive function and nervous system development. Reduced SCFAs may contribute significantly to these alterations.

Keywords: Gastrointestinal microbiome; short-chain fatty acid (SCFA); neuron-specific enolase (NSE); ionized calcium binding adaptor molecule-1 (IBA1); myelin basic proteins (MBP)

Submitted Mar 31, 2024. Accepted for publication Aug 06, 2024. Published online Aug 28, 2024.
doi: 10.21037/tp-24-128
View this article at: https://dx.doi.org/10.21037/tp-24-128

Introduction

The composition of the gut microbiome undergoes changes across different stages of life and has been implicated in the pathophysiology of various health conditions, including inflammatory and metabolic diseases, as well as neurological disorders (1). Microbial metabolites not only provide nutritional value to the host but also serve as substrates for enzymes such as acetylases, methylases, and

glucuronidases (2). These enzymes can modify the availability and function of the epigenetic machinery (2). The colonization of the gut in early life is a crucial process that has been linked to the long-term composition of the gut microbiome. Epigenetic processes during early life play a regulatory role in gene expression and have a significant impact on lifelong health, serving as a primary mechanism for developmental programming (3).

Short-chain fatty acids (SCFAs) are products of microbial fermentation, primarily composed of acetate, propionate, and butyrate. Acetate is a commonly produced SCFA by most gut microbiota, while specific families of Firmicutes have demonstrated the ability to generate butyrate. Propionate, on the other hand, originates from succinate, which is found in Bacteroidetes and certain Firmicutes (4). SCFAs have the capacity to adhere to the gut mucosa, bind to cell receptors, and transmit signals, exerting significant effects on various biological processes. This includes regulating gut metabolism, influencing proliferation and differentiation, as well as modulating homeostasis within organisms.

Neurons, the primary cells involved in nerve conduction, are initially immature at birth and undergo functional and structural development during the early postnatal stage. Neuron-specific enolase (NSE) is an exceptional glycolytic enzyme that is a highly acidic soluble protein found exclusively within neurons (5). It serves as a neuron-specific marker (6). In the embryonic stage, the expression of NSE in the brain is minimal, while non-neuronal enolase (NNE), an isoenzyme of NSE present in glial cells, is prominently produced. However, it has been observed that there is a transition from NNE to NSE during neuronal development. Over time, NSE levels increase while NNE levels decrease. This finding suggests a correlation between NSE and the differentiation as well as the functional maturation of neuronal cells (5).

The function of neurons in the central nervous system (CNS) relies on the presence of myelin, which facilitates rapid and efficient nerve conduction. Myelin is produced by specialized glial cells called oligodendrocytes (7,8). However, both humans and mice have mostly unmyelinated axons at birth, with a rapid onset of myelination occurring shortly after birth through the action of oligodendrocytes (9). The regulation of myelination plays a crucial role in determining the extent of myelin formation and is closely associated with brain plasticity and function (10). Consequently, any disruptions in myelination can have detrimental effects on brain function. The microbiota is essential for myelination and the maintenance of myelin plasticity, and the administration of antibiotics can lead to impaired myelination due to dysbiosis (11). Myelin basic proteins (MBP), which are integral components of CNS myelin, have been used as accurate markers of myelination (12).

Microglia are long-lived immune cells within the brain (13) that appear early during embryonic development and have limited capacity for self-renewal (14). During early postnatal development, microglia undergo a transition from an activated phenotype with high proliferative activity to a state of low homeostatic proliferation. As phagocytes, they play a critical role in removing debris during CNS development and disease (14), which is essential for plasticity and learning. Ionized calcium binding adaptor molecule-1 (IBA1) is a calcium-binding protein that exhibits specific localization in microglia, causing membrane ruffling and phagocytosis among activated microglia (15). The absence of IBA1 has been identified as a marker of dysfunctional microglia (13), indicative of functional impairment in microglia. Thus, the evaluation of IBA1 expression is a suitable criterion for assessing the status of microglia.

The most opportune timeframe for nutritional intervention to effectively support brain development occurs during the

Highlight box

Key findings

- In this study, we observed that early antibiotic exposure in neonates affects the generation of short-chain fatty acids (SCFAs) in feces. In mouse model of intestinal dysbiosis, reduced production of short-chain fatty acids was found, along with impacts on mouse brain development and cognitive function. Importantly, these changes were ameliorated by interventions targeting the microbiota.

What is known and what is new?

- It is well recognized that early antibiotic use during birth affects the establishment of gut microbiota, consequently impacting brain development via the gut-brain axis.
- This study assessed alterations in fecal SCFAs, cognitive function, and markers of brain development in a murine model of intestinal dysbiosis.

What is the implication, and what should change now?

- Early antibiotic administration can disrupt the establishment of gut microbiota, potentially impacting brain development by reducing SCFA production. In clinical practice, there should be stricter criteria for antibiotic use, and SCFAs may serve as important targets for further investigation into early neonatal brain protection.

late stages of fetal development and early postnatal life, which coincides with the establishment of the gut microbiota. There may be a bidirectional communication between the microbiota and the brain. In this context, we propose that SCFAs play a role in mediating the interaction between the gut microbiota and brain development during the early postnatal period.

In this study, we initially investigated the alterations in intestinal microbiota and SCFAs levels in infants during early life. We evaluated whether these changes were associated with clinical physiological function scores, categorized according to the administration or non-administration of antibiotics. Subsequently, we established a model of antibiotic-induced dysbiosis and assessed the effects of changes in intestinal SCFAs and expression levels of three marker proteins in the CNS. We also evaluated the protective effects of fecal bacteria transplantation and probiotics. We present this article in accordance with the ARRIVE reporting checklist (available at https://tp.amegroups.com/article/view/10.21037/tp-24-128/rc).

Methods

Participant selection

In this study, a total of 60 neonates born via cesarean section were enrolled from newborn ward of the Second Affiliated Hospital of Anhui Medical University after obtaining informed consent from their parents. Among the cohort of 60 infants, a subgroup of 30 infants exhibited high-risk factors for infection, such as prolonged rupture of membranes exceeding 18 hours, exposure to contaminated amniotic fluid or meconium during labor, and clinical manifestations suggestive of early infection. As per the clinical guidelines for antibiotic use, these infants were administered antibiotics. The remaining 30 infants did not exhibit any infectious factors and were not subjected to antibiotic treatment. The exclusion criteria for this study included severe birth asphyxia, neonatal respiratory distress syndrome (NRDS), genetic metabolic disorders, and other malformations. Based on antibiotic utilization, the newborns were divided into two groups, and the Neonatal Behavioral Neurological Assessment (NBNA) tests were performed on the infants during the second week of their lives. For discharged patients, follow-up NBNA tests were conducted during the outpatient visit scheduled at the second-week appointment. Stool samples were collected on postnatal days 1–3 and postnatal days 7–10, and the levels of SCFAs were measured using liquid chromatography-mass spectrometry (LC-MS) analysis. This study was conducted in accordance with the Declaration of Helsinki (as revised in 2013) and was approved by the Ethics Committee of The Second Affiliated Hospital of Anhui Medical University (approval No. Pj2016-05-01).

Animals

A total of 40 wild-type male C57BL/6J neonatal mice at 7 days of age were included in this study. The mice were housed in group cages with five mice per cage, and the cages were lined with chip bedding. Throughout the study, the mice had ad libitum access to standard chow and water. The bedding, standard chow, and water were autoclaved prior to the experiment. The vivarium maintained a lighting schedule of 12 hours of light and 12 hours of darkness each day, with a constant temperature of 21±1 ℃. The health status of the mice was monitored daily during the course of the treatment, and no adverse events were observed throughout the experiment. The experimenters processing the samples were blinded to the specific pharmacological therapy being administered. Euthanasia of the mice was performed either by CO_2 asphyxiation followed by cervical dislocation or by isoflurane inhalation followed by perfusion with 4% paraformaldehyde (PFA). All mice used in the study were provided by the Animal Experiment Center of Anhui Medical University, and the experiments were performed under a project license (No. LLSC20211513) granted by the Biology Committee of Anhui Medical University, in compliance with Anhui Medical University guidelines for the care and use of animals. A protocol was prepared before the study without registration.

Animal study design

In this study, 40 seven-day-old C57BL/6J mice, housed in a total of 8 cages, were randomly assigned to one of four groups: three experimental antibiotic groups and one control group, with 10 mice per group (*Figure 1*). The mice in the antibiotic group received oral ceftriaxone via gavage, administered twice daily for 7 days, at a dose of 80 μg/g. Simultaneously, the control group received an oral gavage of the vehicle (water) twice daily for the same duration. On the 8th day, the antibiotic-treated mice were subjected to a 14-day gut microbiota intervention phase, consisting of three groups: the *Bifidobacterium* transplantation group (administered at a dose of 1×10^7 CFU/g; Shanghai Shangyao Xinyi Pharmaceutical Factory Company Limited,

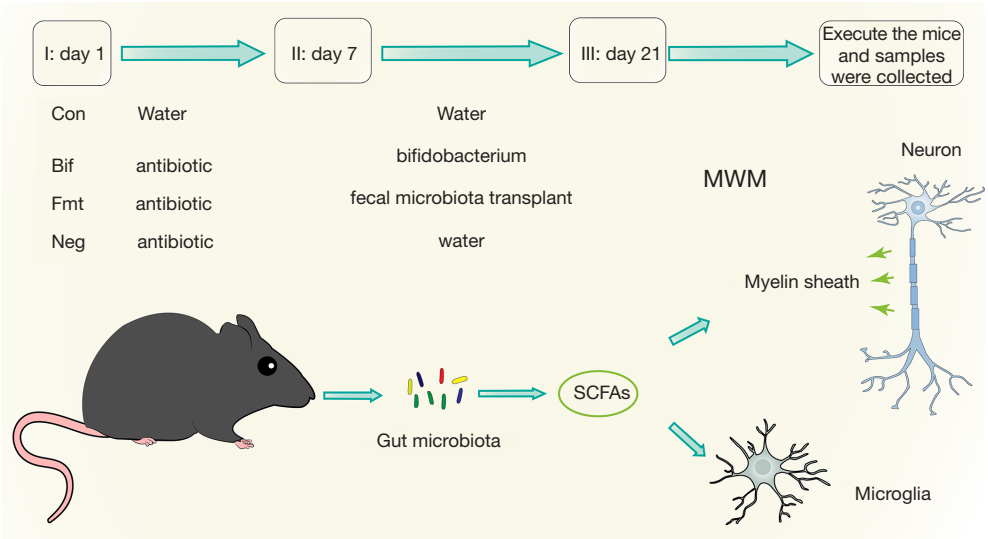

Figure 1 Study design. C57BL/6J mice were oral antibiotic by gavage or vehicle (water) from days 1 to 7. From day 8 to day 21, mice were given *Bifidobacterium* suspension, fecal bacteria suspension or vehicle (water). Con group: mice oral vehicle (water) by gavage twice daily for 21 days; bif group: mice oral antibiotic by gavage twice daily for 7 days and *Bifidobacterium* suspension for 14 days; fmt group: mice oral antibiotic by gavage twice daily for 7 days and fecal microbiota suspension for 14 days; neg group: mice oral antibiotic by gavage twice daily for 7 days and vehicle (water) for 14 days. MWM, Morris water maze; SCFA, short-chain fatty acid.

S10970104, China), the fecal microbiota transplantation group, and a negative control group receiving water intervention. The fecal bacteria transplantation solution comes from normal healthy adult mice. The physical growth and development of mice in each group were monitored to ensure normal progress throughout the experiment. Following completion of behavioural testing by Morris water maze (MWM), all mice were euthanized. Faecal pellets were collected on the 1st day, 7th day, and 21st day for 16S Illumina sequencing and LC-MS analysis. The hippocampus is a key structure involved in memory and learning, thus we chose hippocampal tissues from these mice for western blotting (WB) and immunohistochemistry (IHC) to detect brain NSE, MBP, and IBA1. Adequate and consistent feed was provided to all groups to minimize any confounding effects. During the experiment, the operator was unaware of the group assignments to minimize bias introduced by their actions.

Preparation of fecal microbiota transplantation suspension

We collected fresh feces from two normal adult mice early in the morning on the day of fecal microbiota transplantation and weighed them. The feces were immediately mixed with sterile saline (1:5, w/v) and sealed. The mixture was thoroughly homogenized in an anaerobic environment and then filtered through successive layers of gauze (10 mesh, 30 mesh, 60 mesh, 100 mesh) to remove large particles. The filtrate was collected into sterile centrifuge tubes. The suspension was centrifuged at 3,000 rpm for 3 minutes at 4 ℃ in a refrigerated centrifuge. The supernatant was discarded, and the pellet was resuspended in an equal volume of saline, vortexed, and centrifuged again. This washing step was repeated three times. The final pellet was resuspended in saline to obtain the fecal microbiota filtrate. All steps were performed at low temperatures as much as possible, with the entire process completed within one hour. Based on the clinical dosage used for human fecal microbiota suspension (1.67 g/kg/day), the fecal microbiota transplantation dose was calculated to be 8.4 μL/g/day, administered once daily by gavage (16).

Gut microbiota sequencing

Immediately after collection, the faecal samples were stored at −80 ℃. Genomic DNA extraction from the faecal samples was performed using cetyl trimethyl ammonium bromide. Subsequently, polymerase chain reaction (PCR) amplification of specific regions of the 16S rRNA gene (16S V3-V4) was conducted using primers with barcodes

(341F-806R, 5'-CCTAYGGGRBGCASCAG, and 5'-GGACTACNNGGGTATCTAAT). The amplified products were then purified by excising the target bands and subjected to library construction. Sequencing was performed on the IonS5TMXL sequencing platform (Thermo Fisher Scientific, Waltham, MA, USA). The obtained sequencing data underwent quality control, filtering, and removal of chimeras using UPARSE software (UPARSE v7.0.1001) to generate cleaned data. Representative sequences for each operational taxonomic unit (OTU) were selected from the cleaned data. Taxonomic information for the representative sequences was annotated using the Silva Database based on the Mothur algorithm. Standardization of OTU abundance information was performed, followed by subsequent data analysis.

Mass spectrometry (MS)

Fresh mouse faecal samples were collected and immediately stored at −80 ℃ until further analysis. To prepare the samples, the feces were homogenized in nanopure water (25 mg/mL) for 5 minutes, followed by centrifugation at 14,000 rpm and 4 ℃ for 10 minutes. Then, 160 µL of the resulting supernatant was incubated with 20 µL of 0.1 M BHA in MeOH and 20 µL of 0.25 M EDC in MeOH at 25 ℃ for 1 hour. After the incubation, the faecal extract was diluted 3-fold in 50% aqueous MeOH. Next, 200 µL of the diluted sample was extracted with 600 µL of dichloromethane through vigorous shaking for 10 minutes. Following centrifugation, 200 µL of the lower organic layer was transferred and evaporated using an SPE-dryer at 40 ℃. The resulting residue was reconstituted in 200 µL of 50% aqueous MeOH, briefly vortexed, and centrifuged prior to a 5-µL injection for LC-MS/MS. Chromatographic separations were performed on a Waters C18 stationary phase column (2.1 mm × 50 mm, 1.8 µm). The mobile phases consisted of 100% MeOH (B) and nanopure water (A). A gradient elution method was used with the following conditions: 0 to 4 minutes, 32% to 60% B; 4 to 4.8 minutes, 60% to 65% B; 4.8 to 4.9 minutes, 65% to 98% B; 4.9 to 5.2 minutes, 98% B; and 5.3 minutes to 32% B for re-equilibration. The flow rate was set at 0.40 mL/min, and the column temperature was maintained at 45 ℃. Throughout the analysis, the autosampler was kept at 5 ℃. MS was performed in selected reaction monitoring mode and positive ionization mode, with optimized parameters using SCFAs standards. The optimized ion source parameters were as follows: collision gas (CAD) 7, curtain gas (CUR) 35, ion spray voltage (IS) 5000, and source temperature (TEM) 550 ℃ (17). A calibration curve was generated using SCFAs standards.

WB

Tissue samples were stored at −80 ℃ until further analysis. For brain tissue, it was homogenized in RIPA buffer, and the protein concentration was measured using a bicinchoninic acid (BCA) protein quantification assay. The resulting suspension was diluted in 5X buffer and boiled for 15 minutes. Electrophoresis was performed using a voltage of 75 V for the concentrated gel phase and 120 V for the separation gel phase. The proteins were subsequently transferred onto PVDF membranes using transfer buffer at a constant current of 300 mA for 30 minutes. To prevent nonspecific binding, the membranes were blocked with 5% milk (w/v) in TBST (50 mM Tris, 150 mM NaCl, 1% Tween) for 30 minutes. Next, the membranes were incubated overnight at 4 ℃ with specific mouse antibodies or mouse anti-β-actin. On the following day, the membranes were washed with TBST and then incubated with a secondary antibody for 30 minutes. After another round of washing, the membranes were incubated with an enhanced chemiluminescence (ECL) WB substrate. Finally, the membranes were imaged using the Bio-Rad Image Lab system.

IHC

Brain tissue samples were fixed overnight in 4% PFA and then sliced into 5 mm-thick sections. These sections underwent deparaffinization and dehydration using a gradient of ethanol. To block endogenous peroxidase activity, the sections were treated with 3% H_2O_2 and then underwent high-pressure antigen retrieval using citrate buffer solution (pH =6.0). Subsequently, the sections were blocked with goat serum. For immunostaining, sections were incubated overnight at 4 ℃ with primary antibodies, including Anti-NSE Neuronal Marker (ab53025, Abcam, Cambridge, UK), recombinant Anti-Iba1 antibody (ab178846, Abcam), and Anti-MBP antibody (ab40390, Abcam). After washing with TBST, the sections were incubated with biotinylated secondary antibody (goat anti-mouse-rabbit IgG) for 2 hours. For visualization, the sections were stained with DAKO Liquid DAB+ substrate chromogen system for 30 seconds. Following a water wash, the slides were counterstained with haematoxylin

Table 1 Baseline characteristics of each group

Group	Antibiotic group (n=30)	No-antibiotic group (n=30)	P value
Gestational age (weeks)	38.57±0.80	38.55±0.83	0.92
Male:female, n	16:14	17:13	0.79
Weight (g)	3,270.37±328.37	3,346.77±342.12	0.38
NBNA score	38.50±0.73	38.77±0.68	0.14
AA (days 1–3)	5.17±1.23	5.15±1.07	0.95
PA (days 1–3)	0.32±0.34	0.38±0.37	0.52
BA (days 1–3)	0	0	–
AA (days 7–10)	131.27±10.90	179.44±35.04	<0.001
PA (days 7–10)	101.06±6.81	187.71±42.94	<0.001
BA (days 7–10)	3.30±1.06	16.71±6.04	<0.001

The data are presented as mean ± standard deviation unless otherwise indicated. NBNA, neonatal behavioral neurological assessment; AA, acetic acid; PA, propanoic acid; BA, butyric acid.

and then mounted. We conducted immunohistochemical quantification analysis of IBA1-positive microglial cells, NSE-positive neurons, and MBP-positive oligodendrocytes using Image J software, focusing primarily on three regions of the hippocampus: cornu ammonis 1 (CA1), cornu ammonis 3 (CA3), or dentate gyrus (DG). These regions play pivotal roles in neuroscience and cognitive research.

MWM

The MWM is a well-established method used to assess cognitive processes in mice, specifically spatial learning and memory. In this study, we employed the MWM paradigm to evaluate spatial learning and memory in a circular swimming arena with a diameter of 120 cm. The arena was filled with a nontoxic white pigment suspension maintained at a temperature of 26±1 ℃. To locate a submerged platform with a diameter of 10 cm, the mice relied on distal visual cues. The MWM protocol included two days of learning trials, followed by five days of visual cue tests, and finally, one day of probe tests. Each daily session consisted of four swimming trials, with a 15-minute interval between trials. The starting positions for the learning trials and visual cue tests were randomly assigned from four different locations. Video hardware and Ethovision software from Noldus (the Netherlands) were utilized to record the swimming paths of the mice. If a mouse failed to find the platform within 120 seconds, it was gently guided to the platform and allowed to remain there for 10 seconds. The probe trials, which assessed reference memory, were conducted on the ninth day when the platform was absent. In these trials, the swimming trial began from the quadrant opposite to the target quadrant where the platform had been located.

Statistical analysis

Statistical analysis was performed using SPSS version 23.0 (IBM Corp., Armonk, NY, USA). The data were presented as mean ± standard deviation. To determine differences between the groups, a one-way analysis of variance followed by the Bonferroni post-test was employed. The data obtained from the MWM were analyzed using repeated-measures analysis of variance (ANOVA). Statistical significance was considered when the P value was less than 0.05. For data visualization, ImageJ version 1.53 (National Institutes of Health), GraphPad Prism version 9.0 (GraphPad Software, La Jolla, CA, USA), and Adobe Illustrator CC 2021 (Adobe Software, San Jose, CA, USA) were used.

Results

The association between antibiotic use, NBNA scores, and fecal SCFAs levels in newborns (Table 1)

The neonates who did not receive antibiotics demonstrated higher NBNA scores compared to those who did receive antibiotics, although the difference was not statistically

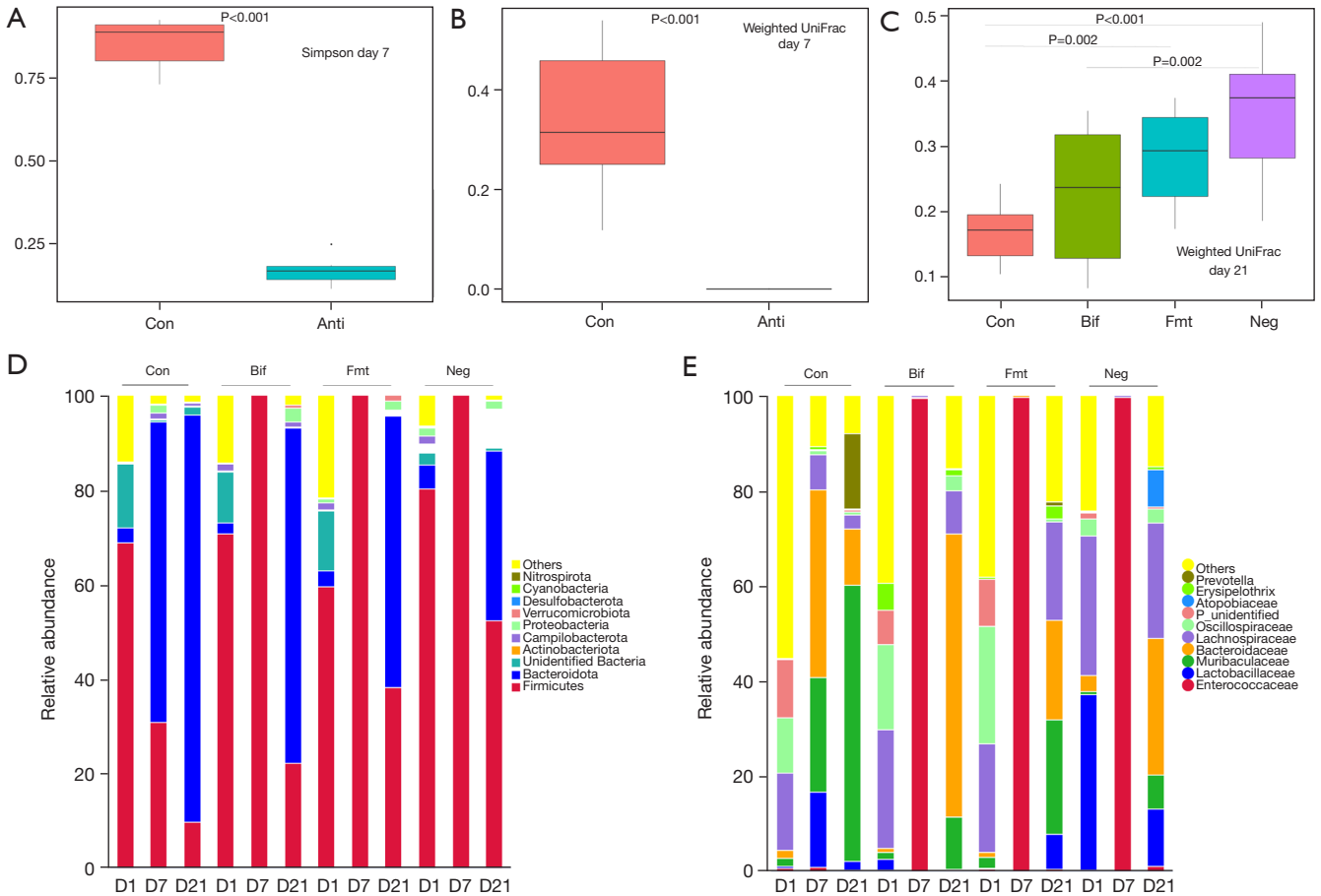

Figure 2 After one week of antibiotic treatment in mice, gut dysbiosis occurred. Subsequent administration of *Lactobacillus* via gavage and fecal microbiota transplantation for two weeks resulted in varying degrees of gut microbiota restoration. (A) α-diversity; (B) β-diversity (day 7); (C) β-diversity (day 21); (D) phylum abundance; (E) family abundance. Con group: mice oral vehicle (water) by gavage twice daily for 21 days; anti group: mice oral antibiotic by gavage twice daily for 7 days, including the con group and the neg group; bif group: mice oral antibiotic by gavage twice daily for 7 days and *Bifidobacterium* suspension for 14 days; fmt group: mice oral antibiotic by gavage twice daily for 7 days and fecal microbiota suspension for 14 days; neg group: mice oral antibiotic by gavage twice daily for 7 days and vehicle (water) for 14 days.

significant. No significant differences were observed in the levels of acetate, propionate, and butyrate in the first fecal samples between the two groups of newborns. On days 7–10, the levels of all three SCFAs were higher in the group of neonates who did not receive antibiotics.

Differential colonization of the gut microbiota between different groups

16S Illumina sequencing was employed to analyze the bacterial community and identify changes in the gut microbiota among neonatal mice treated with antibiotics compared to control mice at day 7. The ten most abundant phyla were examined to assess the recovery of the gut microbiota following interventions such as *Bifidobacterium* transplant, fecal bacteria transplant, and water treatment. Antibiotic administration resulted in significant alterations in bacterial communities, indicating a disturbance in microbiota-gut homeostasis. Alpha-diversity metrics, were significantly lower (P<0.001) in the antibiotic-treated group, indicating a less diverse bacterial community compared to controls (*Figure 2A*). Phylogenetic (weighted UniFrac) beta-diversity indices on day 7 confirmed the significant disparity between the antibiotic and control

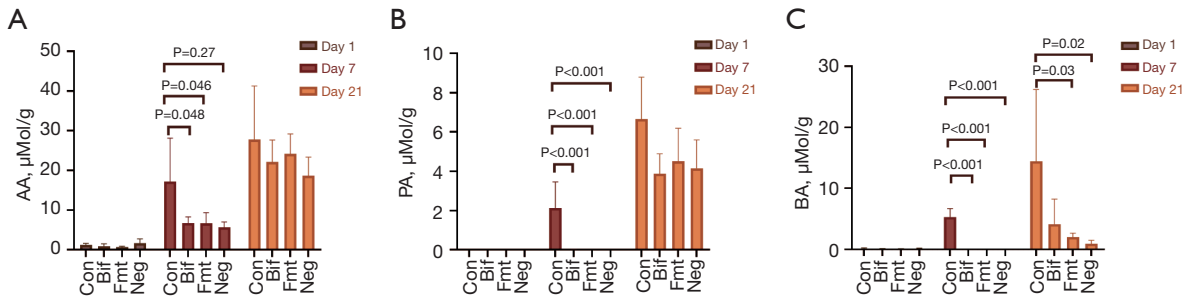

Figure 3 SCFAs concentrations in fecal pellets measured by mass spectrometry indicate that gut microbiota imbalance affects SCFA production, with butyrate being the most severely impacted and least readily restored. (A) The concentration of AA in the feces of different groups of mice at various time points; (B) the concentration of PA in the feces of different groups of mice at various time points; (C) the concentration of BA in the feces of different groups of mice at various time points. The blanks indicated that the concentrations of PA and BA were too low to be detected on day 1 and day 7. Con group: mice oral vehicle (water) by gavage twice daily for 21 days; bif group: mice oral antibiotic by gavage twice daily for 7 days and *Bifidobacterium* suspension for 14 days; fmt group: mice oral antibiotic by gavage twice daily for 7 days and fecal microbiota suspension for 14 days; neg group: mice oral antibiotic by gavage twice daily for 7 days and vehicle (water) for 14 days. SCFA, short-chain fatty acid; AA, acetic acid; PA, propionic acid; BA, butyric acid.

groups (*Figure 2B*). Furthermore, the beta-diversity indices on day 21 revealed significant dissimilarity among the four groups (*Figure 2C*). In control mice, Firmicutes dominated on the first day (68%), followed by rapid establishment and predominance of *Bacteroidota* on the 7th day (63%). By the 21st day, *Bacteroidota* accounted for 86% of the bacterial community. Over time, additional microbial species colonized the intestines, leading to a balanced and abundant intestinal microecology. In contrast, in the antibiotic-treated groups, Firmicutes was the sole surviving phylum after one week of antibiotic administration, comprising 99% of the microbiota, while the relative abundances of Bacteriodetes and Actinobacteria decreased to nearly undetectable levels (*Figure 2D*). This established an ideal dysbiosis model. Detailed bacterial changes are depicted in *Figure 2E*, where *Bacteroidaceae*, *Muribaculaceae*, *Lactobacillaceae*, *Lachnospiraceae*, and *Oscillospiraceae* were detected in all groups on the 21st day but were absent in the antibiotic administration groups on the 7th day.

The levels of SCFAs different groups

LC-MS was utilized to quantify the levels of SCFAs, including acetic acid (AA), propanoic acid (PA), and butyric acid (BA), in mouse feces on days 1, 7, and 21 (*Figure 3*). In the control mice, SCFAs were expected to be present at a low level at birth and gradually increase over time, playing a role in establishing gut microbiota homeostasis. However, following antibiotic administration, the production of SCFAs significantly decreased, particularly PA and BA, which were nearly undetectable (P<0.001; P<0.001) (*Figure 3B,3C*). By day 21, the levels of SCFAs in all groups exhibited varying degrees of increase after gut microbiota intervention, consistent with the recovery of the gut microbiota.

Cognitive differences between different groups

We employed the MWM to evaluate the impact of neonatal gut dysbiosis on cognitive processes. By utilizing the MWM, neonatal mice with gut dysbiosis demonstrated a significantly lower exploration ratio in the target quadrant compared to the control group, indicating impaired learning ability. Over the course of 5-day training, all groups showed a decrease in escape latency (*Figure 4A*). Additionally, throughout the training period, the percentage of swimming time spent in the target quadrant increased for all groups (*Figure 4B*). Overall, the cognitive function of the control group exhibited the highest performance, while the negative intervention group displayed the poorest cognitive function. In the probe trial, it was observed that the control group mice spent the longest time in the target quadrant (*Figure 4C*) and crossed the platform the most frequently compared to the other groups (*Figure 4D*). The *Bifidobacterium* group and the fecal microbiota transplantation group spent more time in the target quadrant (*Figure 4C*) and crossed the platform more frequently than the negative group (*Figure 4D*), with all differences being statistically significant.

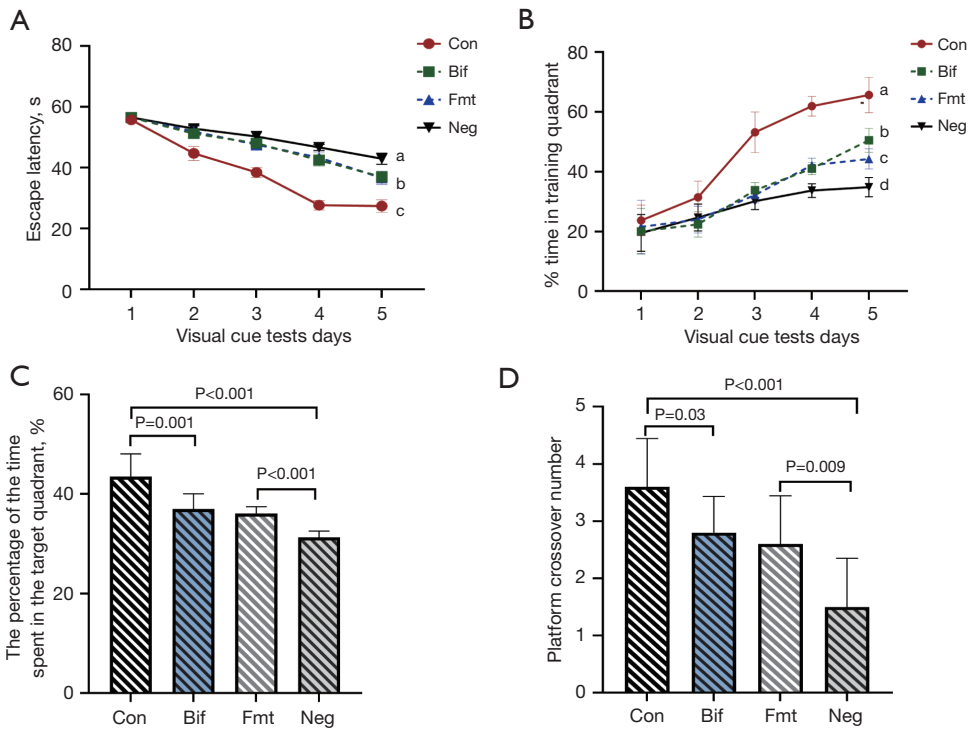

Figure 4 Gut dysbiosis mice exhibit learning disability in the MWM, which was remitted by gut microbiota rebuild intervention. Spatial learning was assessed as a function of training day with respect to the following parameters: (A) escape latency and (B) percentage of time spent in the target quadrant. Repeated-measures ANOVA analyzed data of MWM. (A) a: P<0.001, con versus bif or fmt; b: P<0.001, bif or fmt versus neg; c: P<0.001, neg versus con. (B) a: P<0.001, con versus bif; b: P=0.001, bif versus fmt; c: P<0.001, con versus fmt; d: P<0.001, fmt versus neg. Spatial memory is evaluated as a function of detection tests on the following parameters: (C) the percentage of the time spent in the target quadrant and (D) platform crossover number. Repeated-measures ANOVA analyzed data of MWM. Con group: mice oral vehicle (water) by gavage twice daily for 21 days; bif group: mice oral antibiotic by gavage twice daily for 7 days and *Bifidobacterium* suspension for 14 days; fmt group: mice oral antibiotic by gavage twice daily for 7 days and fecal microbiota suspension for 14 days; neg group: mice oral antibiotic by gavage twice daily for 7 days and vehicle (water) for 14 days. MWM, Morris water maze; ANOVA, analysis of variance.

The expression of brain-related proteins in gut microbiota dysbiosis

We examined the impact of gut dysbiosis on brain function by assessing neuronal development, myelin formation, and microglial changes. The hippocampus, known for its role in memory and learning, was selected for investigation.

Neuronal development was evaluated through the measurement of NSE expression in all groups (*Figures 5*,*6*). NSE is considered an indicator of neuronal maturation. Protein blot and immunohistochemical (IHC) analyses revealed no significant differences in NSE expression among the groups.

To assess myelin's relevance to memory formation and cognitive function, we examined MBP expression using both WB and IHC. MBP deposition serves as a reliable marker for evaluating myelin synthesis. In comparison to the control group, the antibiotic-treated groups (*Bifidobacterium* transplantation group, fecal microbiota transplantation group, and negative intervention group) exhibited lower MBP expression in the WB analysis (*Figure 5*). Similarly, the IHC results demonstrated that the control group displayed a substantial increase in MBP-immunoreactive fibers compared to the antibiotic groups, with a consistent trend observed across all three groups (*Figure 6*).

Reduced expression of IBA1 in microglia is indicative of dystrophic microglial loss of IBA1 immunoreactivity. Results from IHC showed that the negative intervention

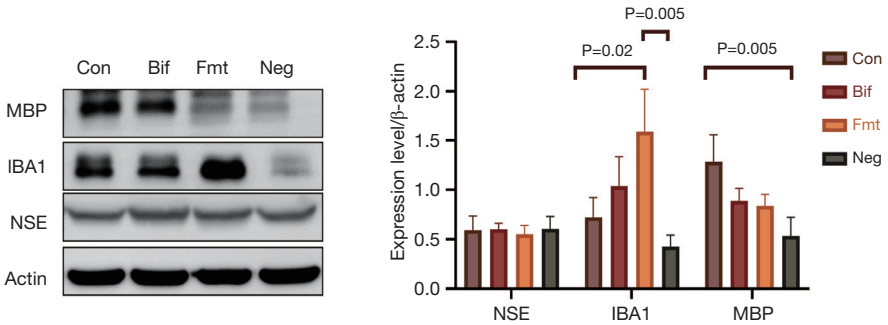

Figure 5 Expression level of NSE, IBA1 and MBP *in vivo* experiment. NSE expression showed no significant differences among groups, while IBA1 and MBP were affected by gut microbiota dysbiosis. Con group: mice oral vehicle (water) by gavage twice daily for 21 days; bif group: mice oral antibiotic by gavage twice daily for 7 days and *Bifidobacterium* suspension for 14 days; fmt group: mice oral antibiotic by gavage twice daily for 7 days and fecal microbiota suspension for 14 days; neg group: mice oral antibiotic by gavage twice daily for 7 days and vehicle (water) for 14 days. MBP, myelin basic proteins; IBA1, ionized calcium binding adaptor molecule-1; NSE, neuron-specific enolase.

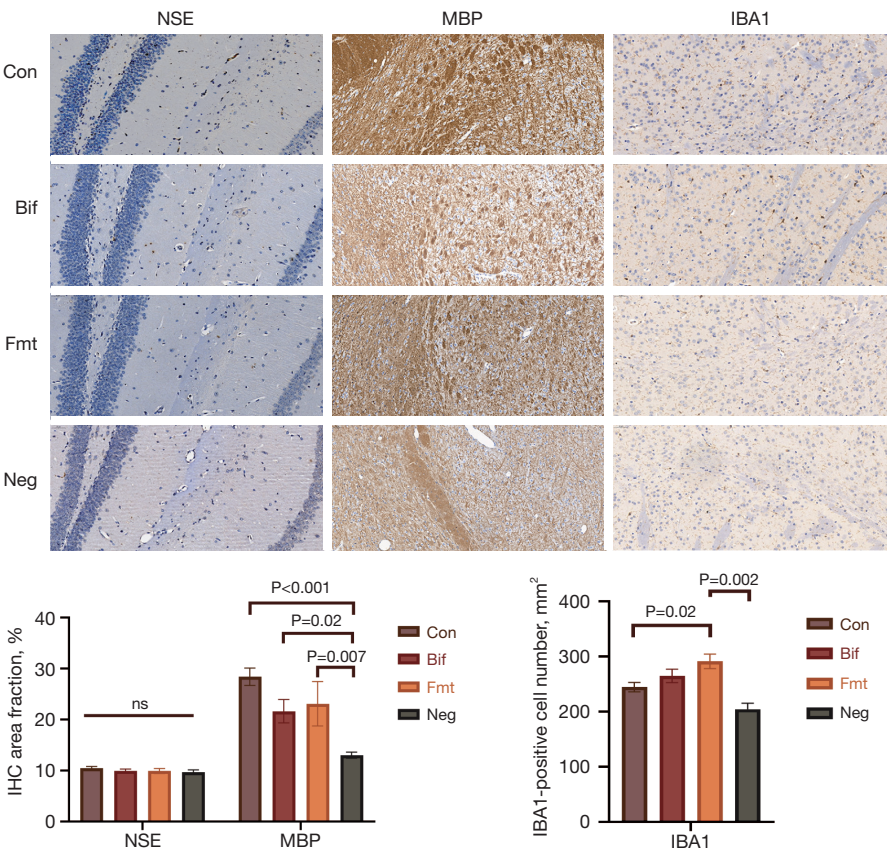

Figure 6 Photomicrographs of immunohistochemical markers showing NSE positivity, IBA1 positivity, and MBP positivity (20×). NSE expression showed no significant differences among groups, while IBA1 and MBP were affected by gut microbiota dysbiosis. Con group: mice oral vehicle (water) by gavage twice daily for 21 days; bif group: mice oral antibiotic by gavage twice daily for 7 days and *Bifidobacterium* suspension for 14 days; fmt group: mice oral antibiotic by gavage twice daily for 7 days and fecal microbiota suspension for 14 days; neg group: mice oral antibiotic by gavage twice daily for 7 days and vehicle (water) for 14 days. IBA1, Ionized calcium binding adaptor molecule-1; MBP, myelin basic proteins; NSE, neuron-specific enolase; ns, none significant.

group had lower expression levels compared to the control group (*Figure 6*), implying that neonatal dysbiosis may lead to IBA1 loss and dystrophic microglia. In contrast, WB analysis demonstrated that gut microbiota restoration interventions, fecal microbiota transplantation, effectively alleviated the damage, leading to even overexpression of IBA1 (P=0.02) (*Figure 5*). Furthermore, IHC analysis supported these findings, revealing a similar trend in the distribution of IBA1-positive microglia. Comparative analysis indicated that the gut microbiota intervention groups exhibited the highest density of IBA1-positive cells, while the negative intervention group had the lowest (*Figure 6*).

Discussion

The period immediately following birth is widely recognized as a critical window for development, during which multiple organs and physiological functions of the body remain immature. During early life, epigenetic processes play a crucial role in regulating gene expression and exert significant influence on long-term health outcomes (3). These processes serve as the primary mechanism for developmental programming. The highest degree of epigenetic imprinting occurs within the initial two years of life and is closely linked to early-life nutrition as well as the development of diseases such as asthma, allergies, obesity, and diabetes later in life (2). The epigenetic mechanisms responsible for early-life inflammation and maintaining intestinal barrier integrity are modulated by the gut microbiota (2). Throughout the critical phase of early development, infant nutrition plays a pivotal role in shaping the composition and maturation of both the gut microbiota and the infant immune system, steering them towards a healthy adult-like state (3). The brain undergoes rapid developmental changes during the initial year after birth, and early nutrition plays a critical role in providing the necessary building blocks for proper brain structure formation. Early weaning can also affect brain development in mice, potentially leading to behavioral disorders and microglial activation (18). Additionally, through epigenetic mechanisms involving chromatin modifications, nutrition can influence gene expression and modify genes associated with synaptic plasticity. In this context, the gut microbiota and its metabolites play a pivotal role. SCFAs not only serve as energy sources for the body but can also traverse the blood-brain barrier to interact with freely expressed fatty acid receptors in the brain or exert their effects through epigenetic mechanisms.

Previous studies have shown that early-life microbial disruption alters the expression of myelin-related genes in the prefrontal cortex and changes the morphology of basolateral amygdala microglia (19). Maternal antibiotic use and changes in gut microbiota can also affect the social abilities and cognitive behaviors of newborns, leading to the development of depression (20,21). In our previous investigations, we have observed that the administration of antibiotics disrupts the establishment of gut microbiota colonization in the intestines of newborns (22). In the current study, we have also identified a notable decrease in the levels of SCFAs detected in the fecal samples of newborns receiving antibiotics, accompanied by lower NBNA scores. Although these differences did not reach statistical significance, it is plausible that the relatively brief duration of the study might not have provided sufficient time to discern substantial disparities in macroscopic functional assessments. Our study aims to examine the interplay between SCFAs, brain neurodevelopment, and cognitive function in neonatal mice experiencing dysbiosis of the gut microbiota. To accomplish this, we have established a mouse model of gut dysbiosis.

The gut microbiota plays a crucial role in the development of the nervous system in neonates, facilitating nerve growth and myelination. In the course of this investigation, we observed cognitive deficits in mice exhibiting dysbiosis of the gut microbiota when subjected to the water maze experiment. In our study, we identified a notable elevation in Firmicutes and an absence of Bacteroidetes in newborn mice treated with antibiotics. Firmicutes have been associated with the maintenance of cognitive function (23) and gut physiology (24), and they play a critical role in the bidirectional communication between the microbiota, gut, and brain axis (MGB axis). The use of antibiotics has been observed to result in a deficiency of Bacteroidetes, which is crucial for the production of SCFAs. Moreover, at the family level, certain microbiota groups such as *Bacteroidaceae*, *Muribaculaceae*, and *Lachnospiraceae*, which contribute significantly to the production of SCFAs, were found to be diminished in the antibiotic-treated group. Interestingly, these microbiota groups were partially restored through the administration of bifidobacteria or fecal transplants, leading to an increase in the production of SCFAs in the mouse feces. This finding aligns with the overall trend observed in the gut microbiota. Notably, the antibiotic-treated group exhibited a significant reduction in AA, PA, and BA levels in the feces, suggesting

that alterations in the gut microbiota may account for these changes. However, it is important to mention that there was a partial recovery of the gut microbiota and an increase in SCFAs production in mice following bifidobacteria and fecal transplants.

SCFAs have the capacity to interact with various G protein-coupled receptors (25), exerting diverse effects through the regulation of gene expression in immune cells and inhibition of histone deacetylase as an epigenetic factor (26). These actions influence the CNS by modulating neuroplasticity, epigenetics, and gene expression. Notably, BA has demonstrated anti-inflammatory properties within the body. Furthermore, it has been found to synergize with glutarate in promoting neuronal maturation and directly regulating nerve function (27). In the context of neurodegenerative diseases, such as multiple sclerosis, the impact of BA on myelin formation in adults is a crucial element, whereas PA shows potential benefits for addressing CNS abnormalities like demyelination in these conditions. SCFAs also have the capacity to influence the nervous system by regulating the release of hunger hormones from adipocytes in the gastrointestinal tract, thereby potentially improving cognition and memory (28). Reduced production of SCFAs is frequently observed in models of intestinal disorders (29). In our study, newborn mice treated with antibiotics exhibited varying degrees of cognitive impairment compared to the control group. However, upon administration of probiotics or fecal transplants, the gut microbiota was restored, endogenous levels of SCFAs increased, and cognitive function showed some signs of improvement. Furthermore, exogenous supplementation of SCFAs also demonstrated therapeutic effects in addressing the disease (29,30), with a positive correlation observed between SCFAs supplementation and behavioral outcomes (30).

The appearance of NSE is correlated with the differentiation and functional maturation of neuronal cells. In this study, the expression of neuronal NSE in mouse brain tissues among different groups showed no statistically significant differences, suggesting that early dysbiosis of gut microbiota and abnormal production of SCFAs may not exert an impact on neuronal differentiation and functional maturation. Yu *et al.* suggested that BA could reverse impairments in cognitive function and synaptic plasticity caused by a maternal low-fibre diet (31). Research has already proven that sodium-butyrate treatment can recover the expression of synaptic-related proteins and ameliorate synaptic plasticity to maintain nervous system function (32), which shows the critical roles SCFAs play in the function of synapses. Considering the significant decreases in SCFAs during dysbiosis and the role of SCFAs in the function of synapses, the influence of dysbiosis-related neuronal function is still worthy of further study.

During the early stages of life in both humans and mice, the majority of CNS myelination takes place (33). This process coincides with the expression of MBP, which is primarily observed in mature oligodendrocytes (33). Mice with dysbiosis during early-stage exhibit reduced levels of MBP, which can be restored through intervention targeting the gut microbiota. In this study, neonatal mice with dysbiosis exhibited notable immaturity in myelinization, indicating a potential impact on cognitive function. This effect, however, could be mitigated by implementing a gut microbiota recovery intervention. Keogh suggested that myelin dysregulation caused by antibiotic-induced dysbiosis could be prevented by SCFA supplementation (11). Our study yielded contrasting results from previous findings, which demonstrated that dysbiosis-induced reduction in SCFAs actually stimulated the expression of genes associated with myelination. It is possible that there are alternative approaches yet to be explored for regulating the intricate process of myelination, which were not investigated in our study. However, we observed that mice with restored gut microbiota exhibited a more normalized expression of MBP compared to those in the negative intervention group. This expression pattern closely mirrored the variation trend of SCFAs, particularly butyrate.

Microglia, as proficient immune cells of the brain, play a crucial role in preserving brain function. As previously demonstrated, microglia exhibit a response to the maternal microbiota during prenatal development (34). The influence of the gut microbiota on the brain exhibits spatial and temporal variations during the early stages of life (34). These effects on microglial function have the potential to modulate synaptic pruning, a critical process in which microglia play a significant role (35). The gut microbiota potentially plays a role in mediating the maturation and activation of microglia in the CNS. The absence of gut microbiota, particularly those related to SCFAs, can result in disruptions in the maturation, differentiation, and function of microglia (36). SCFAs serve as crucial signaling molecules in the development of microglia within the CNS (36). In this study, we have presented evidence demonstrating that neonatal gut dysbiosis disrupts the functionality of microglia, as evidenced by altered expression of IBA1, accompanied by a reduction in SCFAs. It has been suggested that restoring gut microbiota homeostasis and

increasing SCFA levels may enhance microglial function. However, the precise mechanisms underlying the response to SCFA signaling remain poorly understood and require further investigation.

Early nutrition is closely related to the establishment of intestinal homeostasis, which may influence organismal development and is potentially linked to epigenetic regulatory pathways mediated by SCFAs. This is particularly significant in the context of neurodevelopment, as the establishment of early intestinal homeostasis is crucial throughout the lifespan. Our study indicates that SCFAs may play a vital role in the various potential mechanisms by which early life intestinal homeostasis affects the development of the CNS.

Conclusions

This research demonstrates that the establishment of gut microbiota homeostasis in neonates has an impact on the generation of SCFAs, which may be a contributing factor affecting cognitive function. In mice with disrupted gut homeostasis, a decrease in SCFA production and impaired cognitive function were observed, along with aberrant expression of proteins related to brain functional cells. Although no mechanistic investigations were undertaken in this study, future studies could aim to elucidate the mechanisms underlying the impact of SCFAs on the development of different brain functional cells.

Acknowledgments

Funding: This study was supported by Anhui Medical University 2022 Peak Discipline Construction Project (No. 9101001818).

Footnote

Reporting Checklist: The authors have completed the ARRIVE reporting checklist. Available at https://tp.amegroups.com/article/view/10.21037/tp-24-128/rc

Data Sharing Statement: Available at https://tp.amegroups.com/article/view/10.21037/tp-24-128/dss

Peer Review File: Available at https://tp.amegroups.com/article/view/10.21037/tp-24-128/prf

Conflicts of Interest: All authors have completed the ICMJE uniform disclosure form (available at https://tp.amegroups.com/article/view/10.21037/tp-24-128/coif). The authors have no conflicts of interest to declare.

Ethical Statement: The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. This study was conducted in accordance with the Declaration of Helsinki (as revised in 2013) and was approved by the Ethics Committee of The Second Affiliated Hospital of Anhui Medical University (approval No. Pj2016-05-01). Informed consent was obtained from the infants' parents. The animal experiments were performed under a project license (No. LLSC20211513) granted by the Biology Committee of Anhui Medical University, in compliance with Anhui Medical University guidelines for the care and use of animals.

References

1. Cusick SE, Barks A, Georgieff MK. Nutrition and Brain Development. Curr Top Behav Neurosci 2022;53:131-65.
2. Finn S, Culligan EP, Snelling WJ, et al. Early life nutrition. Sci Prog 2018;101:332-59.
3. Ames SR, Lotoski LC, Azad MB. Comparing early life nutritional sources and human milk feeding practices: personalized and dynamic nutrition supports infant gut microbiome development and immune system maturation. Gut Microbes 2023;15:2190305.
4. Martin-Gallausiaux C, Marinelli L, Blottière HM, et al. SCFA: mechanisms and functional importance in the gut. Proc Nutr Soc 2021;80:37-49.
5. Marangos PJ, Schmechel DE, Parma AM, et al. Developmental profile of neuron-specific (NSE) and non-neuronal (NNE) enolase. Brain Res 1980;190:185-93.
6. Isgrò MA, Bottoni P, Scatena R. Neuron-Specific Enolase as a Biomarker: Biochemical and Clinical Aspects. Adv Exp

Med Biol 2015;867:125-43.
7. Ntranos A, Casaccia P. The Microbiome-Gut-Behavior Axis: Crosstalk Between the Gut Microbiome and Oligodendrocytes Modulates Behavioral Responses. Neurotherapeutics 2018;15:31-5.
8. Sharon G, Sampson TR, Geschwind DH, et al. The Central Nervous System and the Gut Microbiome. Cell 2016;167:915-32.
9. Williamson JM, Lyons DA. Myelin Dynamics Throughout Life: An Ever-Changing Landscape? Front Cell Neurosci 2018;12:424.
10. Almeida RG, Lyons DA. On Myelinated Axon Plasticity and Neuronal Circuit Formation and Function. J Neurosci 2017;37:10023-34.
11. Keogh CE, Kim DHJ, Pusceddu MM, et al. Myelin as a regulator of development of the microbiota-gut-brain axis. Brain Behav Immun 2021;91:437-50.
12. Bjelke B, Seiger A. Morphological distribution of MBP-like immunoreactivity in the brain during development. Int J Dev Neurosci 1989;7:145-64.
13. Lier J, Winter K, Bleher J, et al. Loss of IBA1-Expression in brains from individuals with obesity and hepatic dysfunction. Brain Res 2019;1710:220-9.
14. Prinz M, Jung S, Priller J. Microglia Biology: One Century of Evolving Concepts. Cell 2019;179:292-311.
15. Ohsawa K, Imai Y, Sasaki Y, et al. Microglia/macrophage-specific protein Iba1 binds to fimbrin and enhances its actin-bundling activity. J Neurochem 2004;88:844-56.
16. Secombe KR, Al-Qadami GH, Subramaniam CB, et al. Guidelines for reporting on animal fecal transplantation (GRAFT) studies: recommendations from a systematic review of murine transplantation protocols. Gut Microbes 2021;13:1979878.
17. Zeng M, Cao H. Fast quantification of short chain fatty acids and ketone bodies by liquid chromatography-tandem mass spectrometry after facile derivatization coupled with liquid-liquid extraction. J Chromatogr B Analyt Technol Biomed Life Sci 2018;1083:137-45.
18. Zhang W, Zhang Y, Zheng Y, et al. Progress in Research on Brain Development and Function of Mice During Weaning. Curr Protein Pept Sci 2019;20:705-12.
19. Lynch CMK, Cowan CSM, Bastiaanssen TFS, et al. Critical windows of early-life microbiota disruption on behaviour, neuroimmune function, and neurodevelopment. Brain Behav Immun 2023;108:309-27.
20. Morel C, Martinez Sanchez I, Cherifi Y, et al. Perturbation of maternal gut microbiota in mice during a critical perinatal window influences early neurobehavioral outcomes in offspring. Neuropharmacology 2023;229:109479.
21. O'Connor R, Moloney GM, Fulling C, et al. Maternal antibiotic administration during a critical developmental window has enduring neurobehavioural effects in offspring mice. Behav Brain Res 2021;404:113156.
22. Gong C, Yang L, Liu K, et al. Effects of Antibiotic Treatment and Probiotics on the Gut Microbiome of 40 Infants Delivered Before Term by Cesarean Section Analysed by Using 16S rRNA Quantitative Polymerase Chain Reaction Sequencing. Med Sci Monit 2021;27:e928467.
23. Kaakoush NO. Insights into the Role of Erysipelotrichaceae in the Human Host. Front Cell Infect Microbiol 2015;5:84.
24. Forbes JD, Van Domselaar G, Bernstein CN. Microbiome Survey of the Inflamed and Noninflamed Gut at Different Compartments Within the Gastrointestinal Tract of Inflammatory Bowel Disease Patients. Inflamm Bowel Dis 2016;22:817-25.
25. Brown AJ, Goldsworthy SM, Barnes AA, et al. The Orphan G protein-coupled receptors GPR41 and GPR43 are activated by propionate and other short chain carboxylic acids. J Biol Chem 2003;278:11312-9.
26. Canani RB, Costanzo MD, Leone L, et al. Potential beneficial effects of butyrate in intestinal and extraintestinal diseases. World J Gastroenterol 2011;17:1519-28.
27. Bourassa MW, Alim I, Bultman SJ, et al. Butyrate, neuroepigenetics and the gut microbiome: Can a high fiber diet improve brain health? Neurosci Lett 2016;625:56-63.
28. Xiong Y, Miyamoto N, Shibata K, et al. Short-chain fatty acids stimulate leptin production in adipocytes through the G protein-coupled receptor GPR41. Proc Natl Acad Sci U S A 2004;101:1045-50.
29. Vieira EL, Leonel AJ, Sad AP, et al. Oral administration of sodium butyrate attenuates inflammation and mucosal lesion in experimental acute ulcerative colitis. J Nutr Biochem 2012;23:430-6.
30. van de Wouw M, Boehme M, Lyte JM, et al. Short-chain fatty acids: microbial metabolites that alleviate stress-induced brain-gut axis alterations. J Physiol 2018;596:4923-44.
31. Yu L, Zhong X, He Y, et al. Butyrate, but not propionate, reverses maternal diet-induced neurocognitive deficits in offspring. Pharmacol Res 2020;160:105082.
32. Jiang Y, Li K, Li X, et al. Sodium butyrate ameliorates the impairment of synaptic plasticity by inhibiting the neuroinflammation in 5XFAD mice. Chem Biol Interact

2021;341:109452.
33. Mitew S, Hay CM, Peckham H, et al. Mechanisms regulating the development of oligodendrocytes and central nervous system myelin. Neuroscience 2014;276:29-47.
34. Neufeld KM, Kang N, Bienenstock J, et al. Reduced anxiety-like behavior and central neurochemical change in germ-free mice. Neurogastroenterol Motil 2011;23:255-64, e119.
35. Paolicelli RC, Bolasco G, Pagani F, et al. Synaptic pruning by microglia is necessary for normal brain development. Science 2011;333:1456-8.
36. Erny D, Hrabě de Angelis AL, Jaitin D, et al. Host microbiota constantly control maturation and function of microglia in the CNS. Nat Neurosci 2015;18:965-77.

Cite this article as: Zhang Q, Li H, Yin S, Xiao F, Gong C, Zhou J, Liu K, Cheng Y. Changes in short-chain fatty acids affect brain development in mice with early life antibiotic-induced dysbacteriosis. Transl Pediatr 2024;13(8):1312-1326. doi: 10.21037/tp-24-128