Journal of Neuroinflammation

RESEARCH

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Gut microbiota modulates depressive-like behaviors induced by chronic ethanol exposure through short-chain fatty acids

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Abstract

Background Chronic ethanol exposure (CEE) is recognized as an important risk factor for depression, and the gut-brain axis has emerged as a key mechanism underlying chronic ethanol exposure-induced anxiety and depression-like behaviors. Short-chain fatty acids (SCFAs), which are the key metabolites generated by gut microbiota from insoluble dietary fiber, exert protective roles on the central nervous system, including the reduction of neuroinflammation. However, the link between gut microbial disturbances caused by chronic ethanol exposure, production of SCFAs, and anxiety and depression-like behaviors remains unclear.

Methods Initially, a 90-day chronic ethanol exposure model was established, followed by fecal microbiota transplantation model, which was supplemented with SCFAs via gavage. Anxiety and depression-like behaviors were determined by open field test, forced swim test, and elevated plus-maze. Serum and intestinal SCFAs levels were quantified using GC-MS. Changes in related indicators, including the intestinal barrier, intestinal inflammation, neuroinflammation, neurotrophy, and nerve damage, were detected using Western blotting, immunofluorescence, and Nissl staining.

Results Chronic ethanol exposure disrupted with gut microbial homeostasis, reduced the production of SCFAs, and led to anxiety and depression-like behaviors. Recipient mice transplanted with fecal microbiota that had been affected by chronic ethanol exposure exhibited impaired intestinal structure and function, low levels of SCFAs, intestinal inflammation, activation of neuroinflammation, a compromised blood-brain barrier, neurotrophic defects,

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alterations in the GABA system, anxiety and depression-like behaviors. Notably, the negative effects observed in these recipient mice were significantly alleviated through the supplementation of SCFAs.

Conclusion SCFAs not only mitigate damage to intestinal structure and function but also alleviate various lesions in the central nervous system, such as neuroinflammation, and reduce anxiety and depression-like behaviors, which were triggered by transplantation with fecal microbiota that had been affected by chronic ethanol exposure, adding more support that SCFAs serve as a bridge between the gut and the brain.

Keywords Chronic ethanol exposure, Short-chain fatty acids, Gut-brain axis, Neuroinflammation, Anxiety and depression-like behaviors

Background

The overall upward trend in ethanol use is a social and health policy issue [1–3]. In previous comparative global risk assessments, alcohol abuse has been universally recognized as one of the top ten risk factors for disease burden, accounting for 3 million alcohol-attributable deaths [4, 5]. Ethanol is particularly toxic to the brain [6–8], with mental health ramifications such as anxiety and depression [9, 10]. Chronic ethanol exposure (CEE) poses a rising risk for depression disorder, but the involved mechanism is not clear.

Depressive disorder ranks among the most disabling mental health conditions driven by a combination of genetic factors and environmental influences [11]. Depressive and anxiety-like behaviors resulting from chronic ethanol exposure are closely associated with brain pathological changes, including neuroinflammation, neurotrophic deficiency, and dysfunction of the blood-brain barrier. Gut is the crucial organ closely associated with CEE-triggered neuroinflammation [12], blood-brain barrier (BBB) dysfunction [13] and neurotrophic deficiency [14], which are the main mechanisms causing anxiety-depressive behavior anxiety-like and depressive-like symptoms. Variations in gut function may play a role in the development of brain abnormalities, indicating a bidirectional relationship known as the gut-brain axis [15]. Particularly, the link between gut microbiota imbalance caused by chronic ethanol exposure and the development of depression and anxiety-like symptoms highlights this association [16, 17].

The gut microbiota is composed of trillions of microorganisms, mainly dividing into six phyla: Firmicutes, Proteobacteria, Bacteroidetes, Verrucomicrobia, Actinobacteria, and Fusobacteria [18, 19]. The previous study demonstrated that depression is largely correlated with a decrease in the richness and diversity of the gut microbiota, characterized by a diminished abundance of Bacteroidetes, Verrucomicrobiae, Bacteroidia [20].

While the existence of the gut-brain axis has been established, the precise mechanisms connecting the gut and brain remain unclear. Increasing research has suggested that miRNA, the vagus nerve, and microbial metabolites serve as potential intermediaries connecting the gut to the brain [21–23]. Short-chain fatty acids (SCFAs), mainly butyrate, propionate, and acetate, represent the primary fermentation products of dietary fiber in the gut [24, 25]. SCFAs are produced in the intestine and have a protective effect on the intestines. SCFAs can reduce intestinal inflammation [26, 27], and increase intestinal barrier integrity [28, 29]. In addition, SCFAs not only enhance the integrity of the BBB and protect the central nervous system (CNS) from peripheral inflammatory cytokines and toxins, but they also reduce excessive activation of microglia and the synthesis of pro-inflammatory cytokines [30–32]. Moreover, the concentration of SCFAs resulting from a diet rich in soluble fiber influences the levels of brain-derived neurotrophic factor (BDNF) in the hippocampus of mice [33]. Additionally, SCFAs play a positive role in preventing depression [34]. Therefore, SCFAs are generally viewed as key players in the communication between the microbiome and the gut-brain axis, given their capacity to maintain gut equilibrium and efficiently cross the blood-brain barrier to impact central nervous system function [35–37].

The impact of gut dysbiosis induced by CEE on SCFAs production and its relationship to depression remains uncertain. In order to determine if the symptoms observed in donor mice due to chronic ethanol exposure could be transferred to recipient mice through changes in the intestinal microbiota of the donors and whether administering SCFAs via gavage could mitigate those symptoms in the recipient mice, feces were extracted from both the control group and the chronic ethanol exposure group for fecal transplantation to exclude the direct effects of ethanol exposure on the brain, along with additional SCFAs supplements through oral gavage. Changes in behavior, blood and gut SCFAs levels, abnormal alterations in the gut, PFC, and hippocampus were detected. Our findings suggested that SCFAs alleviated depression-like and anxiety-like behaviors induced by CEE-triggered microbiota dysbiosis through gut-brain axis.

Materials and methods

Animals

Male C57BL/6 N mice utilized in the present study were all sourced from the Animal Center (China Medical University, Shenyang, China). Every mouse was kept in a regulated room (20 to 23 °C temperature, 40–60% humidity), with unrestricted access to food and water. All animal experiments were performed according to the Guide for the Care and Use of Laboratory Animals of China Medical University, followed the guidelines set forth in the National Institutes of Health Guide for the Use and Care of Laboratory Animals, and approved by China Medical University Animal Care and Use Committee (CMU2020103).

Chronic ethanol exposure

The mice were separated into two separate groups (Fig. S1A): the ethanol group (Et group) and the control group (Con group). For 2 days, the Et group received a single bottle with a 10% ethanol solution, which was then followed by a 15% ethanol solution for 5 days, and after that, a 20% ethanol solution was administered over the final 87 days (the sole water source for the Et group was a single bottle containing ethanol solution). The actual ethanol exposure of Et group over the duration the study was about 25 g/kg. Con group exposed to a single bottle with drinking water. Gather mouse droppings once the modeling process was completed.

Fecal microbiota transplantation (FMT)

As performed by Leclercq et al. [16], transplantation of fecal microbiota was performed with subtle modifications (Fig. S1B). Recipient mice were administered an oral gavage containing a broad-spectrum antibiotic mix (50 mg/kg, and vancomycin 100 mg/kg metronidazole, ampicillin, neomycin sulfate) once daily for ten days, along with 1 mg/kg amphotericin B for the first three days. Subsequently, the mice underwent an intestinal cleanse using a cleaning solution composed of PEG4000 (59 g/L), KCl (0.75 g/L), NaCl (1.46 g/L), NaHCO3 (1.68 g/L), and Na2SO4 (5.68 g/L) to eliminate any residual antibiotics present in their intestines. This cleaning solution was administered via gavage to the recipient mice three times: two doses of 500μL were given on the day prior to the FMT at one-hour intervals, followed by a single dose of 500μL four hours before the FMT. Each fecal sample was carefully suspended in sterile PBS in a ratio of 1 gram of the sample to 10 milliliters of PBS. This mixture underwent vortexing for a duration of 5 min to ensure thorough integration. Following this, the mixture was allowed to sit undisturbed for an additional 5 min, enabling sedimentation of solid particles. After sedimentation, the slurry was filtered through a 100 μm cell strainer to yield a clear suspension. Subsequently, 200 μl were promptly delivered to the mice through oral gavage every other day for a total of three administrations. Concurrently, the mice received an oral gavage of either SCFA mixtures or simply PBS daily for a duration of 14 days. A mixture of SCFAs was prepared, consisting of 0.36 g of sodium propionate, 0.6 g of sodium butyrate, and 0.05 g of sodium valerate per kilogram of mouse body weight. Thus, recipient mice were divided into four groups, namely the F-Con group, the F-Con+SCFAs group, the F-Et group and the F-Et+SCFAs group.

Open field test (OFT)

The OFT apparatus consisted of a square arena that measured 40 cm by 40 cm with a height of 30 cm, featuring a white base. Each mouse was placed in a corner of the apparatus individually, monitored, and recorded for a duration of 10 min using the SMART™ tracking program. Following each test, the arena was thoroughly cleaned with 75% ethanol to remove any odor cues that could potentially affect the behavior of the mice.

Elevated plus-maze (EPM)

The EPM test was conducted to assess the mice's anxiety-like behaviors. The EPM apparatus consisted of a cross-shaped maze featuring two 50×10 cm open arms, two 50×10 cm closed arms, and a 10×10 cm central area. The height of the EPM apparatus is 50 cm. Each mouse was placed in the central region and given 5 min to explore the maze. The time spent in each arm was monitored and recorded using the SMART™ tracking program.

Forced swimming test (FST)

FST was conducted to evaluate behaviors similar to those exhibited in depression. The test was carried out in a tall cylindrical plastic container (10 cm in diameter, depth of 22 cm) filled with distilled water. A camera was positioned in front of the subjects to capture their movements during the experiment. The total duration of the FST was 6 min, allowing the mice 2 min for adaptation at the beginning. We noted that immobility was defined as the absence of any movement, except for the necessary actions to keep the mouse's nose above the water surface.

Real-time quantitative polymerase chain reaction (RT-qPCR)

The intestinal tissues from mice were utilized to extract total RNA using TRIzol (Invitrogen, USA), following reverse transcription for produce complementary DNA and qPCR to analysis, in accordance with the provided instructions. ZO-1 primers were 5'-GCCGCTAAGAGC ACAGCAA-3' (forward) and 5'-TCCCCACTCTGAAAA TGA.

GGA-3' (reverse); Occludin primers were 5'-TTGAAA GTCCACCTCCTTACAGA-3' (forward) and 5'-CCGGA TAAAAAGAGTACGCTGG-3' (reverse).

Immunofluorescence

30-μm thick or 4 μm thick brain tissue sections, were initially washed with PBS and blocked using 5% BSA for 2 h. Subsequently, the tissue underwent an overnight incubation with primary antibody. Then, sections were subjected to washing process and treated with Alexa Fluor 488/594 for approximately 2 h, following incubated with DAPI for 5 min.

Sholl analysis

The structure of microglia was assessed using ImageJ to reconstruct Z-scan confocal images and conduct Sholl analysis. In the process of Sholl analysis, circles were drawn in concentric patterns starting from the cell body, and the intersections were then quantified.

Nissl staining and AB-PAS staining

The brain sections were subjected to methylene blue stain for a duration of 15 min, then distinguished by Nissl differentiation solution for 30s, and then processed in ammonium molybdate solution for 3 min. Dehydrate by series of ethanol, transparent by xylene, and seal with a xylene-based non-aqueous mounting medium.

The intestinal tissue sections were immersed in Alcian Blue Staining Solution for 15 min, then oxidated by PAS Oxidant for 5 min, subsequently subjected to Schiff Reagent for 15 min, and finally processed in Heamatoxylin Solution for 1 min. Rinse with distilled water between each step. Dehydrate by series of ethanol, transparent by xylene, and seal with a xylene-based non-aqueous mounting medium.

SCFAs measurement

Weigh and prepare standards for butyric acid, valeric acid in ethyl acetate. Take 200 μl of serum, add 500 μl of PBS solution (pH=2), mix well, then add 1 mL of ethyl acetate, stir for 3 min, and centrifuge to collect the supernatant. The supernatant was mixed and added to the injection bottle for GC-MS detection. When testing intestinal tissue, take intestinal tissue, add 300 μl PBS, grind and ultrasonic, centrifuge and take the supernatant, other steps are the same as above. According to the standard, calculate the corresponding content of SCFA in the sample.

Western blot analysis

Hippocampal and intestinal protein extraction was performed using RIPA lysis buffer, which included 1 mM PMSF for protein protection (Beyotime, China), while the extraction period lasted for 30 min, keeping the samples on ice to maintain protein integrity. Following extraction, the tissues were sonicated to further break down the samples. The resulting lysates were then subjected to centrifugation at 20,000 ×*g* for 15 min to separate the protein extracts from cellular debris. The protein concentration in the supernatant was determined using a BCA protein assay kit (Beyotime).

SDS-PAGE was used to separate the proteins and then transferred onto PVDF membranes. After blocking for 3 h with 8% skim milk, the blots were incubated overnight at 4 °C with an antibody. Following the washing process, blots were incubated with secondary antibodies for a duration of 2 h. Protein band visualization was achieved using ECL reagent from Merck Millipore.

Statistical analyses

Statistical analysis using GraphPad Prism software version 8.0 was conducted to analyze all data. An analysis of variance was conducted with the post hoc Dunnett's test to establish the significance of the statistical findings. The data is displayed in the form of the mean ± standard deviation (SD), with a P value below 0.05 indicating significance.

Results

Chronic ethanol exposure induced anxiety and depression-like behaviors, neuroinflammation, and SCFAs reduction

To evaluate the impact of chronic ethanol consumption on neuropsychiatric behaviors, we conducted elevated plus maze (EPM), the open field test (OFT), and forced swim test (FST). Mice assigned to the ethanol group (Et group) exhibited less time spent in central region of the OFT (Fig. S2A) and the open arms of the EPM (Fig. S2B), along with significantly longer immobility time in the FST (Fig. S2C), compared to mice in the control (Con) group. These findings indicated that the mice of Et group displayed behaviors indicative of anxiety and depressive-like symptoms. Western blot analysis (Fig. S2D and E) showed that NLRP3 and P65 levels increased in the Et group, compared to those assigned to Con group. At the same time, IF analysis (Fig. S2F and G) demonstrated that the number of $IBA1^+$ cells in the hippocampus of Et group was significantly raised. Besides, Western blot analysis (Fig. S3A and B) showed that Et group reduced ZO-1 and Occludin proteins expression, as well as synaptic proteins and neurotrophic protein (Fig. S3C and D). And we observed that average fluorescence intensity of BDNF of Et group was lower than Con group (Fig. S3E). Compared with the Con group, the layers of Nissl bodies in the CA1 and CA3 areas of the Et group were blurred, and the structure was chaotic and loose, suggesting that CEE caused damage to the hippocampal neurons of mice (Fig. S3F). All in all, CEE triggered neuroinflammation, destroyed BBB, and caused neurotrophic deficiency,

resulting in neuronal injury, anxiety-like and depressive-like symptoms.

Here, we explored intestinal morphology and intestinal permeability changes caused by chronic ethanol exposure. Results of qPCR (Fig. S4A) displayed that CEE decreased the mRNA level of ZO-1 and occludin, and Western blot (Fig. S4B and E) showed that occludin was also decreased in protein level. Besides, results of HE (Fig. S4C and D) and AB-PAS (Fig. S4F) showed that CEE resulted in intestinal structure atrophy, along with less mucus. Additionally, Western blot analysis (Fig. S4G) showed that the Et group increased NLRP3, P65, and IL-1β levels, suggesting that CEE induced inflammation in the intestine. On the whole, CEE disrupted intestinal homeostasis.

In order to investigate the potential mechanisms by which CEE destroyed intestinal homeostasis, mice serum was assessed for SCFAs levels. The results (Fig. S4H and I) revealed a decrease in valeric acid and butyric acid, as well as a lower level of propionic acid in the serum of mice from the Et group, suggesting the adverse influence of CEE on SCFAs metabolism.

SCFAs alleviated low levels of SCFAs and intestinal wall integrity of the recipient mice transferred from Et mice by FMT

To investigate whether levels of SCFA of Et group and depression-related behavioral phenotypes were linked to CEE-induced microbiota dysbiosis, we transplanted the gut microbiota from mice in the Con and Et groups into healthy recipient mice receiving an antibiotic cocktail to cleanse their gut microbes prior to transplantation, and additional SCFAs supplements were administered via gavage. Initially, we ran agarose gel of DNA isolated from the feces of donor mice before and after intestinal cleansing and plated them on non-selective agar to assess the intestinal microbial cleaning effect prior to transplantation (Fig. 1A). Results showed an antibiotic cocktail successfully cleansed gut microbes (Fig. 1B and C). SCFAs concentrations were directly assessed using gas chromatography-mass spectrometry (GC-MS). In the intestine (Fig. 1D and E) and the serum (Fig. 1F and G) of mice transplanted with gut microbiota from Et groups (F-Et group), levels of butyric acid and valeric acid were found to be at lower levels compared to those mice received FMT from Con group (F-Con group).

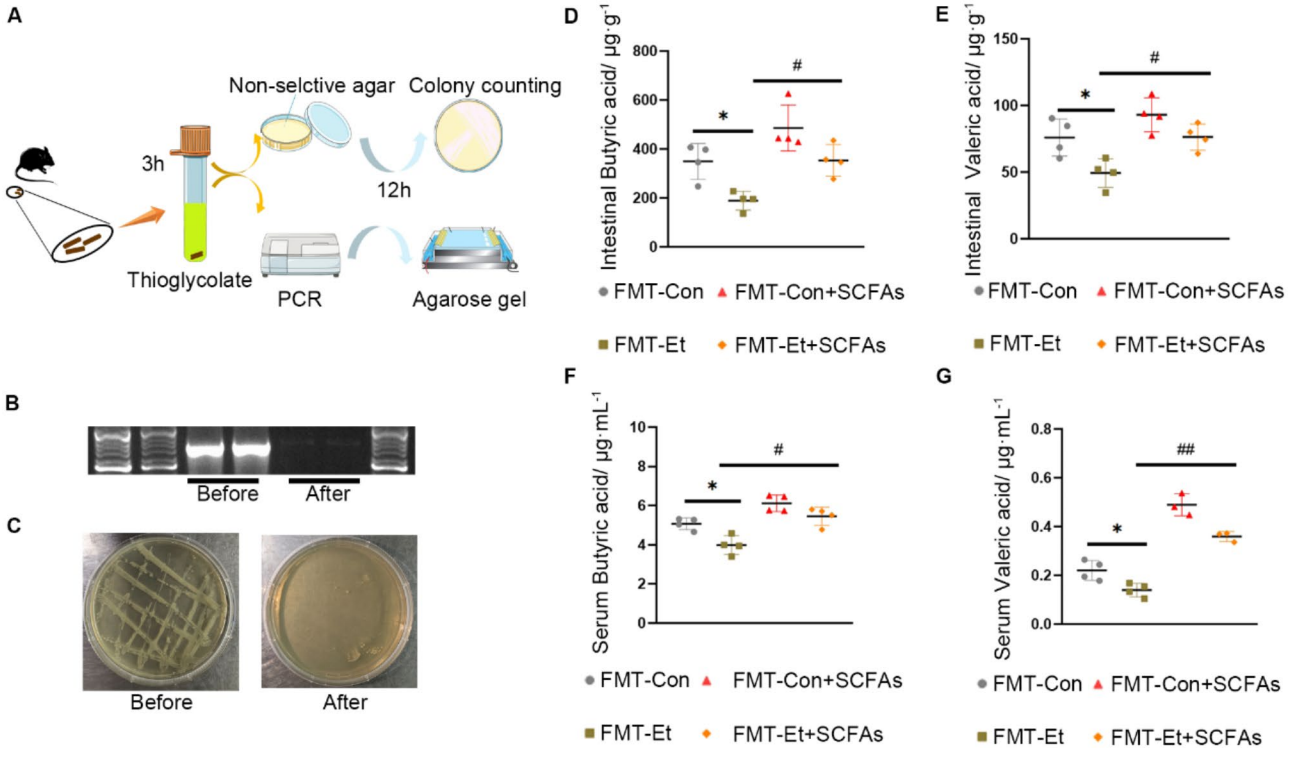

Fig. 1 Effects of intestinal microbial disturbance caused by CEE on short-chain fatty acids. **A** The verification process for the intestinal microbial cleaning effect. **B** DNA was isolated from the stool of mice and were run on an agarose gel. **C** Stools were resuspended in thioglycolate and plated on non-selective agar to measure gut bacterial load. **D** Intestinal butyric acid levels of recipient mice. **E** Intestinal Valeric acid levels of recipient mice. **F** Serum butyric acid levels of recipient mice. **G** Serum Valeric acid levels of recipient mice. Data in the present study are presented as the mean ± SD. $^{*}P < 0.01$, $^{\#}P < 0.01$, $^{\#\#}P < 0.0001$

Gut microbiota dysbiosis is often associated with disturbances in intestinal homeostasis. We observed the intestinal structure of F-Et group atrophy, characterized by diminution of villus height, compared by those of F-Con group (Fig. 2A and B). At the same time, diminution of villus height of F-Et group was recovered by SCFAs supplements. And RT-qPCR results displayed that the mRNA levels of ZO-1 and occludin in F-Et group were significantly lower than those assigned to F-Con group, which could not be significantly relieved by additional SCFAs supplements (Fig. 2C). However, additional SCFAs supplements improved the decreased protein levels of occludin (Fig. 2D). Similarly, IF result showed same trends (Fig. 2E and F). At the same time, F-Et group had less mucus, which could be alleviated by SCFAs (Fig. 2G). In conclusion, recipient mice underwent fecal transplants from the Et group displayed similar patterns of low levels of SCFAs and intestinal wall integrity as seen in the donor mice, and the changes cloud be prevented by SCFAs supplements, indicating that gut microbiota dysbiosis caused by CEE was closely linked with the health status of SCFA levels and the integrity of the intestinal wall, with changes in SCFAs likely contributing significantly.

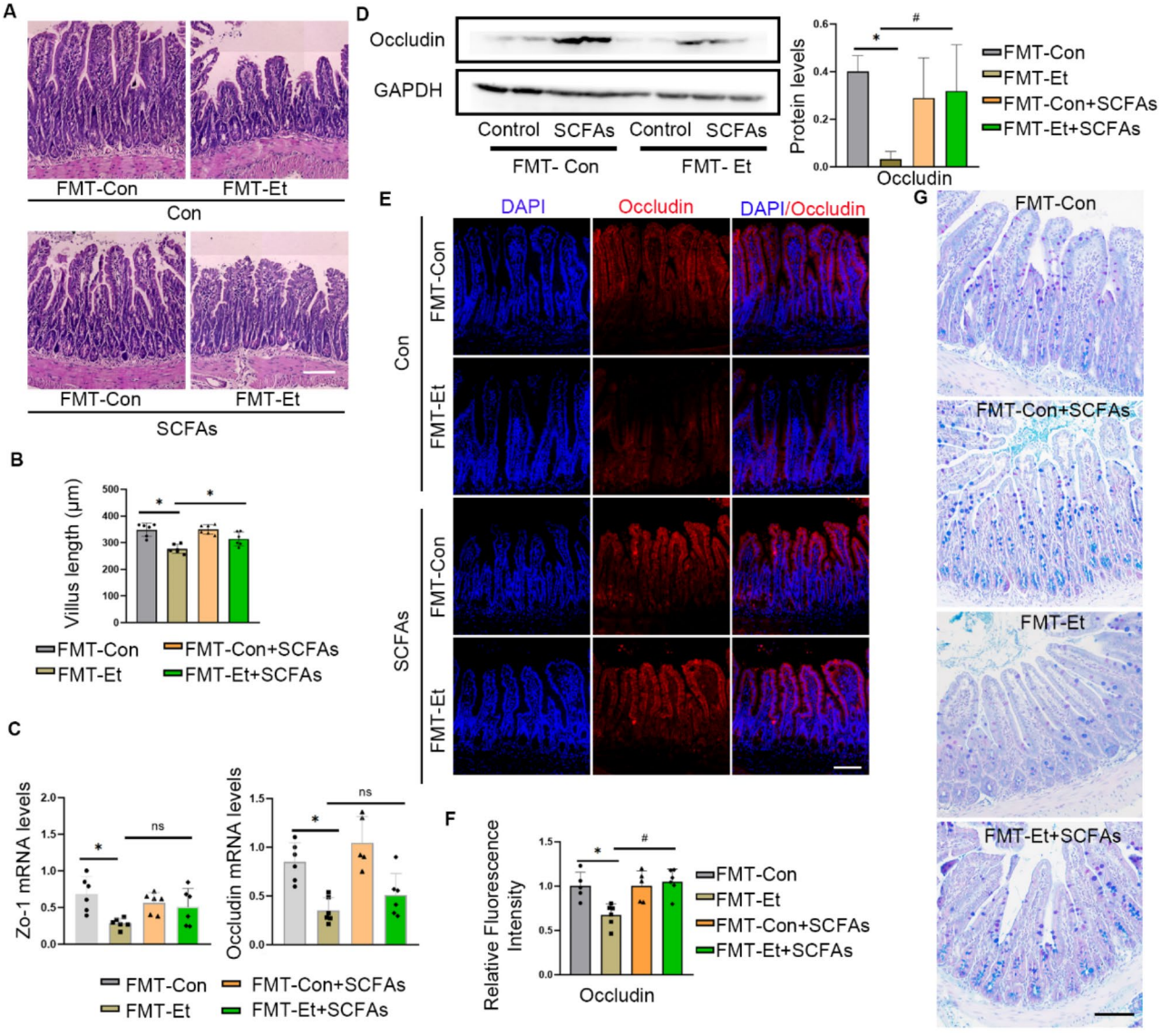

Fig. 2 SCFAs alleviated intestinal wall integrity of the recipient mice transferred from Et mice by FMT. **A** and **B** Representative HE images of intestine tissue of recipient mice along with villus length (scale bar = 100 μm). **C** RT-qPCR analysis of the expression of ZO-1 and occludin in the intestine of recipient mice. **D** Represents images of occludin in the intestine from recipient mice and quantification. **E** and **F** Representative occludin images and relative fluorescence intensity (scale bar = 100 μm). **G** Representative AB-APS images of intestine from recipient mice (scale bar = 100 μm). Data in the present study are presented as the mean ± SD. $^{*}P < 0.01$, $^{\#}P < 0.01$

SCFAs ameliorated intestinal immune response of the recipient mice transferred from Et mice by FMT

Gut microbiota dysbiosis is the key driver leading to intestinal immune response [38]. Western blot analysis (Fig. 3A and B) showed that FMT from Et group increased NLRP3, P65, and IL-1β levels, whereas exogenous SCFAs supplementation reduced these protein levels. Moreover, IHC analysis (Fig. 3C) showed that NLRP3-positive cell of F-Et group increased, which could be significantly prevented by additional SCFAs supplements. The CD11b/CD18 integrin receptor, also referred to as αMβ2, mainly found on immune cells such as macrophages, monocytes, neutrophils [39, 40]. In reaction to inflammatory triggers, leukocyte activation and gathering at the inflammation sites are facilitated by CD11b [41, 42]. In this paper, we found that the F-Et group accumulated more CD11b-positive cells at the inflammatory site than the F-Con group in the intestine (Fig. 3D). Meantime, exogenous SCFAs supplementation decreased the accumulation of CD11b. F-Et group displayed a similar pattern of intestinal inflammation as the Et group, while SCFAs supplementation ameliorated immune response, indicating that gut microbiota dysbiosis caused by CEE was closely linked with intestinal immune response, with alterations in SCFAs potentially playing a key role.

SCFAs ameliorated anxiety and depression-like behaviors of the recipient mice transferred from Et mice by FMT

The F-Et group mice spent less time in the central area in the OFT than the F-Con group, indicating that FMT from the Et group exacerbated anxiety-like behavior and change of gut microbiota triggered by CEE had a significant effect on anxiety-like behavior. Whereas additional SCFA supplements extended the time of central area residence of mice received gut microbiota of the Et group, revealing that supplementation of SCFAs alleviated anxiety-like behavior (Fig. 4A). Additionally, no significant difference was observed in the distance covered in the OFT among groups, suggesting that the other behavioral variations were not due to compromised motor function (Fig. 4B). Similarly, the results of EMP showed the same trend (Fig. 4C). In the FST (Fig. 4D), mice received gut microbiota of the Et group exhibited extended immobility time compared to whose receipts from the Con group,

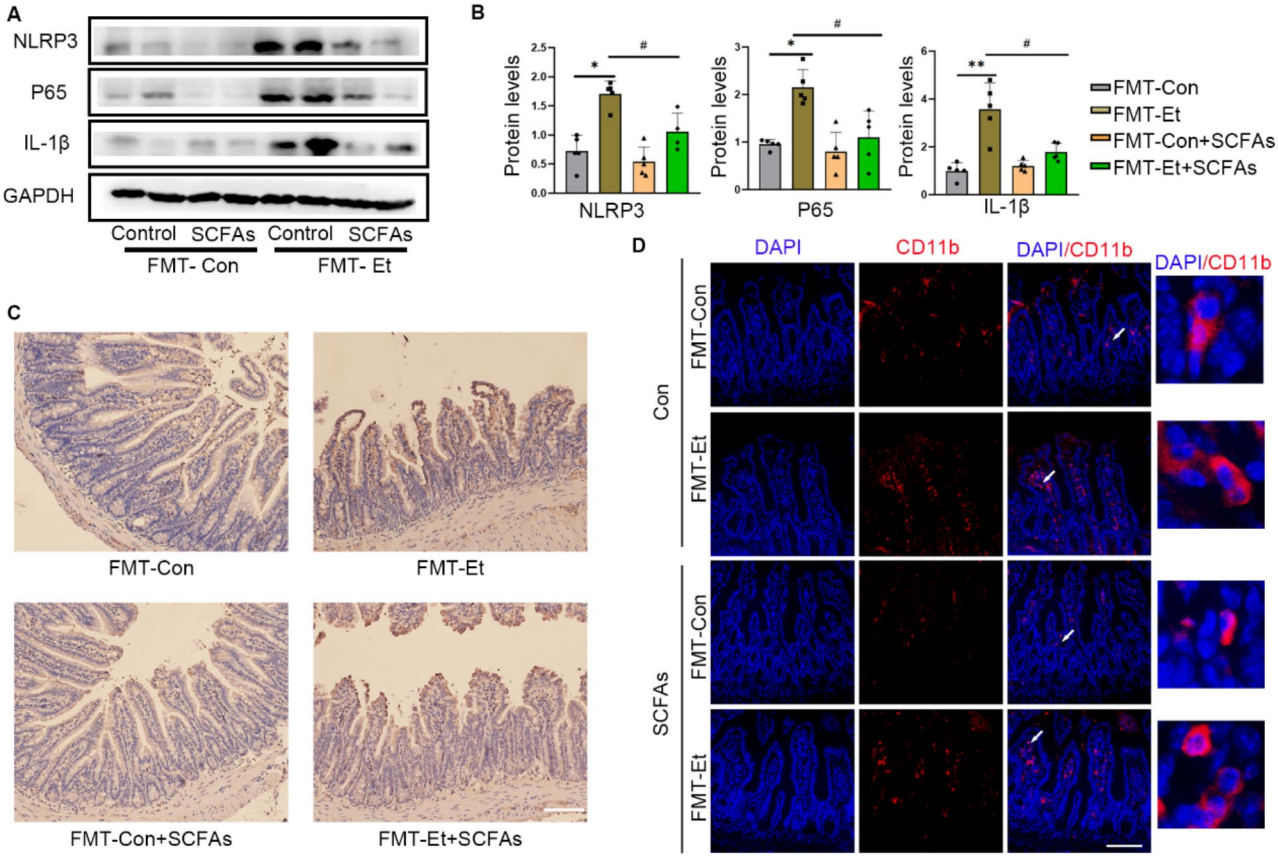

Fig. 3 SCFAs ameliorated intestinal immune response of the recipient mice transferred from Et mice by FMT. **A** and **B** Represents images of NLRP3, P65 and IL-1β in the intestine from recipient mice and quantification. **C** Representative IHC image of NLRP3 in the intestine from recipient mice, scale bar represents 100 μm. **D** Representative IF image of CD11b in the intestine from recipient mice, scale bar represents 100 μm. Data in the present study are presented as the mean ± SD. $^{*}P < 0.01$, $^{\#}P < 0.01$

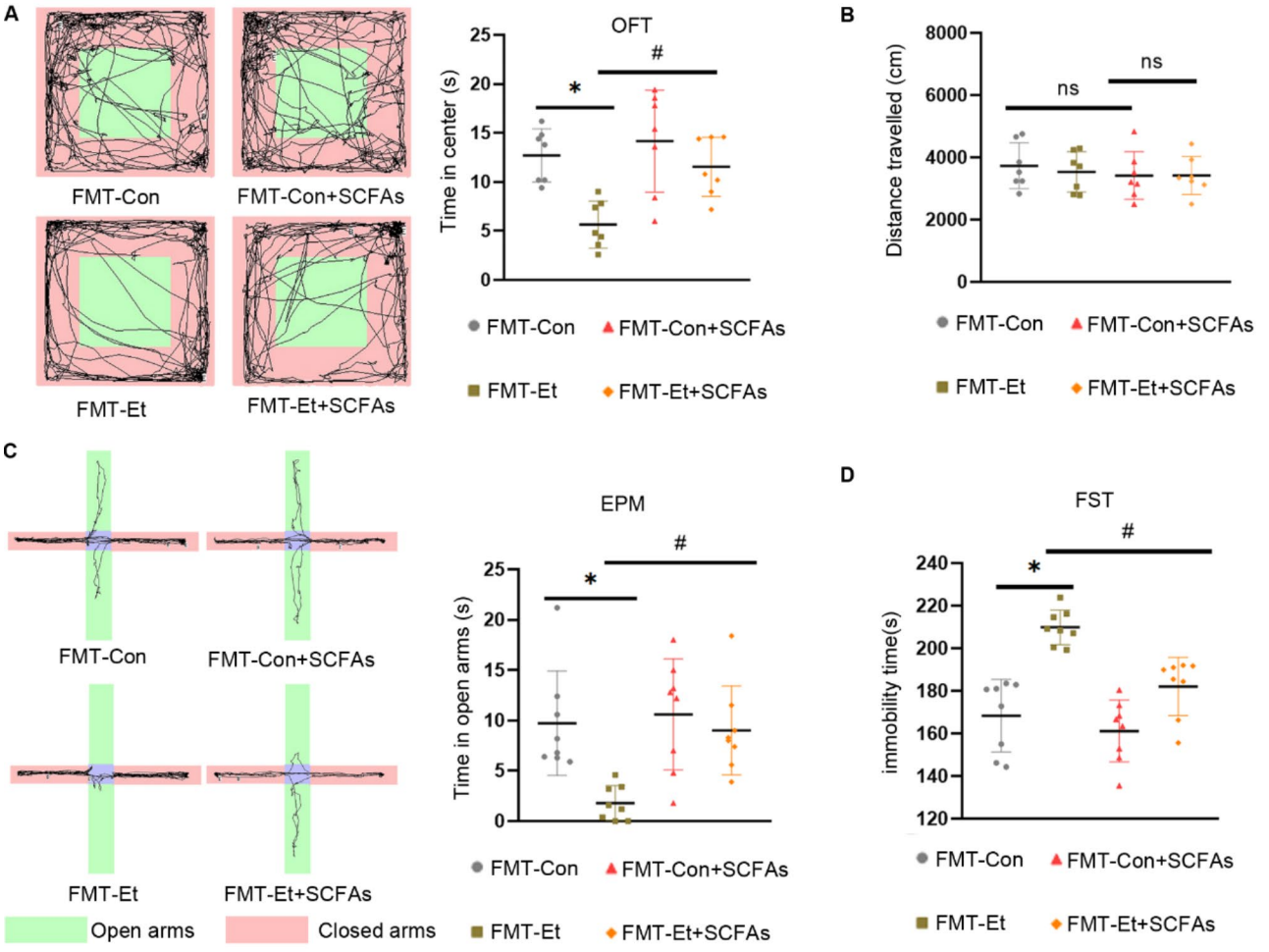

Fig. 4 SCFAs ameliorated anxiety and depression-like behaviors of the recipient mice transferred from Et mice by FMT. **A** Representative tracks and time spent in the central area of the OFT. **B** Distance traveled in the OFT. **C** Representative tracks and time spent in the open arms of the EPM. **D** Immobility time in the FST. Data in the present study are presented as the mean ± SD. $^{*}P < 0.01$, $^{\#}P < 0.01$

while additionally oral gavage of SCFAs decreased the immobility time, suggesting additional SCFAs provision relieved depression-like behaviors caused by fecal microbiota transplantation. In short, the phenotypes resembling anxiety and depression were transferred to the recipient mice through FMT by the Et group mice, and SCFAs supplementation alleviated those behaviors. The behavioral phenotype transfer highlights a considerable impact of the donor microbiota on the emotional and psychological well-being of the recipients, indicating that the gut microbiome and its production of metabolites could be essential in the expression of anxiety and depressive-like behaviors.

SCFAs ameliorated neuroinflammation of the recipient mice transferred from Et mice by FMT

We explore the potential of SCFAs to alleviate neuroinflammation induced by microbiota dysbiosis. In the hippocampus, the elevated levels of NLRP3 and P65 in the F-Et group were impeded by SCFAs (Fig. 5A and B). Similarly, we observed SCFAs alleviated the increased number and decreased the complexity of microglia cells in CA1 and CA3 regions of the F-Et group (Fig. S5A, Fig. 5D, E and F), suggesting additional SCFAs provision relieved the activated state of microglia. Meanwhile, NLRP3-positive and GFAP-positive cells were increased in the F-Et group, and SCFAs reduced the elevated trend (Fig. S5B, Fig. 5C, G, H and I). In the PFC, Western blot results (Fig. 6A and B) showed that FMT from ethanol significantly elevated the levels of NLRP3 and P65. In contrast, the supplementation with SCFAs led to a reduction in these increased levels of NLRP3 and P65. At the same time, we observed that microglia cells of the F-Et group were found to be in an activated state, characterized by increased number and decreased the complexity of microglia cells in the PFC (Fig. 6C, D and E). These results suggested that SCFAs ameliorated neuroinflammation of the recipient mice transferred from Et mice

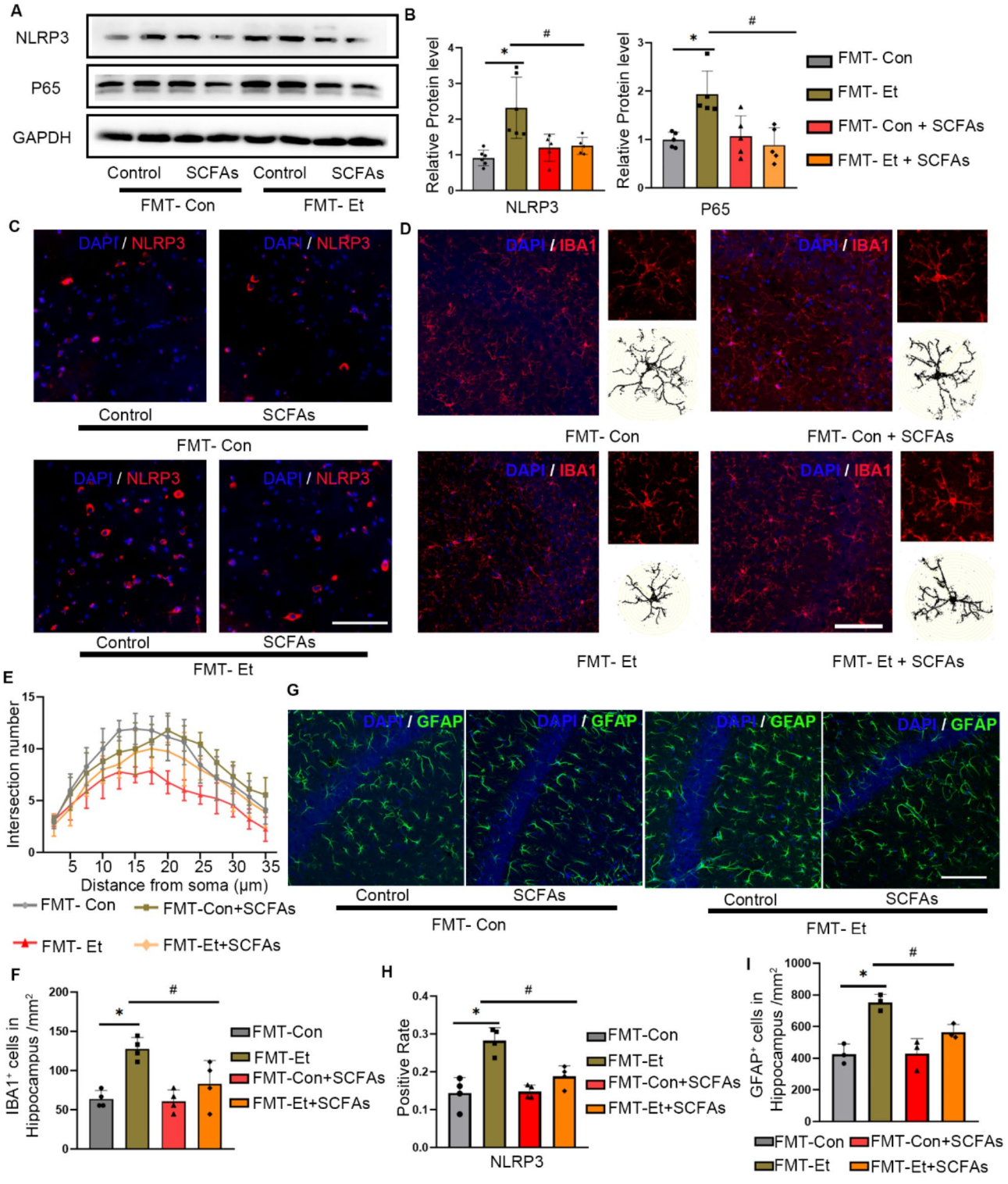

Fig. 5 SCFAs alleviate neuroinflammation in the hippocampus of recipient mice. **A** and **B** Represent images of NLRP3 and P65 in the hippocampus from recipient mice and quantification. **C** and **H** Representative IF image of NLRP3 in the hippocampus from recipient mice and quantification, scale bar represents 100 μm. **D** and **F** Representative IF image and the number of IBA1$^+$ cells in the hippocampal CA3 regions from recipient mice, scale bar represents 100 μm. **E** Sholl analysis showing the morphology of IBA1$^+$ cells in the hippocampus. **G** and **I** Representative IF image and the number of GFAP$^+$ cells in the hippocampus from recipient mice, scale bar represents 100 μm. Data in the present study are presented as the mean ± SD. $^*P < 0.01$, $^\#P < 0.01$

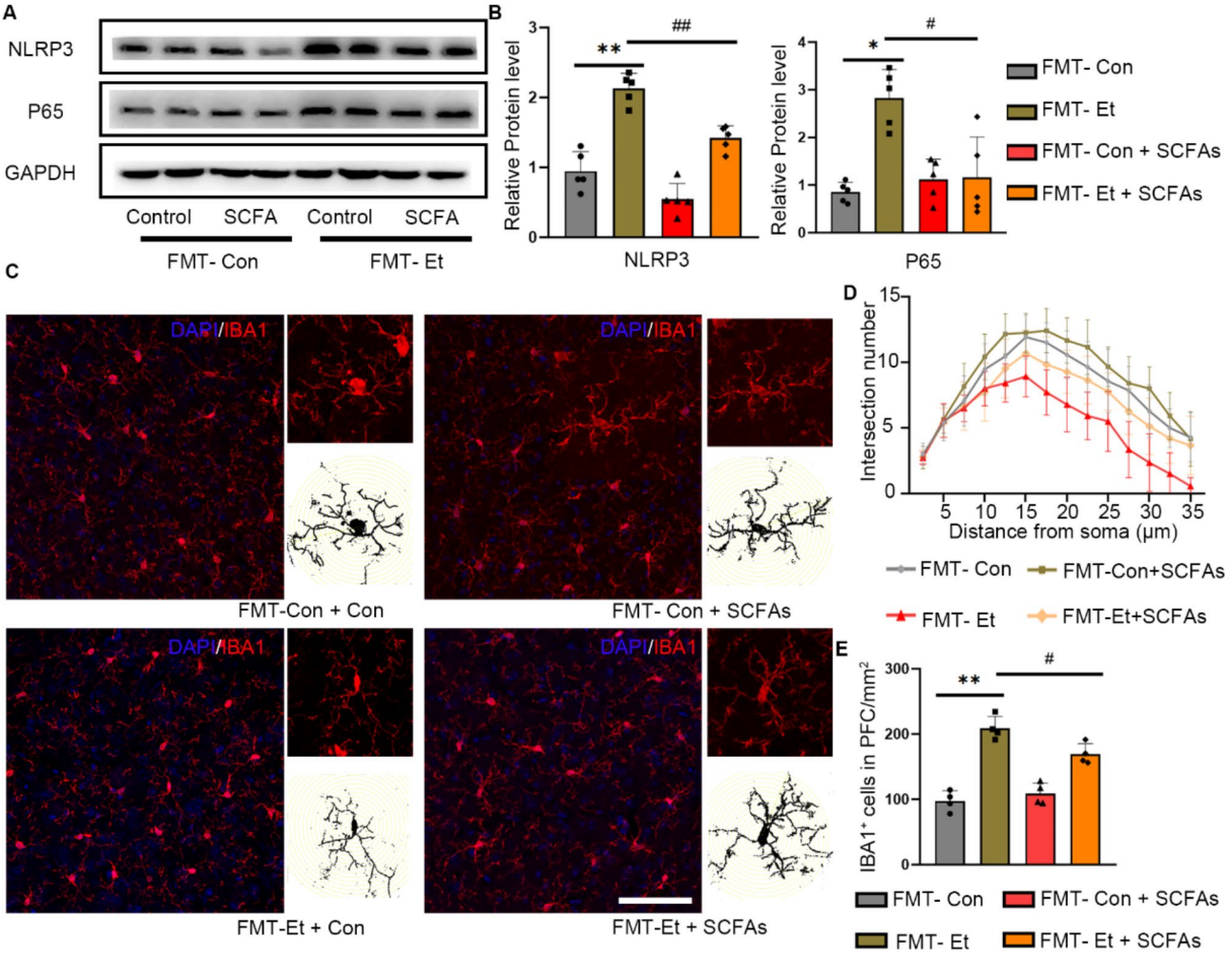

Fig. 6 SCFAs alleviate neuroinflammation in the PFC of recipient mice. **A** and **B** Represents images of NLRP3 and P65 in the PFC of recipient mice and quantification. **C** and **E** Representative IF image and the number of IBA1$^+$ cells in the PFC of recipient mice, scale bar represents 100 μm. **D** Sholl analysis showing the morphology of IBA1$^+$ cells in the PFC. Data in the present study are presented as the mean ± SD. $^*P < 0.01$, $^{**}P < 0.0001$, $^{\#}P < 0.01$, $^{\#\#}P < 0.0001$

by FMT, and SCFAs potentially served as a mediator for relationship between the microbiota dysbiosis and neuroinflammation.

SCFAs improved decreased expression of neurotrophic proteins and dysfunction of blood brain barrier of the recipient mice transferred from Et mice by FMT

CEE is frequently associated with neurotrophic proteins deficiency, a known factor associated with behaviors that mimic anxiety and depression. Here, we explored the effects of microbiota dysbiosis on neurotrophic proteins expression and examined the role of SCFAs in this biological process. In the hippocampus, levels of BDNF, NGF, TrkB, and ERK were decreased in the F-Et group, compared with those of the F-Con group (Fig. 7A and B), suggesting that microbiota dysbiosis could destroy neurotrophic proteins. However, additional SCFAs supplements increased the protein levels. At the same time, we observed the similar trend in the PFC (Fig. 7C and D). IF results of hippocampus and PFC showed that the F-Et group decreased the relative fluorescence intensity of BDNF while administration of SCFAs was shown to slow the decline (Fig. 7E and F). These results demonstrated that microbiota dysbiosis-induced changes in SCFAs could be the potential driver leading to neurotrophic proteins deficiency.

Western blot analysis (Fig. 7G, H, I and J) in the hippocampus and PFC revealed a decline in ZO-1 and occludin levels in the F-Et group compared to the F-Con group. Furthermore, additional SCFAs supplements restored the expression of ZO-1 and occludin, indicating that SCFAs can alleviate the BBB dysfunction induced by CEE-triggered microbiota dysbiosis. Altogether, SCFAs may act as a key factor linking dysbiosis and blood-brain barrier dysfunction.

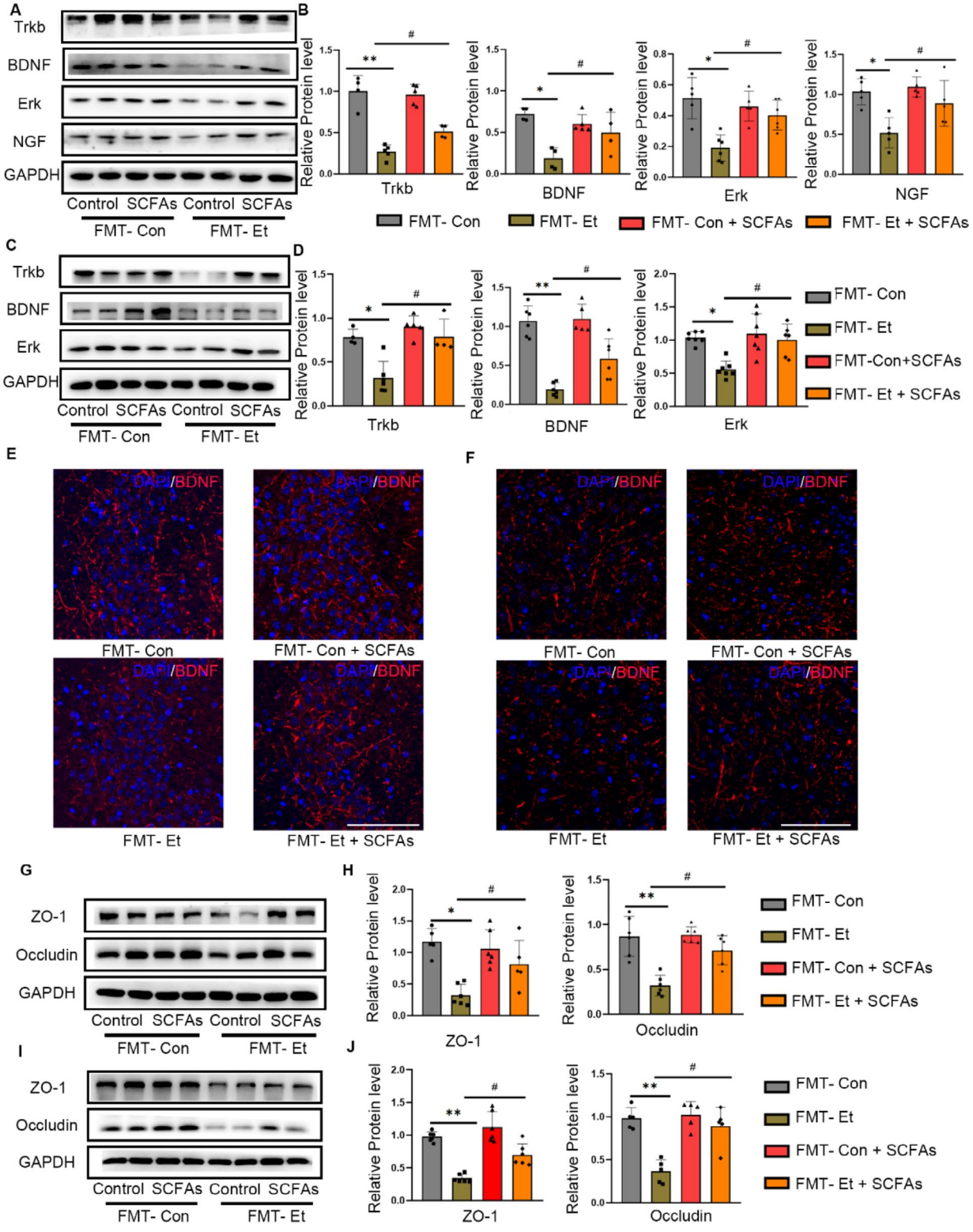

Fig. 7 SCFAs ameliorate DBNF signaling and BBB dysfunction in recipient mice. **A** and **B** Represents images of Trkb, BDNF, Erk and NGF in the hippocampus from recipient mice and quantification. **C** and **D** Represents images of Trkb, BDNF and Erk in the PFC from recipient mice and quantification. **E** Representative IF image of BDNF in the hippocampus of recipient mice, scale bar represents 100 μm. **F** Representative IF image of BDNF in the PFC of recipient mice, scale bar represents 100 μm. **G** and **H** Represents images of ZO-1 and occludin in the hippocampus from recipient mice and quantification. **I** and **J** Represents images of ZO-1 and occludin in the PFC from recipient mice and quantification. Data in the present study are presented as the mean ± SD. $^{*}P < 0.01$, $^{**}P < 0.0001$, $^{\#}P < 0.01$, $^{\#\#}P < 0.0001$

SCFAs restored GABA level of the recipient mice transferred from Et mice by FMT

Alterations in GABA system are significant contributors to anxiety behavior and depression [43, 44]. Previous studies demonstrated that reduced GABRA1 level was correlated with ethanol-induced neuropsychic behaviors, and this correlation might be connected to bacterial dysbiosis [45, 46]. Thus, we explored the changes in the GABA system after FMT and investigated the effect of SCFAs on GABA system. Western blot results in the hippocampus (Fig. 8A and B) and PFC (Fig. 8C and D) showed the obvious decline in the levels of GABRA1 and GAD1 in the F-Et group, compared to the F-Con group. At the meantime, additional SCFAs supplements were found to alleviate the decline of GABRA1 and GAD1. Meanwhile, GAD1-positive and GABRA1-positive cells were decreased in the F-Et group and SCFAs increased the decline in the PFC (Fig. S6A, B, C and D). We observed that the serum GABA levels appeared to be slightly higher in the F-Et group, but there was no statistically significant difference (Fig. 8E). However, GABA levels in the hippocampus (Fig. 8F) and PFC (Fig. 8G) appeared to be significantly higher in the F-Et group. And additional SCFAs supplements reduced the levels of GABA. These results suggested that bacterial dysbiosis of Et group caused by CEE affected the GABA system of the recipient mice, with SCFAs potentially acting as a mediator in this relationship.

SCFAs alleviated neuron damage of the recipient mice transferred from Et mice by FMT

Neuroinflammation, neurotrophic proteins deficiency, and blood-brain barrier dysfunction are identified as crucial elements contributing to neuron damage, which is the pathological change that underlies behavioral changes in mice. F-Et decreased the protein levels of GluA1 and PSD-95, and SCFAs supplements increased the decline levels both in the hippocampus (Fig. 9A) and PFC (Fig. 9D). At the same time, neuronal damage was confirmed through Nissl staining. Nissl staining revealed

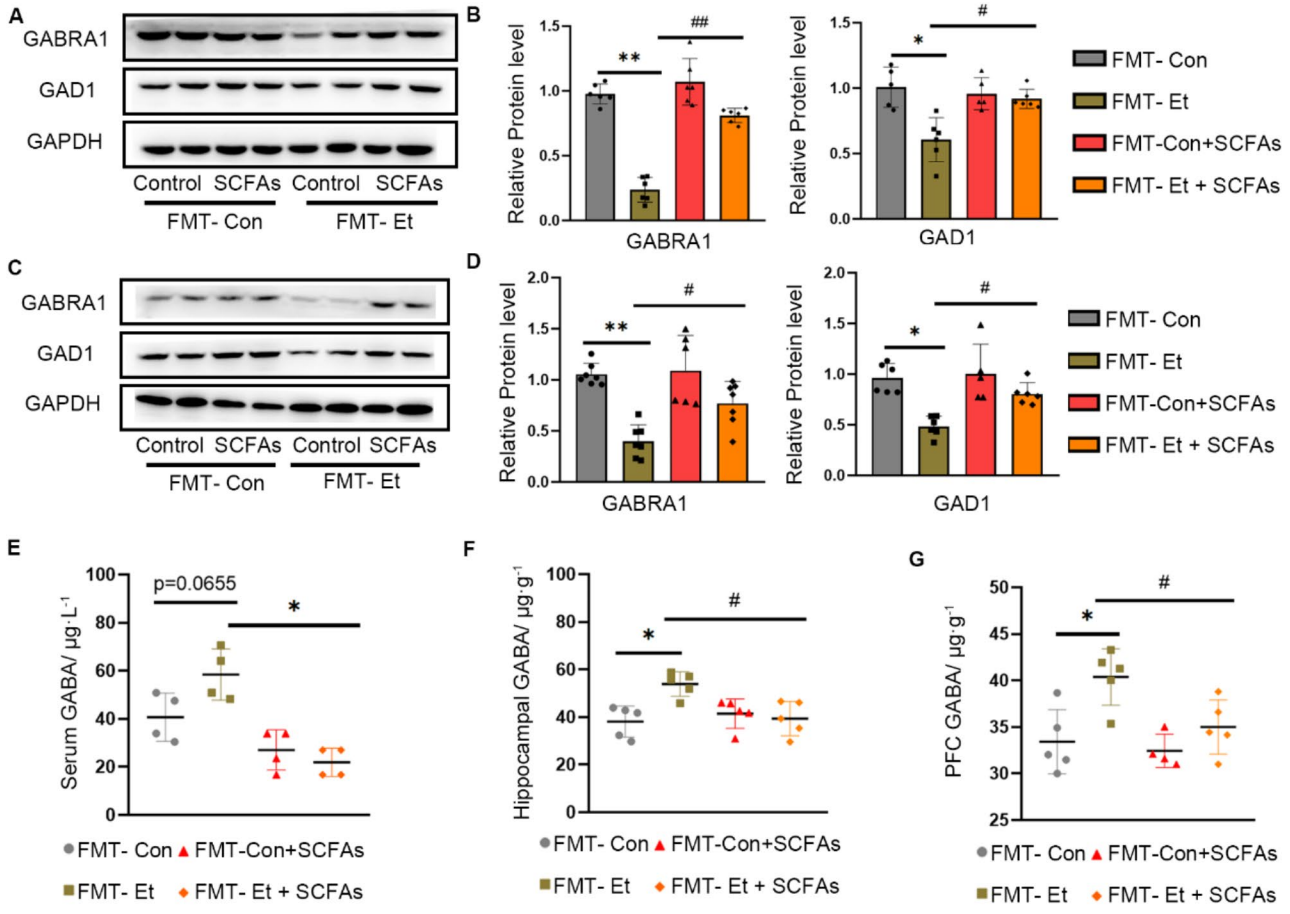

Fig. 8 SCFAs relieve the GABA system. **A** and **B** Represents images of GABRA1 and GAD1 in the hippocampus from recipient mice and quantification. **C** and **D** Represent images of GABRA1 and GAD1 in the PFC from recipient mice and quantification. **E** ELISA showed the level of serum GABA. **F** ELISA showed the level of hippocampal GABA. **G** ELISA showed the level of GABA in the PFC. Data in the present study are presented as the mean ± SD. $^{*}P < 0.01$, $^{**}P < 0.0001$, $^{\#}P < 0.01$, $^{\#\#}P < 0.0001$

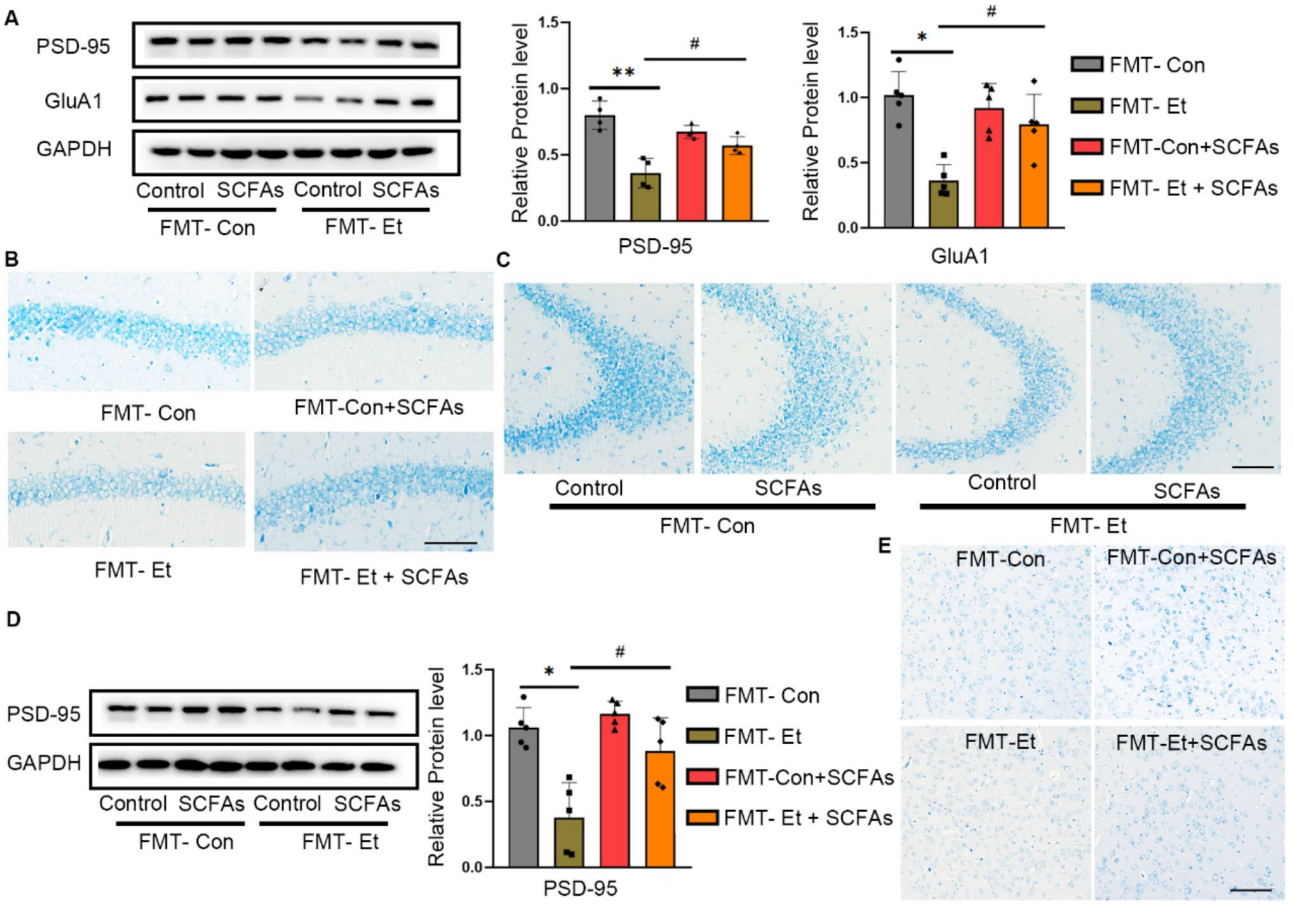

Fig. 9 SCFAs relieve neuronal injury in recipient mice. **A** Represents images of PSD-95 and GluA1 in the hippocampus from recipient mice and quantification. **B** and **C** Representative images of Nissl Staining in the hippocampal CA1 and CA3 regions, scale bar represents 100 μm. **D** Represents images of PSD-95 in the PFC from recipient mice and quantification. **E** Representative images of Nissl Staining in the PFC of recipient mice, scale bar represents 100 μm. Data in the present study are presented as the mean ± SD. $^{*}P < 0.01$, $^{**}P < 0.0001$, $^{\#}P < 0.01$

a decrease in Nissl bodies with fuzzy layering and disorganized, slack pattern in the CA1 and CA3 regions of F-Et group compared to the F-Con group, suggesting bacterial dysbiosis resulted in damage to mouse hippocampal neurons (Fig. 9B and C). However, administration of SCFAs alleviated the manifestations of neuron damage. In the PFC, we observed the similar trend (Fig. 9E). In conclusion, SCFA acts as a bridge connecting bacterial dysbiosis resulting from ethanol exposure to neuron damage in the central nervous system.

Discussion

Our previous sequencing of the 16S rRNA gene indicated notable discrepancies in both the composition and biomarkers of the gut microbiota between the Et group and the Con group. The Et group displayed diminished diversity and abundance in the gut microbiota in comparison to the Con group, suggesting a marked imbalance due to CEE [20]. To delve further into the repercussions of bacterial dysbiosis on the central nervous system, we constructed a 90-day chronic ethanol exposure model as donor mice. We assessed the behavioral performance, intestinal barrier integrity, and inflammation in the donor mice following 90 days of ethanol exposure, in addition to scrutinizing inflammation in the hippocampus, functionality of the blood-brain barrier, and neuronal damage. We observed that CEE impaired integrity of the intestinal barrier and triggered inflammation. The potential mechanisms underlying these alterations in the intestine induced by CEE are unclear. Simultaneously, we observed that the Et group exhibited anxiety and depression-like behaviors, potentially linked to CEE-induced neuroinflammation, dysfunction of the blood-brain barrier, and neurotrophic deficiency resulting in neuron damage.

Neuroinflammation, impairment of the blood-brain barrier, and neurotrophic deficiency are identified as key factors contributing to behavioral changes [47–50]. The relationship between CEE-induced intestinal microbial dysbiosis, inflammation, increased permeability, and subsequent neuroinflammation, blood-brain barrier damage, nutritional deficiency remains unclear. Researchers hypothesize that the microbiome-gut-brain axis may

play a pivotal role in this process [51, 52]. However, the specific mechanisms through which the gut-brain axis communicates alterations in gut microbiota and the central nervous system are not yet fully understood and require further elucidation. Notably, sequencing in this study revealed pronounced changes in the serum levels of SCFAs (propionic acid, butyric acid, and valeric acid) between the Et group and the Con group. SCFAs, derived from intestinal microorganisms [53], and exert effects within the central nervous system [54], suggesting a potential role for SCFAs as intermediaries between gut microbes and the central nervous system. Remolding the gut microbiome holds significant promise for enhancing intestinal functionality and alleviates central nervous system-related symptoms, possibly due to the normalization of SCFAs [55, 56]. Previous research has shown that combining ethanol with butyric acid can relieve anxious-depressive behaviors and alleviate hippocampal neuroinflammation in mice [57].

Microbiota transplantation followed by oral gavage of a SCFA mixture was conducted to investigate the impact of intestinal microbiota changes induced by CEE on the central nervous system and the role of short-chain fatty acids. Serum SCFAs (butyric acid and valeric acid) levels of recipient mice transplanted with fecal microbiota that disrupted homeostasis induced by chronic ethanol exposure decreased, suggesting that bacterial dysbiosis affected the production of SCFAs. As we hypothesized, SCFAs attenuated microbial dysbiosis-induced intestinal structural and functional abnormalities, which are characterized by shortened villus height, intestinal barrier dysfunction, and intestinal inflammatory response. At the same time, behavioral results indicated that treatment with SCFAs alleviated anxiety and depression-like behaviors in mice from the F-Et group. All in all, SCFAs not only impact the structural damage in the intestines caused by dysbiosis resulting from chronic ethanol exposure, but also influence the central nervous system. This aligns with our previous hypothesis that short-chain fatty acids function as bridging molecules in the gut-brain axis.

Then, our research delved into the mechanism by which SCFAs affect the central nervous system. Initial findings indicate that probiotic treatment can alleviate gut microbial imbalances caused by CEE and prevent the development of neuroinflammation [20]. At the same time, fecal transplantation confirmed that intestinal flora imbalance caused by CEE is one of the mechanisms leading to neuroinflammation [20]. In the present study, giving mice additional SCFAs by gavage ameliorated intestinal structural and functional disorders and reduces neuroinflammation. Microglia are one of the main target cells of SCFAs in the brain [58]. We observed that SCFAs reduced microglial activation, exerting anti-inflammatory effects. At same time, we found that the number of astrocytes also decreased after SCFAs treatment.

Neuroinflammation and neurotrophic protein deficiency often occur simultaneously, and improvement in neuroinflammation is often accompanied by melioration in neurotrophic deficiency [59]. BDNF and its receptor, TrkB, expressed extensively in the central nervous system, along with ERK, were associated with anxiety and depression-like behaviors [60–62]. In the study, we observed that additional supplementation of SCFAs increased BDNF, TrkB as well as ERK, suggesting that SCFAs may exert their effects on anxiety and depression through BDNF-related signaling pathways. We did not delve into whether it mainly exerts this effect through BDNF-related pathways such as BDNF-TRKB or BDNF-ERK, which have been linked to depression [63, 64].

GABA and its receptors exert tight control on neuronal activity and functional plasticity, affecting mood behavior profoundly and rapidly [65, 66]. Previous research has shown that CEE affects the GABA system, including reducing the expression of GABRA1 receptors and GAD1, whereas minocycline administration improved the intestinal microbiota and alleviated the trend of decreased GABRA1 and GAD1 expression [46]. In the present study, F-Et exhibited reduced GABRA1 and GAD1, both in the PFC and hippocampus, aligning with prior studies indicating that a reduction in GABRA1 is strongly linked to anxiety and depression. Additional supplementation with SCFAs alleviated the decreased trend of GABRA1 and GAD1.

Neuroinflammation activation, neurotrophic deficiency, and other factors induced anxiety and depressive-like behaviors through neuronal damage resulting from these alterations [67, 68]. This study presented that neuronal damage in F-Et mice underwent histological changes and that SCFA ameliorated neuronal damage. The mechanism by which SCFAs relieve neuronal damage may be related to its anti-inflammation, improvement of BDNF signaling, and enhancement of the blood-brain barrier.

A limitation of this study is our inability to detect the concentration of propionic acid due to instrumentation constraints. Additionally, we did not collect fecal samples following treatment with fecal microbiota transplantation (FMT) and short-chain fatty acids (SCFAs), which prevented us from analyzing their microbial and SCFA content. However, we did assess their concentrations within the intestine.

Conclusions

In summary, our results indicate that CEE leads to dysfunction of intestinal microbial homeostasis and reduction in SCFAs production, then causing intestinal structure deterioration, neuroinflammation, blood-brain

barrier dysfunction, neurotrophic defects, and GABA system changes, thereby resulting in neuronal damage and the manifestation of anxious and depressive behaviors. The modifications were transferred to the recipient mice through FMT by the Et group mice. Notably, supplementation with SCFAs significantly mitigated these adverse effects of the recipient mice. Importantly, SCFAs play a crucial role in the gut-brain axis, providing a foundational theoretical framework for neuroinflammation, anxious and depressive behaviors which are triggered by CEE-induced microbiota dysbiosis.

Abbreviations

BBB	Blood brain barrier
BDNF	Brain derived neurotrophic factor
CEE	Chronic ethanol exposure
EPM	Elevated plus-maze
F-Con	FMT from Con group
F-Et	FMT from Et group
FMT	Fecal microbiota transplantation
FST	Forced swimming test
GC-MS	Gas chromatography-mass spectrometry
OFT	Open field test
RT-qPCR	Real-time quantitative polymerase chain reaction
SCFAs	Short-chain fatty acids

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s12974-024-03282-6.

Supplementary Material 1

Supplementary Material 2

Acknowledgements
We thank Dr. Hui Yao from Sun Yat-sen University of the useful suggestions.

Author contributions
X. Wang., G. Z. H. Yu., H. S. and C. Z.conceived and designed the study. H. S., Q. Z., Q. L. performed the experiments and analyzed the data. S. H. and C. Z. drafted the manuscript. H. L., H. Yuan., F. M., C. W., J. P., and Y. G. J. T., and C.Y. visualization; methodology. H. Yu., G. Z. and X. Wang. provided critical revision. All authors reviewed and approved the final manuscript.

Funding
This research received funding from the National Natural Science Foundation of China (grant number 82101979 to X. Wang, grant number 82271931 to G.Z.); the Natural Science Foundation of Liaoning Province, China (grant number 2022-MS-220 to X. Wang).

Data availability
No datasets were generated or analysed during the current study.

Declarations

Ethics approval and consent to participate
All experimental procedures were approved by China Medical University Animal Care and Use Committee (CMU2020103).

Consent for publication
Not applicable.

Competing interests
The authors declare no competing interests.

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Received: 11 September 2024 / Accepted: 29 October 2024
Published online: 06 November 2024

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