



Research article

Electroacupuncture blocked motor dysfunction and gut barrier damage by modulating intestinal NLRP3 inflammasome in MPTP-induced Parkinson's disease mice

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ABSTRACT

Parkinson's disease (PD) is a neurodegenerative disorder commonly accompanied by gut dysfunction. EA has shown anti-inflammatory and neuroprotective effects. Here, we aim to explore whether EA can treat Parkinson's disease by restoring the intestinal barrier and modulating NLRP3 inflammasome. We applied 1-Methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) to establish a PD mouse model and EA at the GV16, LR3, and ST36 for 12 consecutive days. The open-field test results indicated that EA alleviated depression and behavioral defects, upregulated the expressions of tyrosine hydroxylase (TH) and brain-derived neurotrophic factor (BDNF), and blocked the accumulation of α -synuclein (α -syn) in the midbrain. Moreover, EA blocked the damage to intestinal tissues of PD mice, indicative of suppressed NLRP3 inflammasome activation and increased gut barrier integrity. Notably, the antibiotic-treated mouse experiment validated that the gut microbiota was critical in alleviating PD dyskinesia and intestinal inflammation by EA. In conclusion, this study suggested that EA exhibited a protective effect against MPTP-induced PD by alleviating behavioral defects, reversing the block of motor dysfunction, and improving the gut barrier by modulating intestinal NLRP3 inflammasome. Above all, this study could provide novel insights into the pathogenesis and therapy of PD.

1. Introduction

Parkinson's disease (PD) is a neurodegenerative disease that commonly affects the elderly. PD occurs due to the loss of dopaminergic neurons and the formation of Lewy bodies. Motor dysfunction, including resting tremors, muscle rigidity, bradykinesia, and unstable gait, are the main clinical manifestations of PD. Besides, some PD patients may suffer from a range of non-motor symptoms, such as insomnia, depression, and intestinal dysfunction [1]. Many pharmacological agents, such as levodopa, dopamine agonists,

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monoamine oxidase type B inhibitors, and anticholinergic drugs, have been used to improve PD. Nevertheless, Nausea, vomiting, insomnia, and hepatotoxicity are common side effects of PD treatments. These symptoms can vary from mild to severe [2]. Acupuncture, originating from “acupuncture stones,” is the essence of traditional Chinese medicine and is recognized as a natural treatment without annoying side effects. Acupuncture plays a conspicuous role in the therapy of PD, which not only improves motor symptoms such as tremors and shuffling but also effectively relieves non-motor symptoms such as constipation, depression, and insomnia [3–5]. However, it remains unclear about the mechanism underlying the effect of electroacupuncture (EA) in PD.

It has been reported that 77 %–81 % of PD patients are accompanied by intestinal dysfunction, such as constipation, diarrhea, and inflammatory bowel disease (IBD) [6]. Furthermore, several studies confirmed a close relationship between intestinal inflammation and PD. For example, Devos confirmed that the mRNA expressions of pro-inflammatory cytokines (*IL-6*, *TNF- α* , and *IL-1 β*) in the colon tissues of PD patients were higher than those of healthy controls [7]. Besides, the expressions of inflammation-related genes, such as vascular endothelial growth factor receptor 1 (VEGFR-1), interleukin 1 α (IL-1 α), interleukin 1 β (IL-1 β), and C-reactive protein (CRP), were increased in the feces of PD patients [8]. Moreover, abnormal changes in gut microbiota led to the development of PD [9]. The gut-brain axis is a bidirectional pathway that plays an essential role in maintaining homeostasis. Dysbiosis leads to the activation of inflammatory signals and increases the permeability of intestinal epithelium [10]. Meanwhile, increased intestinal permeability and systemic inflammatory responses can disrupt the blood-brain barrier and the nervous system, leading to impaired brain dopaminergic neuronal function [11]. However, it is unclear whether EA can reverse the motor dysfunction of Parkinson’s disease by restoring the integrity of the gut barrier.

NLRP3 inflammasome is an inflammatory complex consisting of the NOD-like receptor 3 (NLRP3), the apoptosis-associated speck-like protein containing a CARD (ASC), and the effector molecule cysteine protease-1 (Caspase-1). Once the NLRP3 inflammasome is activated, it will initiate a series of inflammatory cascades that cause the subsequent tissue injury. In a recent study, the dysregulation of NLRP3 inflammasome was associated with Alzheimer’s disease and PD [12]. The protein expressions of NLRP3, ASC, Caspase-1, and IL-1 β were increased in the substantia nigra (SN) and striatum of the midbrain in mice treated with 6-hydroxydopamine (6-OHDA) or MPTP [13,14]. It seems that NLRP3 inflammasome might be a potential therapeutic target for PD treatment.

Acupuncture is active in treating neuropsychiatric and gastrointestinal disorders [15,16]. For example, EA-stimulated GV16 acupoint reduced nerve damage [17]. Furthermore, EA intervention at the LR3 acupoint relieved depression [18,19]. In addition, EA stimulation of ST36 acupoint exerted anti-inflammatory, antioxidant, and gastrointestinal recovery effects [20]. Several studies have illustrated that EA could inhibit intestinal inflammation and reduce α -synuclein (α -syn) deposition in the colon, thus alleviating neurogenic damage [21]. In addition, acupuncture treatment reduced protein levels of the NLRP3 complex in the prefrontal cortex and effectively alleviated depression symptoms in a chronic stress rat model [22]. However, it is unclear whether EA can rehabilitate the intestinal barrier function through modulating NLRP3 inflammasome in PD occurrence.

Based on the above, we hypothesize that EA can alleviate PD by restoring intestinal barrier function and modulating NLRP3 inflammasome. In this study, we investigated the therapeutic effect of EA by stimulating GV16, LR3, and ST36 acupoints on MPTP-induced PD mice. We found that EA intervention inhibited the activation of NLRP3 inflammasome in the colon. Also, we confirmed the role of gut microbiota in EA intervention using antibiotic-treated PD mice.

2. Materials and methods

2.1. Chemicals and reagents

1-Methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) was purchased from Sigma-Aldrich (St. Louis, MO, USA). Sterile acupuncture needles (0.16 \times 7 mm) were obtained from Beijing Zhongyan Taihe Medical Equipment Corporation (Beijing, China). SEMZ-II electronic acupuncture device was purchased from Suzhou Medical Supplies Factory Ltd (Suzhou, China). The open field test device was obtained from Wuhan Yihong Technology Corporation (Wuhan, China). FashHS SYBR QPCR mixture and AMeasy 1st Strand cDNA synthesis kit were purchased from Summer Bio (Beijing, China). Primary antibodies were obtained from the following companies: anti-NLRP3 (#15101, 1) from Cell Signaling Technology (Danvers, MA, USA); anti-ASC (E51715) from Elk Biotechnology (Wuhan, China); anti-Tyrosine Hydroxylase (A0028), anti-Caspase-1 (A0964), anti-BDNF (A18129), and anti- α -synuclein (A13354) from Abclonal Corporation (Wuhan, China); anti- β -actin (sc-81178) from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Other chemicals and reagents were of analytical purity grade.

2.2. Dosage regimen

MPTP, a relatively simple compound that causes selective degeneration of the substantiating after systemic administration, has been widely used to create animal models of Parkinson’s disease [23]. After extensive literature research, we found that the PD model was established by intraperitoneal injection of 30 mg/kg MPTP for five days in mice. Therefore, we chose the MPTP to establish the PD model [24,25]. EA treatment was repeated 2 h after MPTP injections and continued once a day for seven days after the last MPTP or saline injection [26].

2.3. Animal experiment

This paper aims to evaluate electroacupuncture’s protective effect and mechanism at specific acupoints in MPTP-induced Parkinson’s disease mice. The MPTP group was designed to demonstrate the success of the animal model establishment. In previous

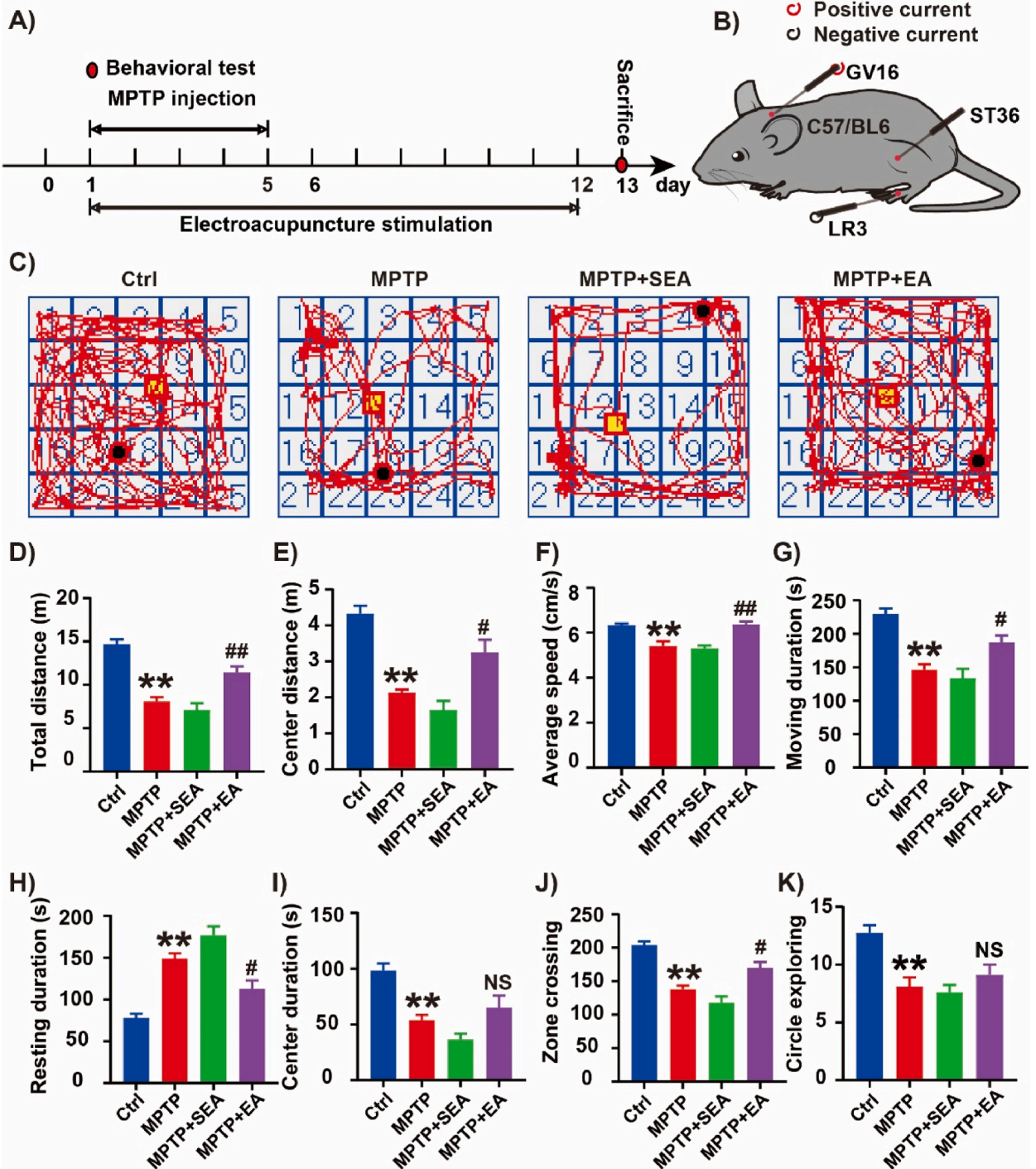


Fig. 1. EA treatment alleviated motor dysfunction in MPTP-induced PD mice. **A** Schedule showing the experimental procedure. **B** Schematics showing the GV16, LR3, and ST36 acupoints. **C** Track of mice in 5 min. **D** Total distance, $F(3, 28) = 28.98, P = 0.0001$. **E** Center distance, $F(3, 27) = 24.89, P = 0.0001$. **F** Average speed, $F(3, 28) = 15.93, P = 0.0001$. **G** Moving duration, $F(3, 27) = 18.13, P = 0.0001$. **H** Resting duration, $F(3, 26) = 29.56, P = 0.0001$. **I** Center duration, $F(3, 27) = 14.61, P = 0.0001$. **J** Zone crossing, $F(3, 27) = 26.57, P = 0.0001$. **K** Circle exploring, $F(3, 28) = 9.748, P = 0.0001$. Data were presented as mean \pm SEM ($n = 8$). ** $P < 0.01$ vs. Ctrl group. # $P < 0.05$, ## $P < 0.01$ vs. MPTP group. NS, not significant.

research, MPTP + sham EA was set as a control group to show that MPTP + EA could ameliorate MPTP-induced Parkinson's disease [26]. In this study, we compared the MPTP + EA group with the MPTP group to observe the protective effect of EA intervention on MPTP-induced Parkinson's disease. We also compared the MPTP + EA group with the MPTP + SEA group to demonstrate the specificity of EA stimulation in blocking Parkinson's disease at specific acupoints.

A total of 64 male C57BL/6 J mice (eight-week-old, 22 ± 2 g) were purchased from the Hubei Provincial Center for Disease Control and Prevention (Wuhan, China). Mice were housed individually at a temperature of 22 ± 2 °C under a 12 h light/dark cycle. After a week of acclimatization, 32C57BL/6 J mice were randomly divided into four groups, each containing eight mice: control group (Ctrl), MPTP-injection group (MPTP), MPTP-injection and sham-electroacupuncture (SEA)-treated group (MPTP + SEA), and MPTP-injection and electroacupuncture (EA)-treated group (MPTP + EA). Prior to starting the study, we conducted a literature review and found that MPTP at a dose of 30 mg/kg given intraperitoneally for five consecutive days was the most commonly used dosage in early studies [27, 28]. So, mice were injected intraperitoneally with saline (0.1 mL/10 g) or MPTP (30 mg/kg, dissolved in saline) for five consecutive days in this study. For example, a mouse weighing 20 g would require an intraperitoneal injection of 0.2 mL MPTP (3 mg/mL). Mice in the MPTP + SEA and MPTP + EA groups were treated with EA (1 mA, continuous wave, frequency 2 Hz) 2 h after the first MPTP injection, once daily until seven days after the last MPTP or saline injection. Fig. 1 A shows the experimental procedure and acupoint position.

For antibiotic treatment, the experimental procedure was shown in Fig. 6 A. 32C57BL/6 J mice were separated into four groups ($n = 8$): Ctrl group, MPTP group, MPTP + Antibiotic mixture group (MPTP + Ab), and MPTP + Antibiotic mixture + electroacupuncture group (MPTP + Ab + EA). In a previous study, we found that using a combination of four antibiotics (ampicillin at 1.0 mg/mL, vancomycin at 0.5 mg/mL, neomycin sulfate at 1.0 mg/mL, and metronidazole at 0.5 mg/mL) dissolved in drinking water was an effective method for depleting gut microbiota [29]. This method has been widely used by researchers and documented in several publications [29–32]. For the Ctrl and MPTP groups, mice were fed with sterile water, and the other two groups were treated with Ab (1.0 mg/mL ampicillin, 0.5 mg/mL vancomycin, 1.0 mg/mL neomycin sulfate, and 0.5 mg/mL metronidazole, dissolved in drinking water) for 12 weeks in this study. From the 13th week, mice were injected intraperitoneally with MPTP or EA stimulation, as mentioned above.

The mice's body weight, water intake, and food intake were recorded weekly during the animal experiment. At the end of the experiment, the mice were anesthetized following the guidelines for the Euthanasia of Animals set by the American Veterinary Medical Association in 2020 (<https://www.avma.org/KB/Policies/Documents/euthanasia.pdf>). After the mice were fully anesthetized, we collected and preserved tissue samples for further analysis.

The animal experiment was performed under the guidance of the Ethical Experimentation Committee of Hubei University of Chinese Medicine (permission number: SYXK (E) 2020–0018) and the National Act on Use of Experimental Animals (China). The work described in our report was conducted following the ARRIVE guidelines.

2.4. Electroacupuncture (EA) treatment

The acupuncture points were shown in Fig. 1 B. This study selected the GV16, LR3, and ST36 acupoints for EA treatment. GV16 acupoint is located in the gap of the trapezius behind the external occipital protuberance. LR3 acupoint is situated in the gap between the first and second metatarsal bones on the dorsal aspect of the hind limb. ST36 acupoint is located on the posterior lateral aspect of the knee, approximately 2 mm below the inferior fibular head. The GV16 and LR3 points were then connected to an electronic acupuncture device (Huatuo, Suzhou, China), with the positive terminal connected to the GV16 point and the negative terminal to the LR3 point. The EA stimulation frequency was 2 Hz, and the current strength was 1 mA with a 15-min duration for each treatment. Our research of relevant literature revealed that the electroacupuncture frequency of 1 mA and 2 Hz was commonly used [33,34]. Therefore, we selected this frequency for our study. Additionally, we conducted a preliminary experiment and found that this frequency is safe for mice. The sham acupoints were located 5 mm apart from the acupoints, and the acupuncture depth was the same as the MPTP + EA group. The EA treatment was performed 2 h after the intraperitoneal injection of MPTP. In this study, we compared the MPTP + EA group with the MPTP group to observe the protective effect of EA intervention on MPTP-induced Parkinson's disease. We also compared the MPTP + EA group with the MPTP + SEA group to demonstrate the specificity of EA stimulation in blocking Parkinson's disease at specific acupoints. As a result, we did not establish a separate EA treatment group.

2.5. Open field test

Mice were acclimatized in the test room for 1 h, then placed in an open box (40 cm long, 40 cm wide, and 35 cm high). The total time was set to 5 min through the computer analysis software (AniLab ver 5.10), and the open-field experiment started after the mice were placed in the middle of the box. The total distance, moving duration, resting duration, average speed, center distance, and zone crossing were counted and used to observe the changes in the behavioral performance of the autonomous movement.

2.6. RNA extraction and real-time quantitative PCR (RT-qPCR)

The total RNA from the striatum or colon tissues was extracted by Trizol reagent (Summer Bio, Beijing, China). Next, cDNA was obtained by reverse transcription from the isolated total RNA (1 μ g) using a First Strand cDNA Synthesis Kit (Summer Bio, Beijing, China). Then, cDNA was amplified on a real-time fluorescence quantitative PCR instrument (BioRad, CA, USA). Finally, the relative expression of the target genes and the internal reference gene Beta-actin was calculated using the $2^{(-\Delta\Delta Ct)}$ method. The sequences of all

primers used in the experiments were summarized in [Supplementary Table 1](#).

2.7. Western blotting analysis

Total proteins from the midbrain or colon tissues were extracted with RIPA buffer (Beyotime, Shanghai, China). The protein concentration of sample lysate was quantified using a bicinchoninic acid (BCA) assay kit (Beyotime Biotechnology, Shanghai, China). The samples were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to polyvinylidene difluoride (PVDF) membranes. The membranes were incubated with primary antibodies at 4 °C overnight, followed by the incubation with horseradish peroxidase (HRP)-conjugated secondary antibodies for 1.5 h at room temperature. Finally, the target protein bands were detected by an enhanced chemiluminescence solution (ECL, Cell Signaling Technology, Danvers, MA, USA). The intensity of protein bands was quantified by Image J2x (National Institute of Health, Bethesda, MD, USA). Western blotting was performed using the following antibodies: NLRP3 (1 : 1000), Caspase-1 (1 : 1000), ASC (1 : 1000), TH (1 : 1000), α -syn (1 : 1000), BDNF (1 : 5000), β -actin (1 : 500).

2.8. Histopathological analysis

Colon and brain tissues were fixed in 4 % paraformaldehyde, paraffin-embedded, and cut into 5 μ m slices. Histological staining was performed using a hematoxylin and eosin (H&E) staining kit (Beyotime, Shanghai, China). The colon tissues were stained using Alcian Blue (Vectorlabs, Beijing, China) and Wheat Germ Agglutinin-FITC (WGA-FITC) conjugate (Sigma Aldrich, St. Louis, MO, USA) to assess the acidic and glycosylated mucin expression, respectively. Immunohistochemical staining was conducted to analyze the expression of TH (1:300) in the SN, and TH (1:300) and α -syn (1:400) in the striatum. The micrographs were photographed by a Leica DMI4000 Blight microscope connected to a Leica DFC310 FX digital camera (Wetzlar, Germany).

2.9. Enzyme-linked immunosorbent assay (ELISA)

All mice were anesthetized, and 0.5–0.6 mL peripheral blood was collected through the eyes. After centrifugation at 3000 \times g for 5 min, the supernatant was stored at -80 °C. Then, ELISA was performed to analyze IL-1 β level in the supernatant using commercially available ELISA kits (Elabscience Biotechnology Co., Ltd. Wuhan, China) according to the manufacturer's instructions. The concentration of IL-1 β was determined based on the standard curve.

2.10. Statistical analysis

The GraphPad Prism 8.3 software (La Jolla, CA, USA) was used for routine data analysis. Data were expressed as mean \pm SEM. In this study, we selected the GV16, LR3, and ST36 acupoints for electroacupuncture treatment of PD, while the sham acupoints were located 5 mm apart from the acupoints. Two factors are involved in the treatment of PD: whether the EA has a therapeutic effect on PD and whether the acupoints except GV16, LR3, and ST36 have a therapeutic effect on PD. Therefore, we used a two-way analysis of variance (ANOVA) to compare multiple groups. P value $<$ 0.05 was considered significant.

3. Results

3.1. Alleviation of motor dysfunction by EA treatment in MPTP-induced PD mice

As shown in [Fig. 1 A](#), mice were injected intraperitoneally with MPTP (30 mg/kg) for five days to establish a PD mouse model. Next, we performed EA interventions on mice for 12 continuous days ([Fig. 1 A and B](#)). We performed an open-field test to determine the effect of EA on MPTP-induced motor function in PD mice. The results revealed that PD mice showed a decrease in range of motion and exploration of open space ([Fig. 1 C](#)). In addition, PD mice exhibited a significant reduction in the total distance ([Fig. 1 D](#)), center distance ([Fig. 1 E](#)), average speed ([Fig. 1 F](#)), moving duration ([Fig. 1 G](#)), center duration ([Fig. 1 I](#)), zone crossing ([Fig. 1 J](#)), and circle exploring ([Fig. 1 K](#)) compared to the Ctrl group ($P <$ 0.01). Meanwhile, the increased resting duration was observed in PD mice ($P <$ 0.01, vs. Ctrl group) ([Fig. 1 H](#)). In contrast, after EA intervention, most of these changed indicators were statistically improved, including the range of movement in the enclosed space, total distance, center distance, average speed, moving duration, resting duration, and zone crossing ($P <$ 0.05 or 0.01, vs. MPTP group) ([Fig. 1 C–K](#)). Notably, the SEA intervention had no improvement in the alterations of motor impairment-related indicators ([Fig. 1 C–K](#)). The above data suggest that EA treatment with GV16, LR3, and ST36 acupoints improved motor dysfunction in PD mice.

3.2. Suppressive effect of EA treatment on physiological parameter changes and brain damage in MPTP-induced PD mice

As depicted in [Fig. 2 A](#), the body weight of mice in the MPTP group decreased significantly compared with the Ctrl group ($P <$ 0.05). However, EA treatment alleviated the MPTP-induced weight loss. In addition, we monitored the dynamic changes in food and water intake during the experiment. The results showed that PD mice ate less than the Ctrl group mice ($P <$ 0.05), whereas EA intervention increased the intake of food ([Fig. 2 B](#)) ($P <$ 0.05). In comparison, there was no difference in water drinking among the four groups ([Fig. 2 C](#)). Then, we carried out routine blood tests to analyze the effect of EA on the blood parameters. Compared to the Ctrl group,

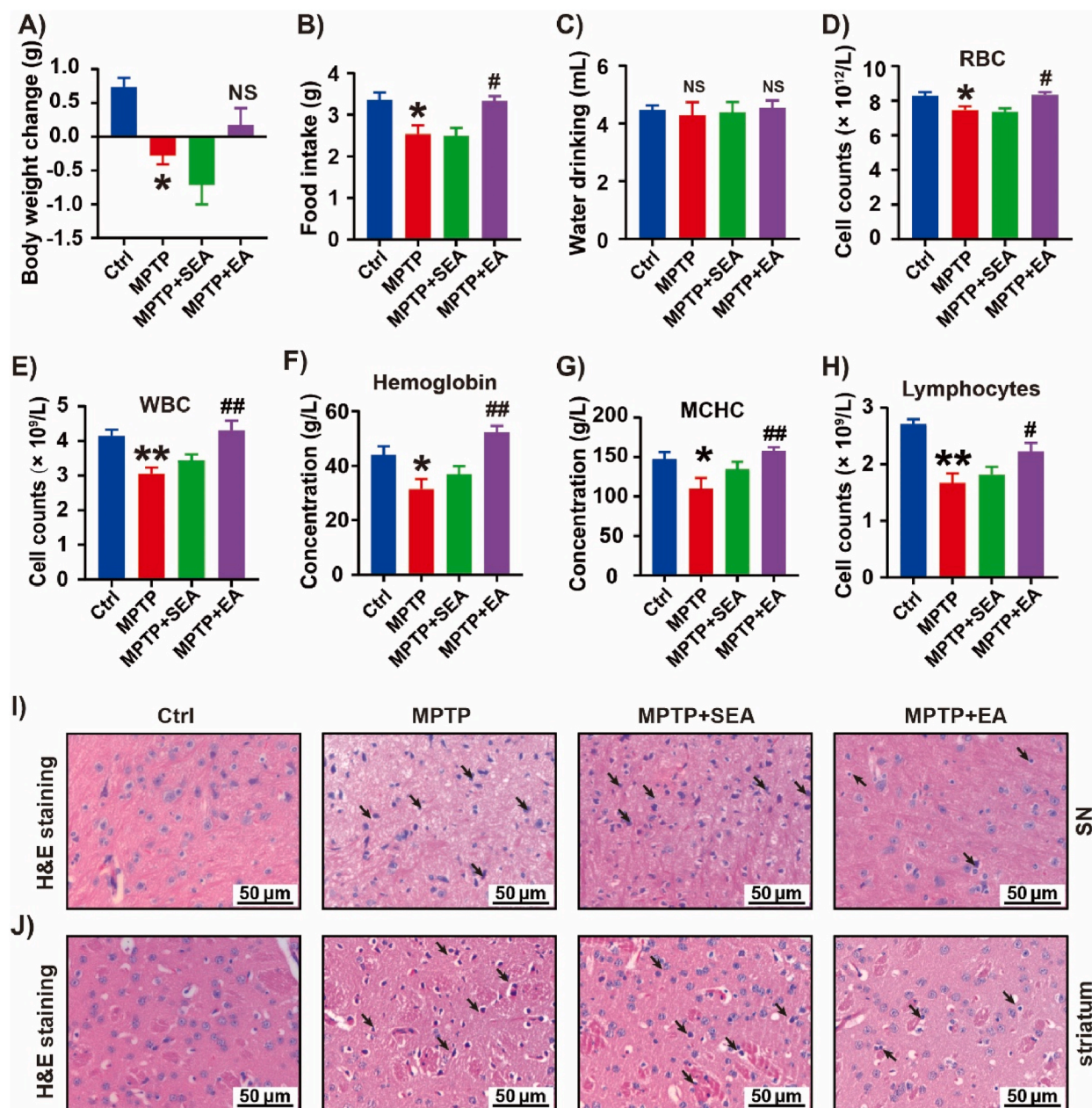


Fig. 2. EA treatment suppressed physiological parameter changes and brain damage in MPTP-induced PD mice. **A)** Body weight change ($F(3, 25) = 8.28, P = 0.0006$). **B)** Food intake ($F(3, 27) = 6.934, P = 0.0013$). **C)** Water drinking ($F(3, 28) = 0.1057, P = 0.9561$). **D)** Red blood cell (RBC) ($F(3, 25) = 7.435, P = 0.001$). **E)** White blood cell (WBC) ($F(3, 28) = 7.866, P = 0.0006$). **F)** Hemoglobin (HGB) concentrations ($F(3, 26) = 8.68, P = 0.0003$). **G)** Mean corpuscular hemoglobin concentrations (MCHC) ($F(3, 25) = 4.995, P = 0.0075$). **H)** Lymphocyte (LYM) count ($F(3, 28) = 11.43, P = 0.0001$). **(I and J)** Hematoxylin & eosin (H&E) staining assay of SN **(I)** and striatum tissues **(J)** (view: $\times 400$). Black arrows indicate degenerated neurons. SEA, sham electroacupuncture. EA, electroacupuncture. Data were presented as mean \pm SEM ($n = 8$). * $P < 0.05$, ** $P < 0.01$ vs. Ctrl group. # $P < 0.05$, ## $P < 0.01$ vs. MPTP group. NS, not significant. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

white blood cell count (WBC), red blood cell count (RBC), hemoglobin (HGB), mean hemoglobin concentration (MCHC), and lymphocyte count (LYM) were reduced in the MPTP group ($P < 0.05$ or 0.01 , which were reversed by EA intervention ($P < 0.05$ or 0.01 , vs. MPTP group) (Fig. 2D–H). On the other hand, SEA intervention did not reverse the abnormal changes in the above-mentioned hematological parameters in PD mice (Fig. 2D–H). In addition, we performed H&E staining to analyze MPTP-induced alterations in the substantia nigra (SN) and striatum tissues of PD mice. As illustrated in Fig. 2I and J, MPTP reduced the number of normal neurons in the SN and striatum. Besides, the neuronal cells in PD mice were reduced, with blurred borders and irregular morphology. On the

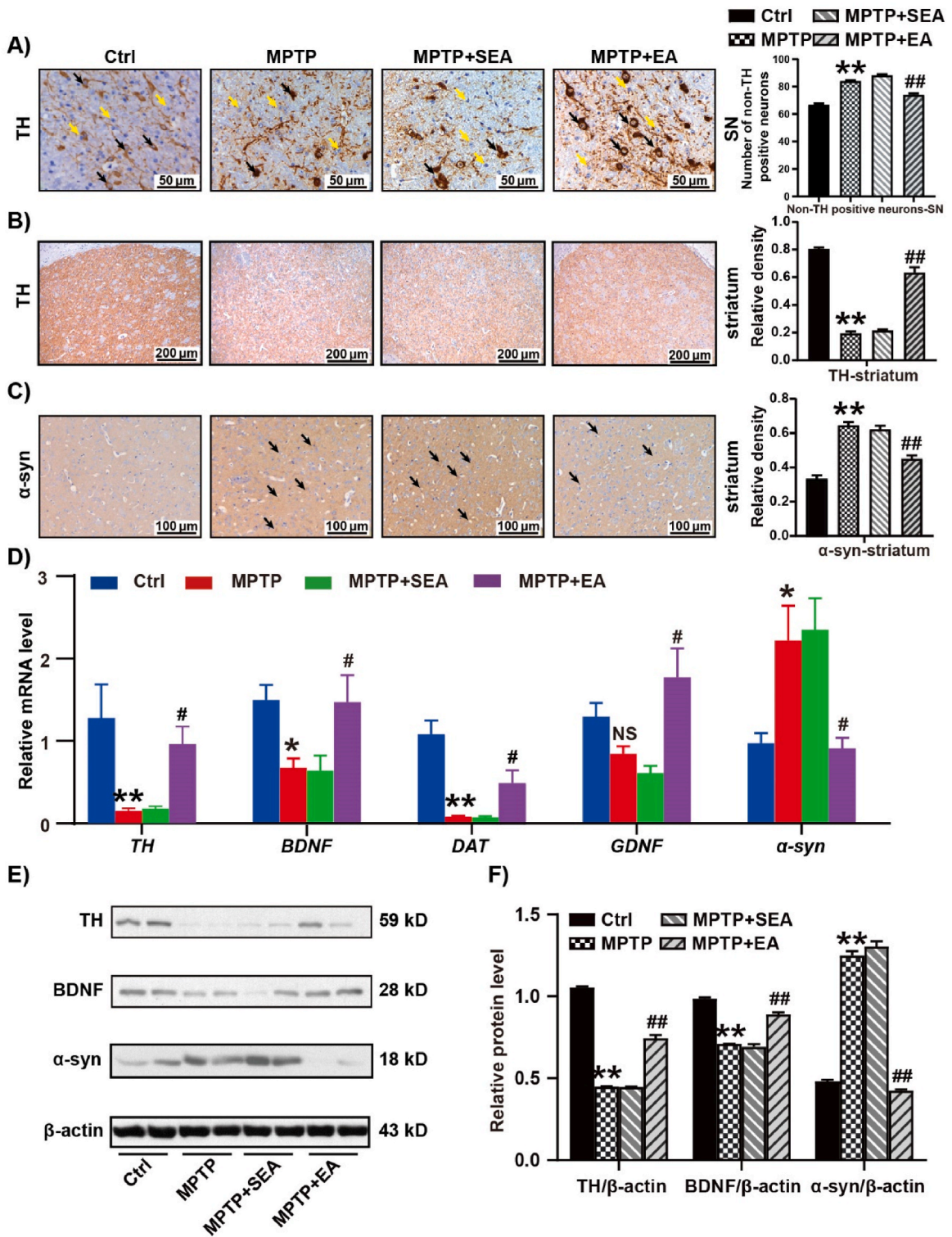


Fig. 3. EA treatment blocked MPTP-induced damage to dopamine neurons and fibers. (A–C) Immunohistochemical staining of TH in substantia nigra (SN) (view: × 400), TH in striatum (view: × 100), and α-syn in the striatum (view: × 200). The number of non-TH labeled neurons in the SN and the quantification of TH and α-syn in striatum were shown in the right panel. (D) Transcriptional expressions of tyrosine hydroxylase (TH), brain-derived neurotrophic factor (BDNF), dopamine transporter (DAT), glial-derived neurotrophic factor (GDNF), and α-syn in the midbrain were analyzed by RT-qPCR. (TH: F (3, 20) = 8.701, P = 0.0007. BDNF: F (3, 28) = 6.193, P = 0.0023. DAT: F (3, 23) = 23.58, P = 0.0001. GDNF: F (3, 23) = 5.444, P = 0.0056. α-syn: F (3, 25) = 6.389, P = 0.0023.) (E and F) Protein levels of TH, BDNF, and α-syn in the midbrain were analyzed by western

blotting (TH: F (3, 28) = 871.8, $P = 0.0001$. BDNF: F (3, 28) = 206, $P = 0.0001$. α -syn: F (3, 28) = 501.5, $P = 0.0001$.). The quantification of band intensity was conducted using Image J2x software. Black arrows indicate TH labeled cells or α -syn-labeled cells; yellow arrows indicate non-TH labeled cells. Data were presented as mean \pm SEM ($n = 8$). * $P < 0.05$, ** $P < 0.01$ vs. Ctrl group. # $P < 0.05$, ## $P < 0.01$ vs. MPTP group. NS, not significant. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

contrary, after EA intervention, the number of normal neurons was increased, and the cytosol was enlarged with clear boundaries (Fig. 2I and J). These results suggest that EA stimulation of GV16, LR3, and ST36 points improved MPTP-induced weight loss, abnormal blood indicators, and neuronal cell degeneration.

3.3. Effect of EA treatment on MPTP-induced damage to dopamine neurons and fibers in PD mice

To examine the effect of EA on neuron repairment in PD mice, we conducted immunohistochemistry, RT-qPCR, and western blotting assay. The immunohistochemical results showed that MPTP induced a decrease in TH expression ($P < 0.01$, vs. Ctrl group) in SN and striatum tissues and an increase in α -syn level ($P < 0.01$, vs. Ctrl group) in striatum tissues (Fig. 3 A–C, Supplementary Fig. 1), indicating that MPTP damaged dopamine neurons and nerve fibers. In contrast, EA intervention promoted TH expression ($P < 0.01$, vs. Ctrl group) and reduced the level of α -syn ($P < 0.01$).

To further examine the repairing effect of EA on neuronal dopamine injury, we analyzed the alterations of dopamine-related genes in midbrain tissues by RT-qPCR and western blotting. As shown in Fig. 3D, EA treatment increased the mRNA levels of tyrosine hydroxylase (TH), brain-derived neurotrophic factor (BDNF), dopamine transporter (DAT), and glial-derived neurotrophic factor (GDNF), but inhibited the expression of α -syn in the midbrain tissues ($P < 0.05$). In addition, EA treatment significantly upregulated the protein levels of TH and BDNF and suppressed the expression of α -syn in the midbrain of PD mice (Fig. 3E and F). These data demonstrated that EA treatment protected against MPTP-induced damage to dopamine neurons and fibers by upregulating TH and BDNF expression.

3.4. EA treatment inhibited colonic apoptosis and NLRP3 inflammasome activation, and repaired intestinal barrier damage in MPTP-induced PD mice

PD patients often have distinct intestinal dysfunction, and the intestinal inflammatory response will exacerbate brain degeneration [35]. Based on the critical role of intestinal inflammation in PD progression, we next investigated the therapeutic effect of EA on inflammation and intestinal dysfunction in PD mice. The H&E staining indicated the destruction of the intestinal muscular layer in the MPTP and MPTP + SEA group, such as cup cell reduction, inflammatory infiltrations, and crypt structure destruction. Compared with MPTP and MPTP + SEA groups, the EA treatment resulted in more goblet cells, intact crypt structure, and less inflammation in the intestine. Meanwhile, Alcian blue and WGA-FITC staining were used to analyze the distribution of glycoprotein and mucin in the colon. As shown in Fig. 4B and C, EA treatment improved the reduction of glycoprotein and mucin.

Also, the mRNA levels of gut barrier-related genes Claudin-1, Occludin, and ZO-1 in colonic tissues were examined, only Claudin-1 was down-regulated in the MPTP group and reversed by EA treatment ($P < 0.05$, vs. MPTP group) (Fig. 4D). The results indicated that no significant changes were observed in the MPTP group and MPTP + EA group for Occludin and ZO-1 (Fig. 3S). Additionally, we examined the relative mRNA expression levels of TH, BDNF, DAT, and GDNF in colon tissues using RT-qPCR. We found that the transcriptional expressions of these factors were down-regulated in the colon tissues of PD mice ($P < 0.01$, vs. Ctrl group) (Fig. 4E). In contrast, EA treatment reversed the down-regulation of the above genes ($P < 0.05$, vs. MPTP group). Finally, EA repressed the mRNA levels of *Bax* and *Caspase-3* in colon tissues ($P < 0.05$ or 0.01 , vs. Ctrl group) (Fig. 4F). Though MPTP stimulation decreased the mRNA level of *Bcl-2*, there was no statistical difference. And the *Bcl-2* mRNA expression tended to be up-regulated by EA (Fig. 4F).

Then, we examined the modulatory effect of EA on intestinal inflammation. The result showed that the mRNA expressions of pro-inflammatory factors (*Caspase-1*, *TNF- α* , *IL-6*, and *IL-18*) in the colon were significantly up-regulated ($P < 0.01$, vs. Ctrl group), and the mRNA expressions of anti-inflammatory factors (*IL-10* and *TGF- β*) were down-regulated ($P < 0.01$, vs. Ctrl group) in the MPTP group. However, the trends of these genes were reversed by EA ($P < 0.05$ or 0.01 , vs. MPTP group) (Fig. 5A). Further, EA inhibited the protein levels of NLRP3, p20, and ASC ($P < 0.05$ or 0.01 , vs. MPTP group) (Fig. 5B). Meanwhile, EA significantly reduced the serum level of IL-1 β ($P < 0.05$, vs. MPTP group) (Fig. 5C). These data suggest that EA treatment repaired intestinal barrier damage by preventing apoptosis and inhibiting NLRP3 inflammasome activation in the colon tissues.

3.5. Gut microbiota was required for EA-mediated alleviation of motor dysfunction in MPTP-induced PD mice

Given the close correlation between gut microbiota and the pathogenesis of PD, we next tested whether EA exerted its therapeutic effects by modulating the gut microbiota. As depicted in Fig. 6A, the mice were pretreated with an antibiotic (Ab) mixture for twelve weeks to remove intestinal microbes before EA treatment. qPCR data confirmed that most gut microorganisms were cleared by antibiotic treatment (Supplementary Fig. 2). Compared to the MPTP group, the MPTP + Ab group showed an increase in body weight ($P < 0.05$). There was no significant difference in body weight change between the MPTP + Ab + EA and MPTP + Ab groups (Fig. 6B).

Further, we conducted an open-field test to analyze EA therapeutics in antibiotic-treated mice. The result showed that Ab treatment partly increased the range of motion in open space, total distance, center distance, moving duration, center duration, zone crossing, and circle exploring while reducing the resting duration in PD mice (no statistical significance) (Fig. 6C–K). However, these indices were not further improved after EA treatment (Fig. 6C–K). These results proved that the protective effect of EA against locomotor

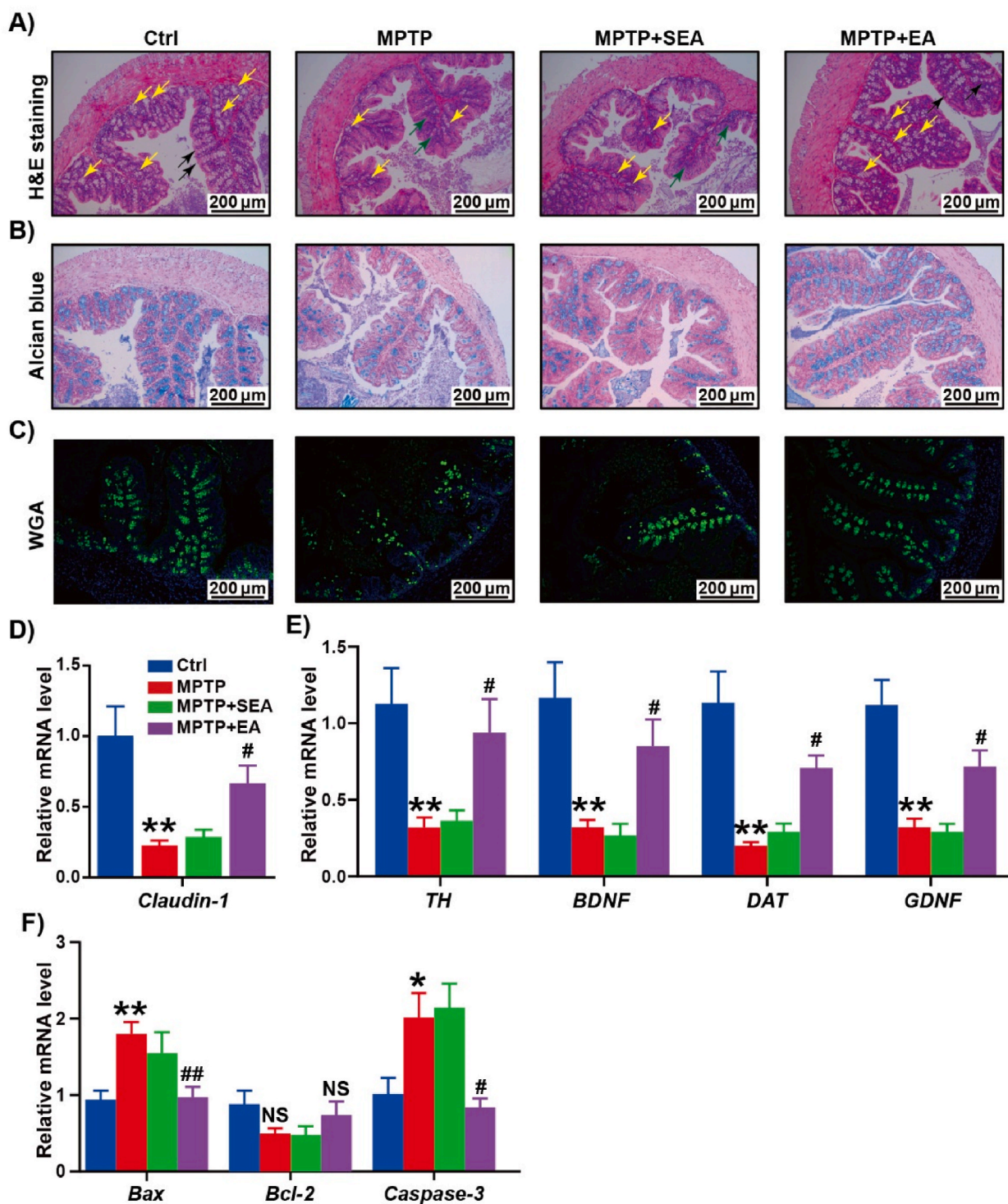
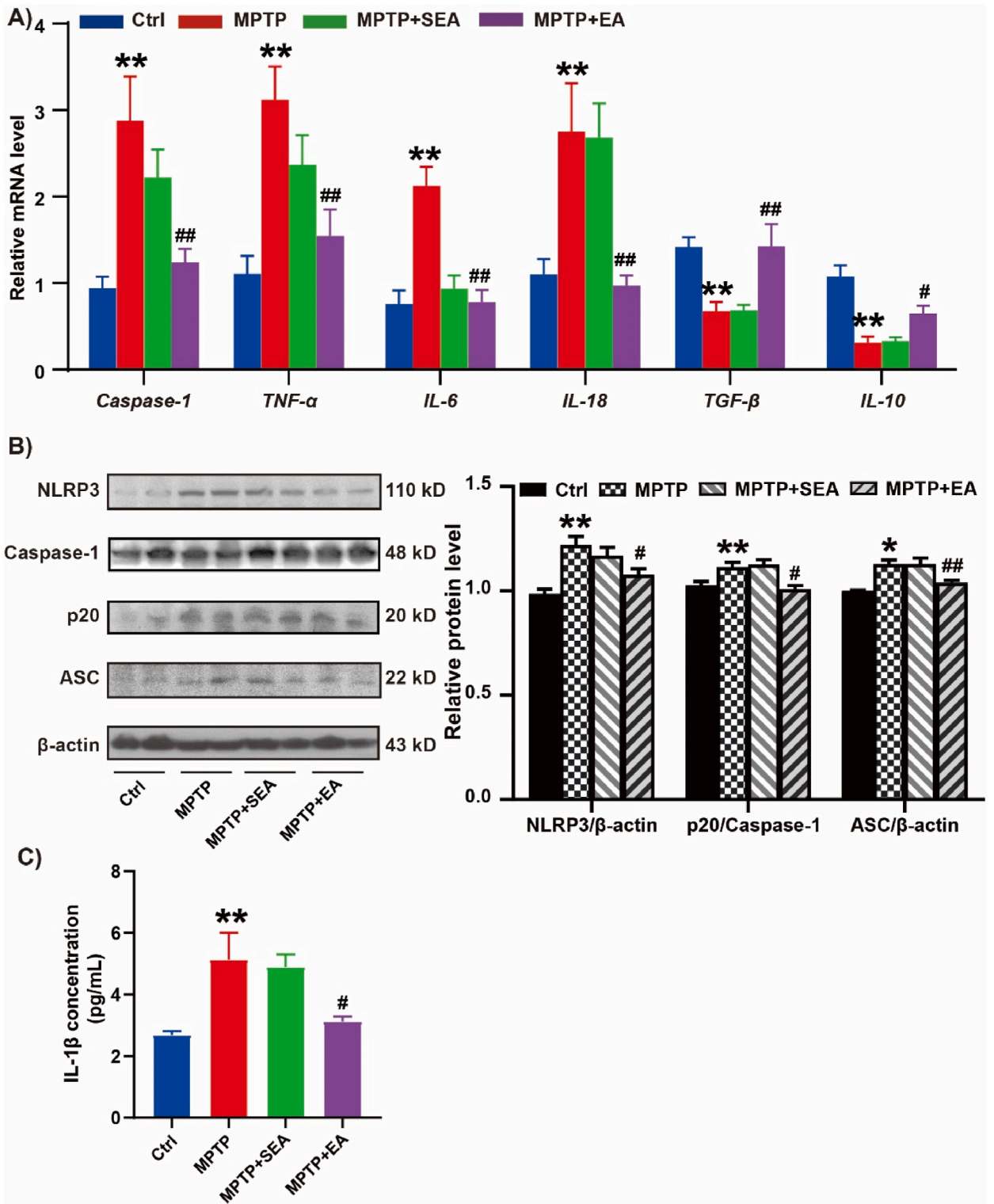


Fig. 4. Inhibitory effect of EA treatment on intestinal barrier damage in MPTP-induced PD mice. (A–C) Morphological analysis of intestinal tissues by H&E staining (A), Alcian blue staining (B), and WGA-FITC staining (C) (view: $\times 100$). (D) mRNA level of Claudin-1 in colon tissues by RT-qPCR (F (3, 28) = 10.64, $P = 0.0001$). (E) The mRNA levels of *TH*, *BDNF*, *DAT*, and *GDNF* in colon tissues by RT-qPCR (*TH*: F (3, 28) = 6.229, $P = 0.0022$. *BDNF*: F (3, 28) = 8.213, $P = 0.0004$. *DAT*: F (3, 26) = 15.4, $P = 0.0001$. *GDNF*: F (3, 27) = 13.36, $P = 0.0001$). (F) The mRNA levels of apoptosis-related genes (*Bax*, *Bcl-2*, and *Caspase-3*) in colon tissues by RT-qPCR (*Bax*: F (3, 22) = 6.903, $P = 0.0019$. *Bcl-2*: F (3, 26) = 1.853, $P = 0.1625$. *Caspase-3*: F (3, 21) = 12.03, $P = 0.0001$). Black arrows indicate crypt structure; yellow arrows indicate goblet cells; green arrows indicate inflammatory infiltration. Data were presented as mean \pm SEM ($n = 8$). * $P < 0.05$, ** $P < 0.01$ vs. Ctrl group. # $P < 0.05$, ## $P < 0.01$ vs. MPTP group. NS, not significant. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)



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Fig. 5. EA treatment attenuated inflammation by inhibiting NLRP3 inflammasome activation in MPTP-induced PD mice. (A) The mRNA expressions of inflammatory cytokines in colon tissues by RT-qPCR (Caspase-1: F (3, 25) = 9.148, $P = 0.0003$. TNF- α : F (3, 20) = 7.844, $P = 0.0012$. IL-6: F (3, 19) = 14.94, $P = 0.0001$. IL-18: F (3, 25) = 8.237, $P = 0.0006$. TGF- β : F (3, 27) = 7.547, $P = 0.0008$. IL-10: F (3, 27) = 20.36, $P = 0.0001$). (B) Protein levels of NLRP3 inflammasome complex by western blotting (NLRP3: F (3, 28) = 9.482, $P = 0.0002$. ASC: F (3, 28) = 8.038, $P = 0.005$. Caspase-1: F (3, 21) = 11.78, $P = 0.0001$). (C) Plasma levels of IL-1 β (F (3, 28) = 6.79, $P = 0.0014$). Data were presented as mean \pm SEM (n = 8). * $P < 0.05$, ** $P < 0.01$ vs. Ctrl group. # $P < 0.05$, ## $P < 0.01$ vs. MPTP group.

dysfunction depended on the presence of intestinal microbes.

3.6. Effect of EA treatment on dopamine neuron degeneration in MPTP-induced PD mice with gut microbiota depletion

H&E staining indicates that the MPTP + Ab group had an increased number of normal neurons with enlarged cytosol and clear borders (Fig. 7A and B). There was no difference between the MPTP + Ab and MPTP + Ab + EA group (Fig. 7A and B). To examine the repairment of neurons in PD mice by EA, we performed an immunohistochemical analysis of the midbrain SN and striatum. As shown in Fig. 7C and D, TH expression was reduced in both tissues of PD mice. A similar result was obtained from the detection of TH protein in the midbrain tissues by Western blotting ($P < 0.01$, vs. Ctrl group), indicating the deficits in dopamine neurons and nerve fibers. Compared to the MPTP group, TH expression in the MPTP + Ab group was upregulated in the midbrain SN and striatum tissues (Fig. 7C–E). There was no difference in TH expression between the MPTP + Ab + EA and MPTP + Ab groups (Fig. 7C–E). The results suggest that EA could not ameliorate dopamine neuron deficiency in intestinal flora-destroyed PD mice.

3.7. EA treatment had no protective effect on intestinal injury in MPTP-induced PD mice with gut microbiota depletion

Our studies have demonstrated that EA intervention ameliorated PD-associated intestinal inflammation and epithelial barrier injury (Fig. 4A–C and Fig. 5A and B). Thus, we performed histopathological analysis to examine the effect of EA on intestinal barrier integrity in antibiotic mixture-treated PD mice. Compared with the MPTP group, Ab and Ab + EA treatment failed to improve the number of cuprocytes and colonic villi deficiency (Fig. 8A). As shown in Fig. 8B and C, our data suggested that EA failed to ameliorate the reduction of glycoproteins and mucins in the colon of PD mice with depleted intestinal flora.

Next, we analyzed the mRNA levels of *TH*, *BDNF*, and *GDNF* in colon tissues among experimental groups by RT-qPCR. The *TH* mRNA level in the MPTP + Ab group was upregulated compared to the MPTP group, while no significant alterations were observed for the mRNA levels of *BDNF* and *GDNF* (Fig. 8D–F). There were no significant differences in the mRNA expressions of the three factors between the MPTP + Ab + EA and MPTP + Ab groups (Fig. 8 D–F).

Finally, we investigated the activation of NLRP3 inflammasome. As depicted in Fig. 8E and F, the protein levels of NLRP3 and Cleaved-Caspase-1 (p20) were decreased in the MPTP + Ab group compared to the MPTP group ($P < 0.01$). However, there was no significant difference in the levels of both proteins in the MPTP + Ab + EA group compared to the MPTP + Ab group (Fig. 8 G–H). These results indicated that EA could not improve colon tissue damage in PD mice with depleted intestinal flora.

4. Discussion

Currently, acupuncture has received widespread attention due to its remarkable efficacy in movement disorders, migraines, and chronic pain [36–38]. Modern medical treatment has further developed acupuncture into EA therapy. Studies show that EA has a therapeutic effect on neurodegenerative diseases [39,40]. Clinical studies have shown that EA could improve motor and non-motor dysfunction in PD patients [41]. However, the effect of EA stimulation on GV16, LR3, and ST36 acupoints to PD has not been reported. Therefore, we selected GV16, LR3, and ST36 as acupuncture points to verify the therapeutic effect of EA intervention on MPTP-induced PD mice in this study.

The occurrence of PD correlates with the patient's body mass index. Studies reported that PD patients with decreased body mass index had more severe autonomic dysfunction and more rapid progression of motor deficits than those with normal, stable, or increased body mass index [42,43]. In this study, we found that MPTP-induced PD mice showed a decreased body weight (Fig. 2A). Meanwhile, the body weight of PD mice increased after EA treatment. Besides, clinical studies have confirmed that WBC, RBC, HGB, MCHC, and LYM in the blood were down-regulated in PD patients [44]. In addition, lower Lym count is related to an increased risk of PD [45]. According to the blood routine test, we found reduced counts of WBC, RBC, HGB, MCHC, and LYM in the MPTP group, which were elevated by EA (Fig. 2D–H). These data indicated a possible dysregulation of the neuro-immune-inflammatory network in MPTP-induced PD mice. In comparison, EA intervention might block weight loss and blood routine dyscrasias in PD mice by modulating the neuro-immune-inflammatory network.

PD is triggered by the degeneration of dopaminergic neurons in the midbrain SN and striatum, and overexpression of α -syn in the midbrain impaired dopamine release and induced nigrostriatal neuronal cell death [46]. Therefore, the pathological accumulation of α -syn in the midbrain is considered a typical pathological feature of PD. In addition, PD occurs with decreased expression of TH, a key enzyme responsible for dopamine synthesis. At the same time, dopamine levels in the nigrostriatal pathway were reduced, leading to motor dysfunction [47]. In this study, we found that EA stimulation restored the motor function of PD mice (Fig. 1C–K). Besides, EA treatment had a protective effect on midbrain TH expression and suppressed the protein levels of α -syn (Fig. 3E and F). Additionally, EA inhibited neuronal death caused by MPTP in the SN and striatum tissues (Fig. 2I and J). Therefore, it was suggested that EA might

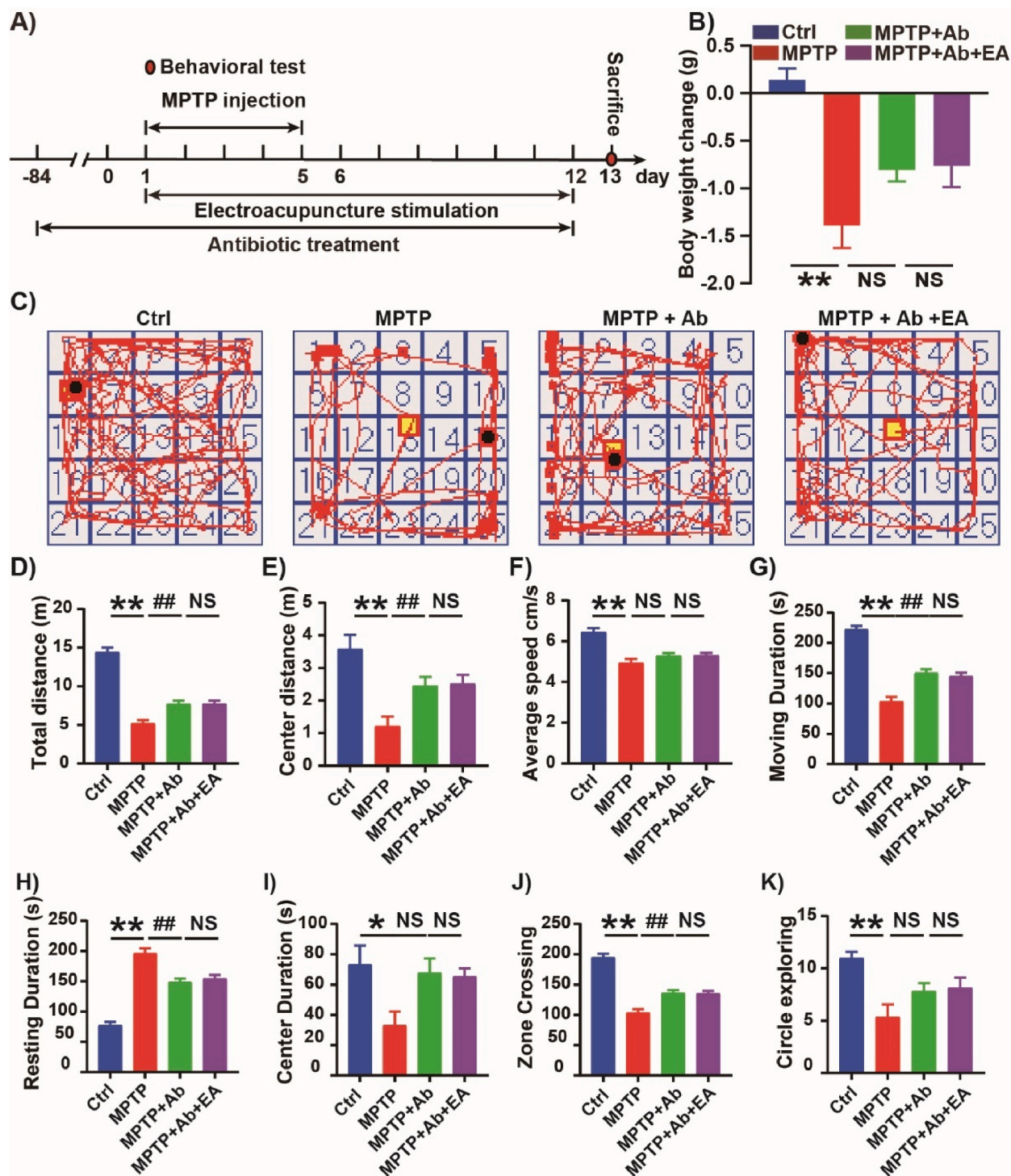


Fig. 6. Gut microbiota was required for EA-mediated alleviation of motor dysfunction in MPTP-induced PD mice. (A) Experimental procedure for antibiotic treatment. (B) Body weight change ($F(1, 28) = 20.49, P = 0.0001$). (C) Track of mice in 5 min. (D) Total distance ($F(1, 28) = 71.07, P = 0.0001$). (E) Center distance ($F(1, 28) = 8.554, P = 0.0068$). (F) Average speed ($F(1, 26) = 23.92, P = 0.0001$). (G) Moving duration ($F(1, 28) = 60.11, P = 0.0001$). (H) Resting duration ($F(1, 28) = 60.11, P = 0.0001$). (I) Center duration ($F(1, 26) = 4.989, P = 0.0343$). (J) Zone crossing ($F(1, 28) = 58.73, P = 0.0001$). (K) Circle exploring ($F(1, 28) = 8.766, P = 0.0062$). Data were presented as mean \pm SEM ($n = 8$). * $P < 0.05$, ** $P < 0.01$ vs. Ctrl group. # $P < 0.05$, ## $P < 0.01$ vs. MPTP group. NS, not significant.

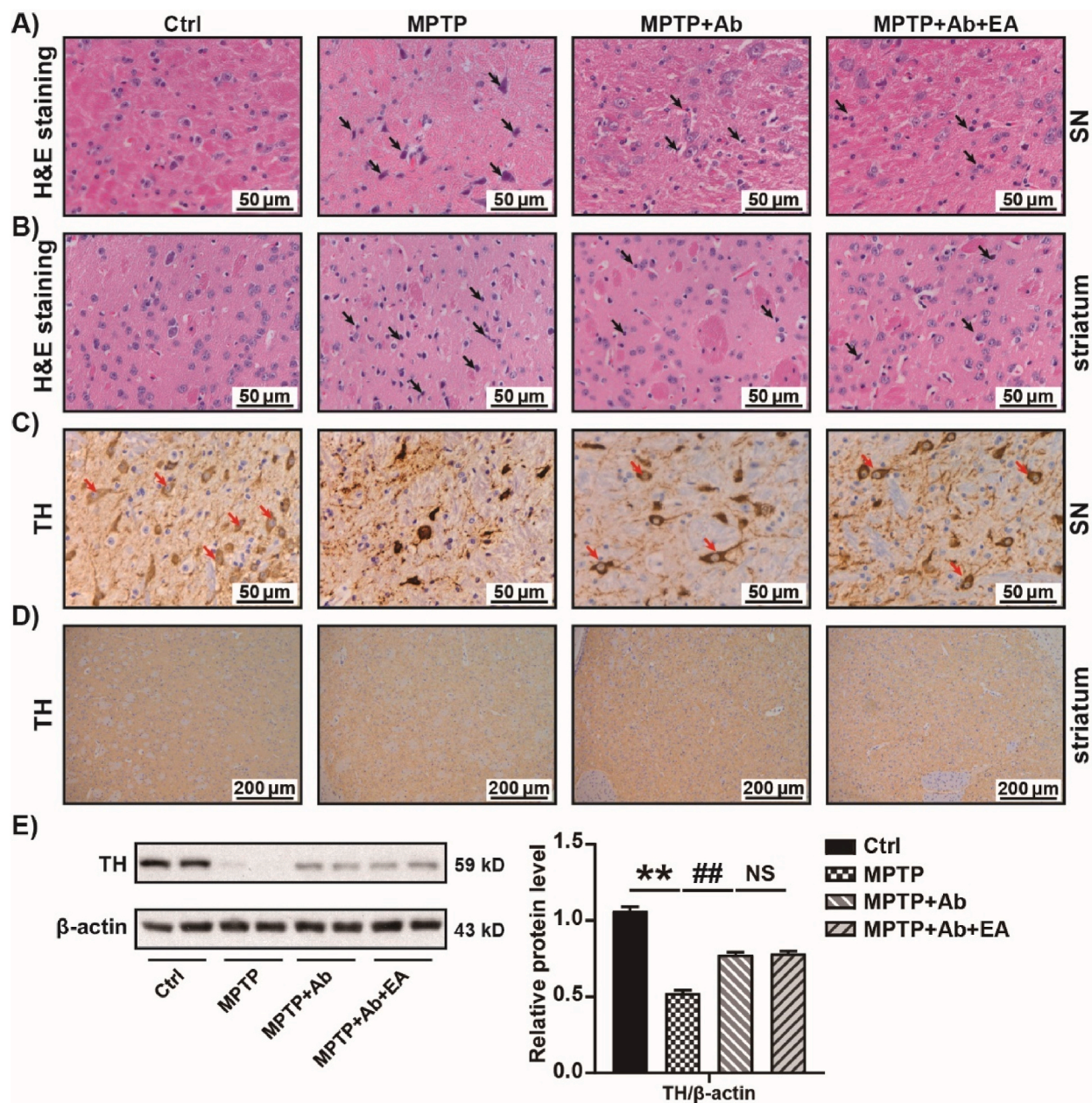
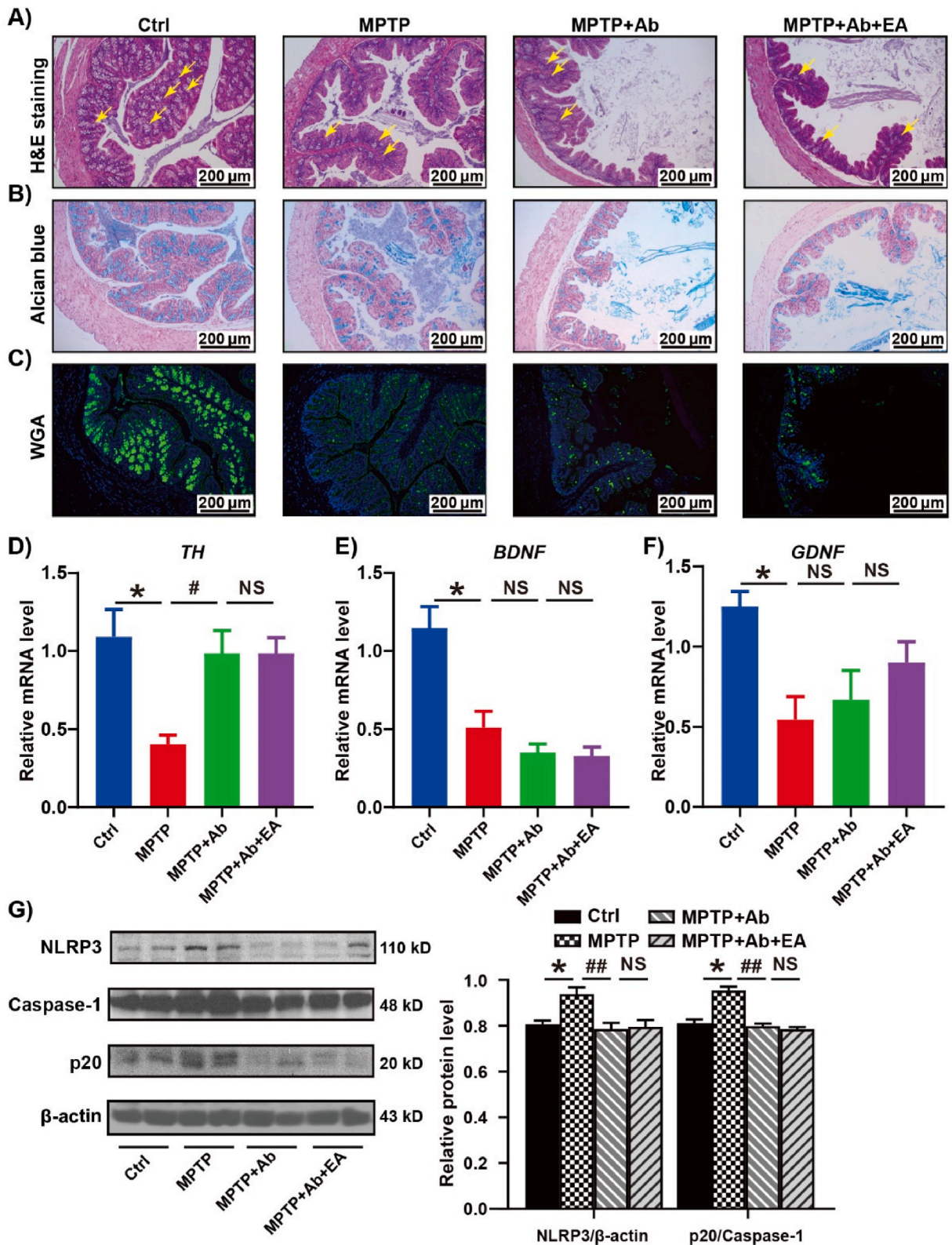


Fig. 7. Absence of gut microbiota counteracted protective effect of EA treatment on MPTP-induced dopamine neuron degeneration in MPTP-induced PD mice. (A–B) H&E staining of SN (A) and striatum (B) tissues (view: $\times 400$). (C) Immunochemical staining of TH in SN (view: $\times 400$). (D) Immunochemical staining of TH in striatum tissues (view: $\times 100$). (E) Protein levels of TH in midbrain tissues by western blotting (F (1, 28) = 190.2, $P = 0.0001$). Black arrows indicate degenerated neurons. Red arrows indicate dopamine neurons. Data were presented as mean \pm SEM ($n = 8$). ** $P < 0.01$ vs. Ctrl group. ## $P < 0.01$ vs. MPTP group. NS, not significant. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

intercept the toxicity of MPTP, and improve motor dysfunction of PD mice by increasing midbrain TH levels, inhibiting disaggregation of α -syn, and protecting dopamine neurons.

Depression, one of the common comorbidities of PD, severely affects the quality of life in 40–50 % of PD patients [48]. In Fig. 1C–K, the PD mice showed depression-like manifestations. BDNF, a member of the neurotrophic factor family, plays a crucial role in neuronal survival, synaptic plasticity, and depression [49]. Additionally, GDNF is closely connected to mood regulation and cognitive function. BDNF and GDNF levels elevated by amitriptyline could promote the survival of dopamine neurons and alleviate depression symptoms in PD [50]. In addition, researchers have also observed that the BDNF and GDNF expressions were reduced in the hippocampus and prefrontal cortex of PD animals [51]. Indeed, we observed that BDNF and GDNF were reduced in the midbrain of PD mice, an effect



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Fig. 8. EA treatment failed to suppress intestinal injury in MPTP-induced mice with gut microbiota depletion. (A–C) Histopathological assay of intestinal tissues by H&E staining (A), Alcian blue staining (B), and WGA-FITC staining (C) (view: $\times 100$). (D–F) mRNA expressions of *TH* (D), *BDNF* (E), and *GDNF* (F) in colon tissues by RT-qPCR. D) *TH* (F (1, 23) = 6.286, $P = 0.0197$). E) *BDNF* (F (1, 25) = 4.709, $P = 0.0397$). F) *GDNF* (F (1, 21) = 10.87, $P = 0.0034$). (G) Protein levels of NLRP3 and Caspase-1 in colon tissues by western blotting (NLRP3: F (1, 28) = 44.92, $P = 0.0001$. Caspase-1: F (1, 28) = 256.1, $P = 0.0001$). Yellow arrows indicate goblet cells. Data were presented as mean \pm SEM ($n = 8$). * $P < 0.05$ vs. Ctrl group. # $P < 0.05$ vs. MPTP group. NS, not significant. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

that was obstructed by EA (Fig. 3D and E). Furthermore, EA treatment could protect the viability and number of neuronal cells (Fig. 2I and J). Also, EA therapy protects against depressive-like behaviors (Fig. 1C–K). Hence, it was implied that EA could interdict the behavioral defects of PD mice by promoting the expressions of brain BDNF and GDNF and neuronal survival.

Another significant complication of PD is intestinal dysfunction, which can start more than twenty years before the onset of motor symptoms. The activation of the NLRP3 inflammasome induces an inflammatory response and disrupts the integrity of intestinal tight junction proteins, resulting in the disruption of the intestinal barrier [52,53]. In the present study, PD mice showed significant intestinal mucin disruption in the colon. Que et al. showed that NLRP3 inflammasome was significantly activated in PD mice [54]; we found the same results, which were reversed by EA treatment (Fig. 5A and B). Our data suggest that EA might block intestinal inflammation and intestinal barrier damage by inhibiting the activation of colonic NLRP3 inflammasome. Nevertheless, the mechanism underlying the effect of EA-initiated NLRP3 inflammasome inhibition remains to be discovered.

Dopamine contributes to gastrointestinal motility in the enteric nervous system. Thus, intestinal dopamine damage may lead to gastrointestinal dysfunction [55]. GDNF protects against MPTP-induced damage to the dopaminergic nervous system by inhibiting intestinal epithelial cell apoptosis, reducing intestinal permeability, inhibiting mucosal inflammation, and promoting intestinal barrier repair [56]. In addition, GDNF could promote the expression of Bcl-2, reduce the expression of Bax, and inhibit the activity of Caspase-3 [57]. In this study, we found that the transcription of GDNF was down-regulated in the colonic tissues of PD mice, which was increased by EA (Fig. 4D). Meanwhile, EA remarkably reduced the mRNA levels of Bax and Caspase-3 (Fig. 4F). Further, the levels of colonic glycoprotein and mucin were restored after EA treatment (Fig. 4A–C). These results suggest that EA could promote intestinal barrier repair by promoting GDNF expression and inhibiting apoptosis in PD mice.

Intestinal inflammation in PD is associated with changes in intestinal permeability and microbiota composition [58]. Lai reported that the intraperitoneal injection of MPTP could induce dysbiosis in mice [59]. At the same time, probiotics alleviated the onset and progression of PD by regulating the structure and quantity of gut microbiota [60]. Han demonstrated that acupuncture stimulation could alter gut microbiota [61]. The alterations of gut microbiota and SCFAs induced by vancomycin pretreatment might improve motor dysfunction, decrease dopamine metabolism, and relieve inflammation in PD mice's brains and gut [62].

Other studies have shown that MPTP did not decrease DAT or TH immunoreactivity in the brains of mice treated with an antibiotic cocktail. Antibiotic treatment significantly decreased the diversity and altered the composition of the host gut microbiota at the genus and species levels. Treatment with an antibiotic cocktail significantly improved MPTP-induced behavior [63,64]. It has also been demonstrated that gut microbiota disorders could promote PD progression. Increased intestinal bacteria, such as *Lachnospira* and *Clostridia*, contribute to weight loss and worsen PD symptoms [65]. Given the close association between PD and gut microbiota dysbiosis [66–68], it is possible to explain the improvement of PD symptoms in mice after microbiota removal by antibiotics. Previous studies have also reported that clearing the intestinal microbiota could alleviate symptoms and pathological damage in PD mice [69]. Compared with the MPTP group, the MPTP + Ab group and the MPTP + Ab + EA group failed to reverse motor dysfunction significantly (Fig. 6B). In contrast, the EA + MPTP group did, suggesting that the block effect of EA on motor dysfunction is related to the gut microbiota.

Cui demonstrated that vancomycin pretreatment ameliorated motor dysfunction in MPTP-induced PD mice [70]. Another study found that the aggregation of alpha-syn and motor deficits was improved in mice with gut microbiota destruction by antibiotics [71]. Therefore, we believe that only a milder PD model can be established in antibiotic-treated mice (Figs. 6 and 7). In this study, we found that EA treatment failed to reverse the protein of NLRP3 and Cleaved-caspase-1 expression in MPTP-induced mice with gut microbiota depletion (Fig. 8 G). However, EA significantly down-regulated the NLRP3 and Cleaved-caspase-1 expression levels (Fig. 5 B). Only EA could improve the motor dysfunction of PD mice (Figs. 2 and 7). The results show that EA failed to secure dopaminergic neurons, motor dysfunction, and intestinal inflammatory response in antibiotics-treated PD mice (Figs. 6–8). It suggests that EA may block motor dysfunction and gut barrier integrity by modulating intestinal NLRP3 inflammasome and gut microbiota in MPTP-induced Parkinson's disease mice.

In this study, MPTP was administered intraperitoneally to mice, while antibiotics were administered orally, so antibiotics had no direct effect on the metabolism of MPTP. In addition, we used multiple antibiotics (ampicillin, vancomycin, neomycin sulfate, and metronidazole) with little or no oral bioavailability to clear intestinal bacteria. Previous studies have shown that the antibiotics used in this study can hardly be absorbed by the intestine and did not enter the brain [72,73], which means that the antibiotics we choose will not affect the metabolism of MPTP or the uptake of MPP⁺ in nerve cells. Indeed, a future study using vagotomy to study the effects of acupuncture in this Parkinson's model is of great interest.

5. Conclusions

In summary, this study demonstrated that EA blocked the effect of MPTP in PD mice. The mechanisms of EA in ameliorating PD include: (1) inhibiting NLRP3 inflammasome activation, (2) increasing neurotrophic factor content, and (3) regulating the brain-gut

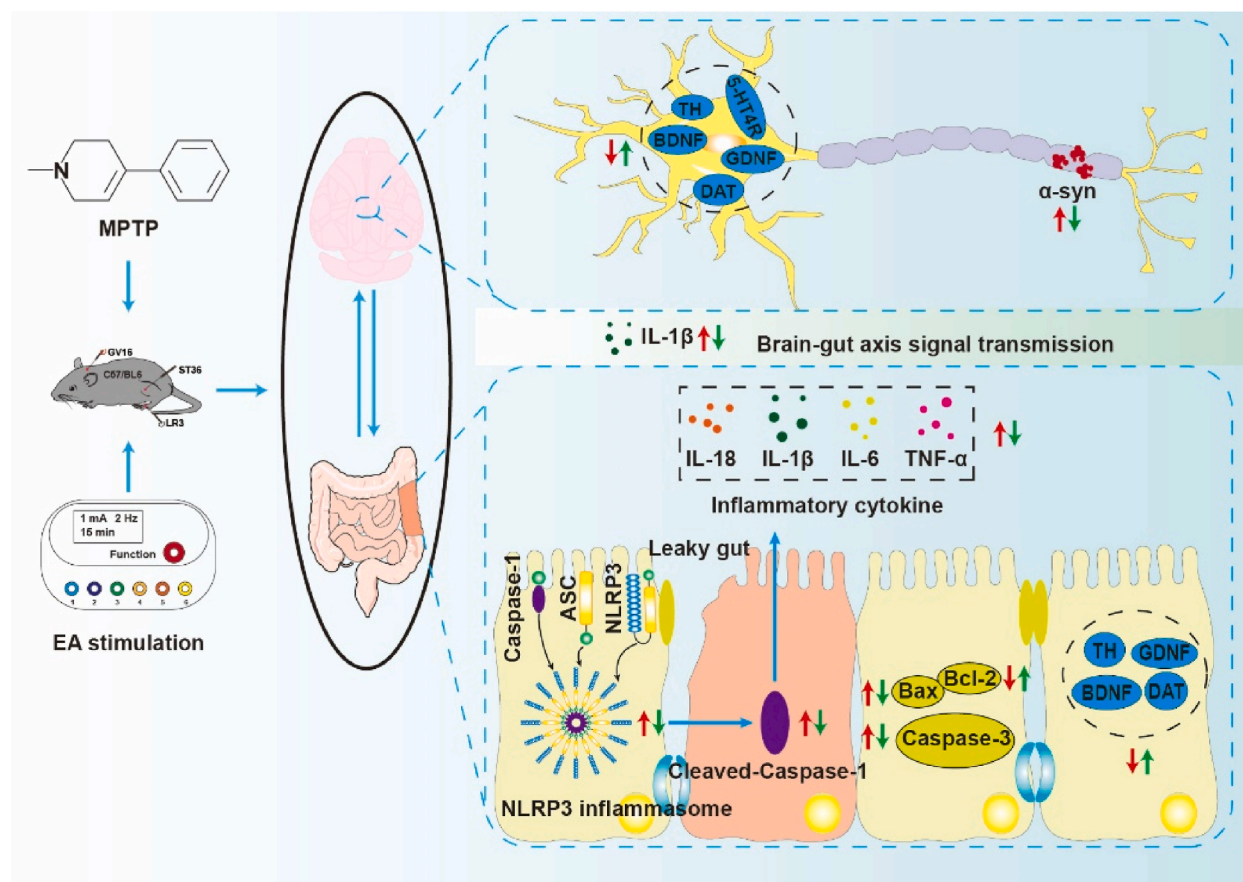


Fig. 9. Schematic diagram showing how EA exerted a protective effect on MPTP-induced PD. Firstly, EA intervention reduced α -syn misfolding in midbrain tissues and increased TH and DAT expression in midbrain and colon tissues. Secondly, EA inhibited apoptosis in colonic tissues. Thirdly, EA reduced the expressions of inflammatory cytokines (IL-1 β , IL-18, IL-6, and TNF- α) in the colon by inhibiting the activation of the NLRP3 inflammasome. Fourthly, EA treatment increased brain-gut axis signaling by increasing the expression levels of neurotrophic factors, such as BDNF and GDNF, in the colon and midbrain tissues. Red arrows indicate changes in the MPTP group compared to the control group. Green arrows indicate changes in the MPTP + EA group compared to the MPTP group. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

axis (Fig. 9). Collectively, our study revealed a new mechanism of EA for the treatment of PD and indicated that EA treatment might be an effective therapy for patients with PD.

Of course, this study also has some limitations. Firstly, a control/AB or EA should be designed as an only control group. Secondly, it is necessary to conduct more in-depth research at the animal and cellular level to determine whether antibiotics have an impact on MPTP metabolism. We plan to conduct further studies to examine the effect of antibiotics on MPTP metabolism. Thirdly, it is recommended to perform fecal bacterial transplantation experiments to explore and verify the impact of EA on the gut flora in MPTP-induced PD mice.

Data availability statement

Data supporting the findings of this study are available within the paper and the supplementary information files. Datasets generated and analyzed in the study are available from the corresponding author upon reasonable request.

CRedit authorship contribution statement

Lei Guo: Writing – review & editing, Writing – original draft, Software, Investigation, Data curation. **Haiming Hu:** Data curation, Methodology, Supervision, Writing – original draft, Writing – review & editing. **Nan Jiang:** Data curation. **Huabing Yang:** Data curation. **Xiongjie Sun:** Data curation. **Hui Xia:** Data curation. **Jun Ma:** Project administration, Methodology, Funding acquisition, Data curation, Conceptualization. **Hongtao Liu:** Writing – review & editing, Resources, Project administration, Funding acquisition, Data curation, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.heliyon.2024.e30819>.

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