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ORIGINAL RESEARCH

Electroacupuncture Alleviates Neuropathic Pain and Negative Emotion in Mice by Regulating Gut Microbiota

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Background: Neuropathic pain (NP) is a prevalent chronic condition frequently accompanied by adverse emotional states. Previous research has demonstrated the clinical efficacy of electroacupuncture (EA) in mitigating neuropathic pain and its associated mood disorders. Recent studies have underscored a correlation between gut microbiota and both NP and negative emotional states. Nevertheless, the relationship between the modulation of gut microbiota by EA and the amelioration of NP remains inadequately understood.

Methods: Mice were randomly assigned to one of the three groups: the Control (Con) group, the EA group, and the Chronic Constrictive Injury (CCI) group (n = 12 each). Starting from the 8th day post-CCI induction, the EA group underwent EA treatment once every two days, for a total of 20 sessions. To investigate the impact of gut microbiota on CCI mice, we employed a variety of methods, including various behavioral tests and 16S ribosomal DNA (rDNA) sequencing.

Results: The results indicated that EA significantly ameliorated mechanical allodynia and emotional dysfunction induced by CCI in mice. Analysis through 16S rDNA sequencing revealed that the gut microbiota of NP model mice exhibited a marked increase in diversity. However, EA could partially reverse changes in the diversity of gut microbiota. The abundance of *Alloprevotella, A2, Roseburia, Muribaculum, Ruminiclostridium*, and *Rikenella* was increased, and the abundance levels of *Bacteroides* were decreased at the *genus* level in CCI mice. Following EA treatment, the relative abundance of *Alistipes, A2, Roseburia*, and *Rikenella* was decreased, whereas the relative abundance of *Alloprevotella* and *Parabacteroides* was increased in EA group when compared with the CCI group.

Conclusion: These findings suggested that EA exerted a significant therapeutic effect on NP, potentially through modulation of the gut microbiota.

Keywords: electroacupuncture, neuropathic pain, negative emotion, gut microbiota

Introduction

Pain is characterized as a distressing sensory and emotional phenomenon often resulting from actual or potential damage to tissue.[1] Neuropathic pain (NP) is a prevalent chronic pain condition, with a prevalence ranging from 3.2% to 17.9% in the general population.[2] NP has been frequently observed in patients with chronic low back pain and is also associated with various inflammatory diseases, including ankylosing spondylitis, rheumatoid arthritis, and osteoarthritis.[3] NP commonly co-occurs with negative emotional states, such as anxiety and depression, with comorbidity rates of depression and NP ranging from 18% to 85%.[4] Anxiety, depression, and other mood disorders are present in approximately 34% of NP patients, significantly impacting their quality of life.[5,6] Negative emotions have the potential to worsen NP.[4]

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The etiology of NP and its related negative emotions is complex and not fully understood. Managing NP continues to pose challenges due to the limited efficacy of pharmacological treatments and the scarcity of novel therapeutic interventions. Additionally, current medications are associated with various adverse effects, including addiction, gastrointestinal ulcers, and bleeding.[7–9] Antidepressants and anticonvulsants are commonly prescribed as initial pharmacological treatments for negative emotions associated with NP, despite their limited efficacy and tolerability.[10] Consequently, there is a pressing need for the exploration and development of novel therapeutic interventions that can effectively address pain and its concomitant mood disturbances in NP patients.

Acupuncture is a widely utilized alternative therapy for the management of diverse pain disorders, including NP.[11,12] A comprehensive review of over 100,000 patients and approximately one million treatments demonstrated that acupuncture is a well-tolerated treatment option for pain management, with fewer adverse effects compared to conventional medical interventions.[13] Electroacupuncture (EA), a contemporary advancement in acupuncture therapy, integrates mechanical and electrical stimulation. Existing studies have indicated that EA is not only able to relieve hyperalgesia and allodynia but also to improve negative emotional states associated with NP.[14,15] However, the mechanisms of EA in treating pain and accompanying negative emotions require further exploration.

The gut microbiota is a complex microbial community that plays an important role in the maintenance of the central nervous system, behavior, host immunity, and metabolism.[16] Recent studies have suggested that gut microbiota are involved in the onset and development of widespread chronic pain.[17,18] A study has shown that the diversity and composition of the gut microbiome were altered in NP model of mice, and probiotic treatment could inhibit nerve injury-induced TNF-α expression in the spinal cord and pain sensitization.[19] Meanwhile, changes in gut microbiota are associated with a broad variety of psychiatric diseases, such as anxiety and depression, cognitive impairment.[20–22] Furthermore, a previous study has proven that transplantation of fecal microbiota from anhedonia-susceptible rats with NP into antibiotics-treated pseudo-germ-free mice significantly exaggerated pain and depression-like phenotypes, suggesting that gut microbiota plays a key role in the pain as well as negative emotions of NP.[23] Several studies have demonstrated that the therapeutic effects of EA were significantly correlated with the regulation of intestinal microflora.[24,25] Therefore, in the present study, we investigated whether the mechanism by which EA ameliorated the pain and negative emotions was associated with the modulation of gut microbiota in chronic constriction injury (CCI) mice.

Materials and Methods

Animals

Adult male C57/BL6 mice weighing about 20–23 g were obtained from Shanghai Slack Laboratory Animal Co., Ltd. (Shanghai, China). All mice were housed in a room with a temperature of 22–24°C and a 12 h light/dark cycle, with access to food and water ad libitum. All mice were allowed to acclimatize to their new environment and were handled daily for one week before initiating the experimental procedures. All experiments followed the National Institutes of Health Guidelines for the Care and Use of Laboratory Animals. Mice were randomly divided into the 3 following groups (n = 12 each): control group (Con), CCI group, and EA group (CCI+EA stimulation).

CCI Model

The CCI model used in this experiment was conducted as previously described.[26] Briefly, the mice were anaesthetized by 2% sodium pentobarbital (30 mg/kg, i.p.) and placed in a prone position. The left sciatic nerve at the mid-thigh level of each mouse was carefully exposed and tied with three knots loosely. The distance between ligatures was approximately 1 mm, and all ligatures were of the same tightness. Finally, the nerve was returned to its original location after ligation, and the muscle and skin layers were sutured. The control group underwent the same procedure without being ligated. A workflow diagram of the experimental design was presented in Figure 1A.

EA Treatment

Three days before EA, the mice were fixed with a homemade fixing device for acclimatization. All groups of mice were subjected to the same restraint as the EA group to rule out the specific effects of non-acupuncture manipulation. The EA

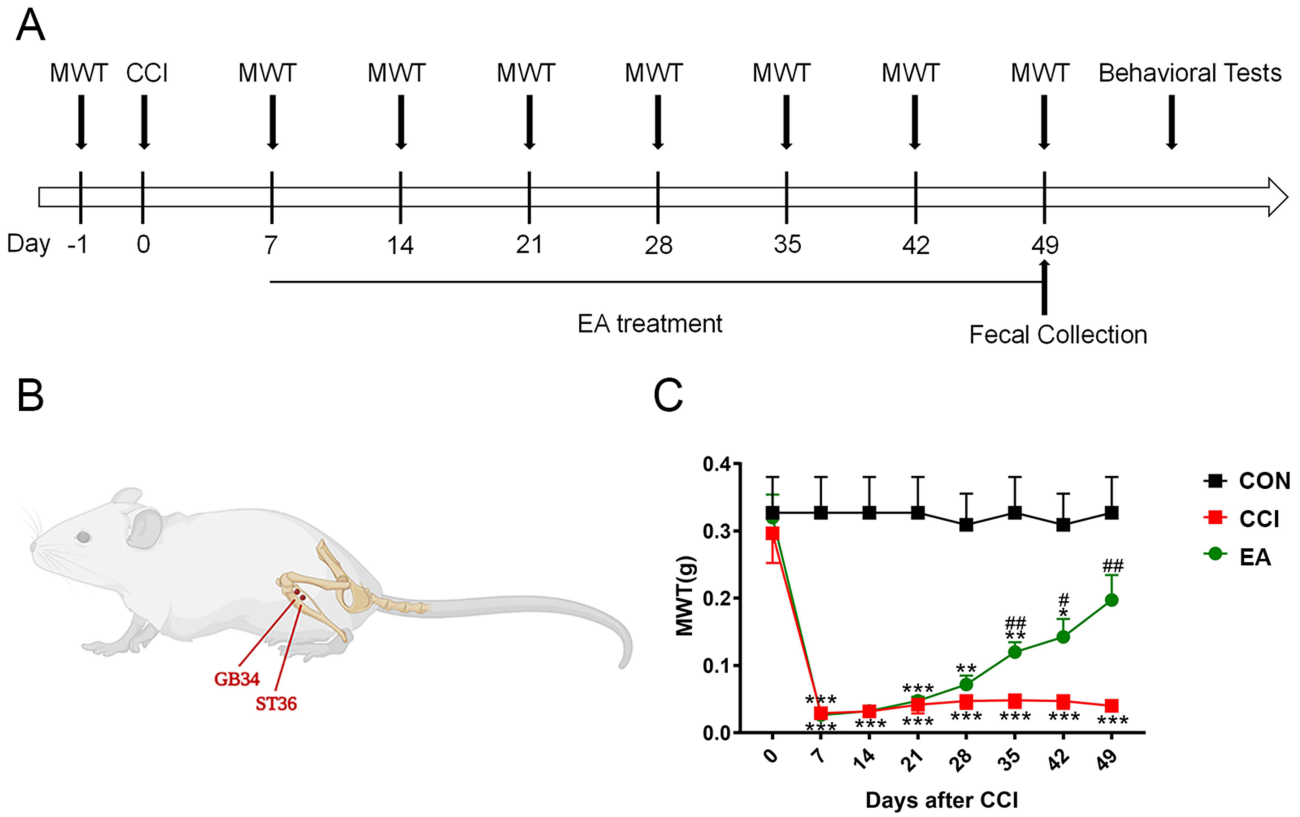

Figure 1 The effects of electroacupuncture (EA) on mechanical allodynia induced by chronic constrictive injury (CCI) in mice. (**A**) Schematic of the experimental procedure. (**B**) Schematics showing the ST-36 and GB-34 acupoints on mouse. (**C**) Evaluation of mechanical withdrawal thresholds (MWT). Data are presented as mean ± SEM (n=11-12 per group at each time point).
Note: *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ vs the control group; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ vs the CCI group.

treatment, which did not involve the use of any anesthetic agents, was administered while the mice were in a conscious state. In this study, we performed two specific acupoints: Zusanli (ST-36), situated approximately 4 mm from the knee joint, 2 mm laterally adjacent to the anterior tibial tubercle, and in close proximity to the common peroneal and tibial branches of the sciatic nerve, and Yanglingquan (GB-34), positioned in proximity to the knee joint, specifically anterior and inferior to the head of the fibula, within the region of the peroneus longus and brevis muscles. The precise locations of these acupoints are delineated in Figure 1B. The acupuncture needle (0.16 mm in diameter, 7 mm in length) was inserted with a depth of 2–3 mm and was linked to a device (Huatuo, Suzhou, China), and the parameter was set as 1.0 mA, 2 hz. Each EA treatment session lasted for 30 minutes and was conducted once every two days starting from the 8th day after CCI, culminating in a total of 20 treatments.

Animal Behavioral Tests

Nociceptive Behavioral Test

Mechanical Withdrawal Threshold (MWT) were evaluated using von Frey filaments.[27] Mice were placed on a metal mesh and von Frey filaments (IITC Life Science, California, USA) were pushed against the plantar surface of the left hind paw for one second with five times. The filament that triggered the mouse to move its hind paw was defined as the mechanical threshold. If a positive response occurred, the next smaller von Frey filament was used; if not, the next higher intensity was used. The threshold force of response was defined as the first filament that evoked at least 3 withdrawals out of 5 applications.

Open Field Test

The open field test (OFT) is used for evolution of the locomotion pattern of anxiety.[28] Mice were placed in the center of the square box, and the exploratory behavior was recorded for 5 min for behavioral analysis.[29] The parameters of the distance and residence time of the mice in the central area and the total distance of the entire open field were recorded.

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O-Maze Test

The O-maze test is used to assess unconditioned anxiety like-behaviors.[30] The O-maze consisted of two open arms and two closed arms alternating in quadrants. During the test, the experimenter places the mice quickly and gently into the central area with their heads facing an open arm and observed for 5 min. The percentage of time spent in the open arms ([time spent in the open arms / (time spent in open arms + time spent in closed arms)] × 100) was calculated for each mouse.

Tail Suspension Test

The tail suspension test (TST) is widely used to test the depression-like behaviors.[31] Briefly, the mice were suspended from the top of the apparatus (25 × 25 × 35 cm) using tape about 1 cm from the tail tip. The duration of immobility was recorded in the last 4 min for a total of 6 min. When mice are passively suspended immobile, they are judged to be stationary.

Forced Swim Test

The forced swim test (FST) is used to evaluate depressive-like behavior.[32] Briefly, mice were placed individually in a clear cylinder, containing 1800 mL of water at 24±1°C. Mice were forced to swim for 6 min, and the immobility time during the last 4 min was manually measured by the observer. Mice were considered immobile when they ceased struggling, remained floating motionless, and only made those movements necessary to keep their head above the water.

Novelty-Suppressed Feeding Test

The novelty-suppressed feeding test (NSFT) is used to assess anxiety- and depression-related behaviors.[33] Mice were food-deprived for 24 hours before testing. Mice were placed in the corner of a square box covered with bedding with a single food pellet placed in the center of the arena. The latency to feed was recorded in 5 min.

Faeces Collection and DNA Extraction

Fecal samples from all groups were collected after the last EA treatment for microbiome analysis and then were frozen at −80°C within three hours after sampling. Total genomic DNA was extracted using DNA Extraction Kit (DNeasy PowerSoil Kit, Cat.12888; QIAGEN, Dusseldorf, Germany) following the manufacturer's instructions. Concentration and purification of DNA were verified with NanoDrop 2000 UV-vis spectrophotometer (Thermo Fisher Scientific, Wilmington, USA), and DNA quality was checked by 1% agarose gel electrophoresis.

Library Construction and Gene Sequencing

The genome DNA was used as template for PCR amplification with the barcoded primers and Tks Gflex DNA Polymerase (R060B; TaKaRa Bio, Beijing, China). The V3-V4 hypervariable regions of the bacteria 16s rRNA gene were amplified with universal primers 343F (5'- TACGGRAGGCAGCAG −3') and 798R (5'- AGGGTATCTAATCCT-3') by thermocycler PCR system (2100; Agilent Bioanalyzer, USA)[34] The PCR reactions were conducted using the following program: 5 min of denaturation at 94°C 26 cycles of 30s at 94°C, 30s for annealing at 56°C, and 20s for elongation at 72°C, and a final extension at 72°C for 4 min (580BR10905; Bio-rad, USA).

Amplicons quality was detected by gel electrophoresis, and then amplicons were purified with AMPure XP beads (Cat. A63880; Agencourt), and amplified for another round of PCR. After a second purification with the AMPure XP beads, the final amplicon was quantified using Qubit dsDNA assay kit (Cat.Q32854; Thermo Fisher Scientific). The purified amplicons were pooled in equimolar concentrations, for subsequent sequencing was performed using an Illumina MiSeq.

Microbiome Analysis

The 16s rDNA raw sequencing data were exported in FASTQ format. Sequencing Paired-end reads were demultiplexed and filtered using Trimmomatic software (version 0.35)[35] to detect and eliminate ambiguous bases (N). Using a sliding window trimming approach, we cut off regions with an average quality score below 20 and removed sequences less than 50 base pairs (bp) in length. After trimming, we used FLASH software (version 1.2.11) to assemble the paired-end reads.

The assembly parameters were set as follows: a minimum overlap of 10 bp, a maximum overlap of 200 bp, and a maximum mismatch rate of 20%. We then employed the split_libraries (version 1.8.0) software in QIIME to remove sequences with bases (N) from the paired-end reads, to eliminate sequences with single base repeats greater than 8, and to discard sequences less than 200 bp in length, thereby obtaining clean tag sequences. Finally, we utilized the UCHIME software (Version 2.4.2) for the removal and clustering of primer sequences, and for the generation of operational taxonomic units (OTUs) at a 97% similarity cutoff. Chimeric sequences were identified and deleted. The representative read of each operational taxonomic unit (OTU) was selected using the QIIME package. All representative reads were annotated and blasted against the Unite database (ITSs rDNA) using blast. In addition, rarefaction was performed on the OTU table to prevent methodological artefacts arising from varying sequencing depth. Alpha-Diversity was measured by species richness from the rarefied OTU table Beta-Diversity was estimated by computing Bray-Curtis and was visualized with principal coordinate analysis.

Statistics

SPSS 24.0 (IBM Corp., Armonk, NY, USA) and GraphPad Prism 8.0 (GraphPad, Inc., La Jolla, CA, USA) were employed for analysis. Behavioral data were presented as mean ± standard error (SEM). Comparison of MWT values between the three groups at multiple time points was performed by two-way repeated-measures analysis of variance (ANOVA) with the Bonferroni post-hoc tests. Differences in other behavioral data between multiple groups were analyzed using one-way ANOVA, and post hoc comparisons were analyzed using Tukey's test. $P < 0.05$ was considered statistically significant.

Results

EA Relieved Mechanical Allodynia Induced by CCI-Induced NP

A two-way repeated-measures ANOVA revealed a significant main effect of treatment [$F_{(2, 32)} = 21.78$, $P < 0.001$] and a significant group × time interaction [$F_{(14, 224)} = 11.11$, $P < 0.001$]. As shown in Figure 1C, no significant difference was observed among the three groups on the baseline before the CCI surgery ($P > 0.05$). After the mice received the CCI surgery, the MWT of their ipsilateral hindpaws decreased significantly from day 7 to day 49 as compared with the CON group values (all $P < 0.001$; Figure 1C). EA treatment significantly increased the MWT from day 35 to day 49 (overall $P < 0.05$, EA versus CCI; Figure 1C). These results indicated that EA ameliorated the mechanical allodynia of NP.

EA Improved Negative Emotion Caused by CCI

In the OFT, the total distance did not differ in all groups [$F_{(2, 32)} = 0.5468$, $P > 0.05$] (Figure 2A). The mice in the CCI group traveled shorter distances [$F_{(2, 32)} = 7.548$, $P < 0.05$], spent shorter time [$F_{(2, 32)} = 7.837$, $P < 0.01$] in the central zone than the CON group (Figure 2B and C). After EA treatment, the mice traveled longer distances ($P < 0.01$), and spent more time ($P < 0.05$) in the central zone than those in the CCI group (Figure 2B and C). In the O-maze test, the mice in the CCI groups also traveled shorter distances [$F(2, 32) = 26.91$, $P < 0.001$], spent shorter time [$F(2, 32) = 6.042$, $P < 0.05$] in the open arms than the mice in the CON group (Figure 2D and E). Compared with the CCI group, mice treated with EA traveled longer distances ($P < 0.001$), and spent more time ($P < 0.05$) in the open arms (Figure 2D and E). In the TST and FST, the immobility time was increased in the CCI group (TST: $F_{(2, 32)} = 7.717$, $P < 0.01$; FST: $F_{(2, 32)} = 19.47$, $P < 0.001$) (Figure 2F and G). EA treatment significantly attenuated an increased immobility time in the CCI mice (TST: $P < 0.05$; FST: $P < 0.001$) (Figure 2F and G). Regarding the NSFT, CCI mice displayed significantly increased latency to feed [$F_{(2, 32)} = 7.174$, $P < 0.05$] (Figure 2H). Compared to the CCI group, this increased latency was significantly attenuated by EA treatment ($P < 0.01$) (Figure 2H). Taken together, these results suggested that EA treatment could attenuate depressive and anxiety-like behaviors induced by NP.

Differential Gut Microbiota Profiling Among the Con, CCI and EA Mice

We then utilized the 16s rDNA sequencing to investigate the alterations of gut microbiota from the three groups. Substantial gut microbiota has been altered among these groups and Figure 3A showed the top 15 dominant microbiota at the genus

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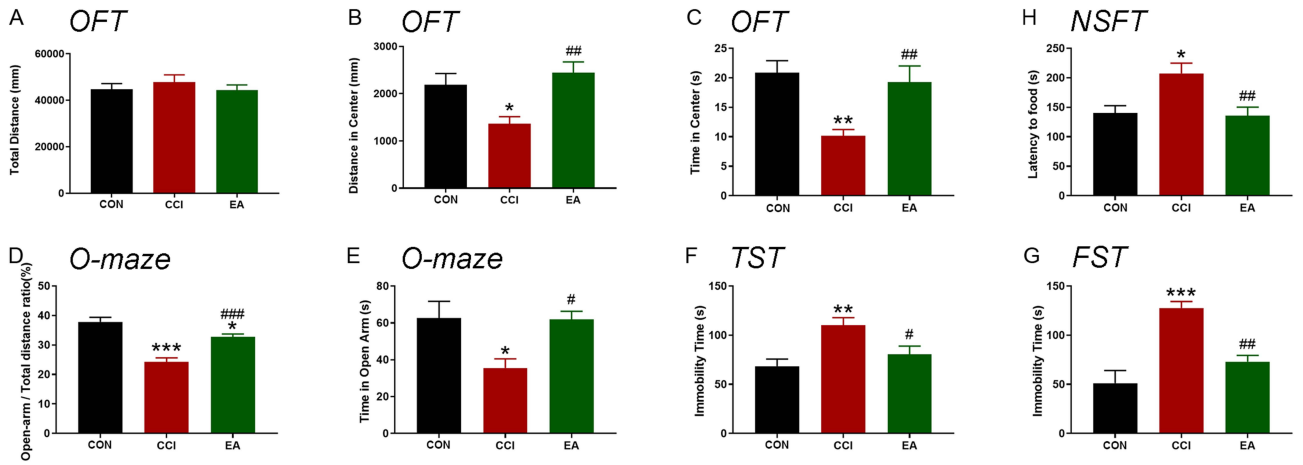

Figure 2 The effects of EA on depressive and anxiety-like behaviors induced by CCI mice. (**A**) total distance in the open field test (OFT); (**B**) Distance in center of the OFT; (**C**) Time spent in center of the OFT; (**D**) Ratio distance of the O-maze test (OMT); (**E**) Time spent in open arm of the OMT; (**F**) Immobility time of the tail suspension test (TST); (**G**) Immobility time of the force swimming test (FST); (**H**) Latency to eat the food of the novelty-suppressed feeding test (NSFT). Data are presented as mean ± SEM (n=11-12 per group at each time point).

Notes: $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ vs the control group; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$ vs the CCI group.

level. The results showed that the α-diversity of gut microbiota was markedly increased, with the increased levels of Chao1 and Shannon index ($P < 0.001$; $P < 0.01$), while EA treatment reversed the Chao1 and Shannon index (all $P< 0.001$, Figure 3B and C). The β-diversity metric, represented by the principal component analysis (PCA) and principal coordinate analysis (PCoA), indicated that gut microbiota was different among the three groups (Figure 3D and E).

Linear discriminate analysis (LDA) coupled with effect size measurements was used to identify relatively higher abundance of each group (Figure 3F). The CON group had a higher abundance of *uncultured_bacterium, Bacteroidia, Bacteroidetes, Bacteroidales, Bacteroidaceae, Bacteroides, CAG_873, Erysipelatoclostridium, Prevotellaceae_Ga6A1_group, Ruminiclostridium.6, Christensenellaceae*, whereas the CCI group had a higher abundance of *Clostridia, Clostridiales, Firmicutes, Lachnospiraceae, Rikenellaceae, Alistipes, Unknown_Family, Acidibacter, A2, Gammaproteobacteria_Incertae Sedis, Ruminococcaceae, uncultured, Rikenella, Roseburia, Romboutsia, Family _XIII_UCG_001, Anaerovorax, Sphingomonadales, Sphingomonadaceae, Ruminiclostridium, ShuttLeworthia, GCA.900066225, Family_XIII, Parasutterella, Ruminococcaceae_UCG_010, Morganella* (Figure 3F). The abundance of *Ambiguous taxa, Alloprevotella, Prevotellaceae, uncultured bacterium, Negativibacillus, Parabacteroides, Tannerellaceae, Ruminococcaceae_UCG_014, Muribaculum*, was enriched in the EA group (Figure 3F). Predicted KEGG pathways from metagenomic sequences were profiled via PICRUSt. Compared to the CON group, the CCI group exhibited significant enrichment of host genes modulating pathways mainly involving xenobiotics biodegradation and metabolism (Caprolactam degradation, Dioxin degradation, Xylene degradation, Styrene degradation, Nitrotoluene degradation), cellular processes (Meiosis-yeast, Bacterial chemotaxis, Flagellar assembly, Bacterial motility proteins, Sporulation, Germination), lipid metabolism (Synthesis and degradation of ketone bodies, and alpha-Linolenic acid metabolism) ($P < 0.05$), but showed decrease of host genes modulating pathways mainly involving glycan biosynthesis and metabolism (Glycosphingolipid biosynthesis– ganglio series, Glycosaminoglycan degradation, Lipopolysaccharide biosynthesis), immune (Cellular antigens), Lipoic acid metabolism, and Adipocytokine signaling pathway ($P < 0.05$) (Figure 3G). Compared to the CCI group, the host genes modulating pathways were exhibited down-regulation in the EA group, mainly including xenobiotics biodegradation and metabolism (1,1,1-Trichloro-2,2-bis(4-chlorophenyl)ethane (DDT) degradation, Caprolactam degradation, Dioxin degradation, Xylene degradation, Styrene degradation, Nitrotoluene degradation), cellular processes (Bacterial chemotaxis, Flagellar assembly, Bacterial motility proteins, Sporulation, Germination), organismal systems (Cardiac muscle contraction, Mineral absorption), lipid metabolism (Steroid hormone biosynthesis, Synthesis and degradation of ketone bodies, alpha-Linolenic acid metabolism).

The *Firmicutes*/*Bacteroidetes* (F/B) ratio is usually used to reflect the health status and the degree of dysbiosis of the gut microbiota.[36] Here, we found that the F/B ratio of the CCI group was higher than the other two groups, suggesting an

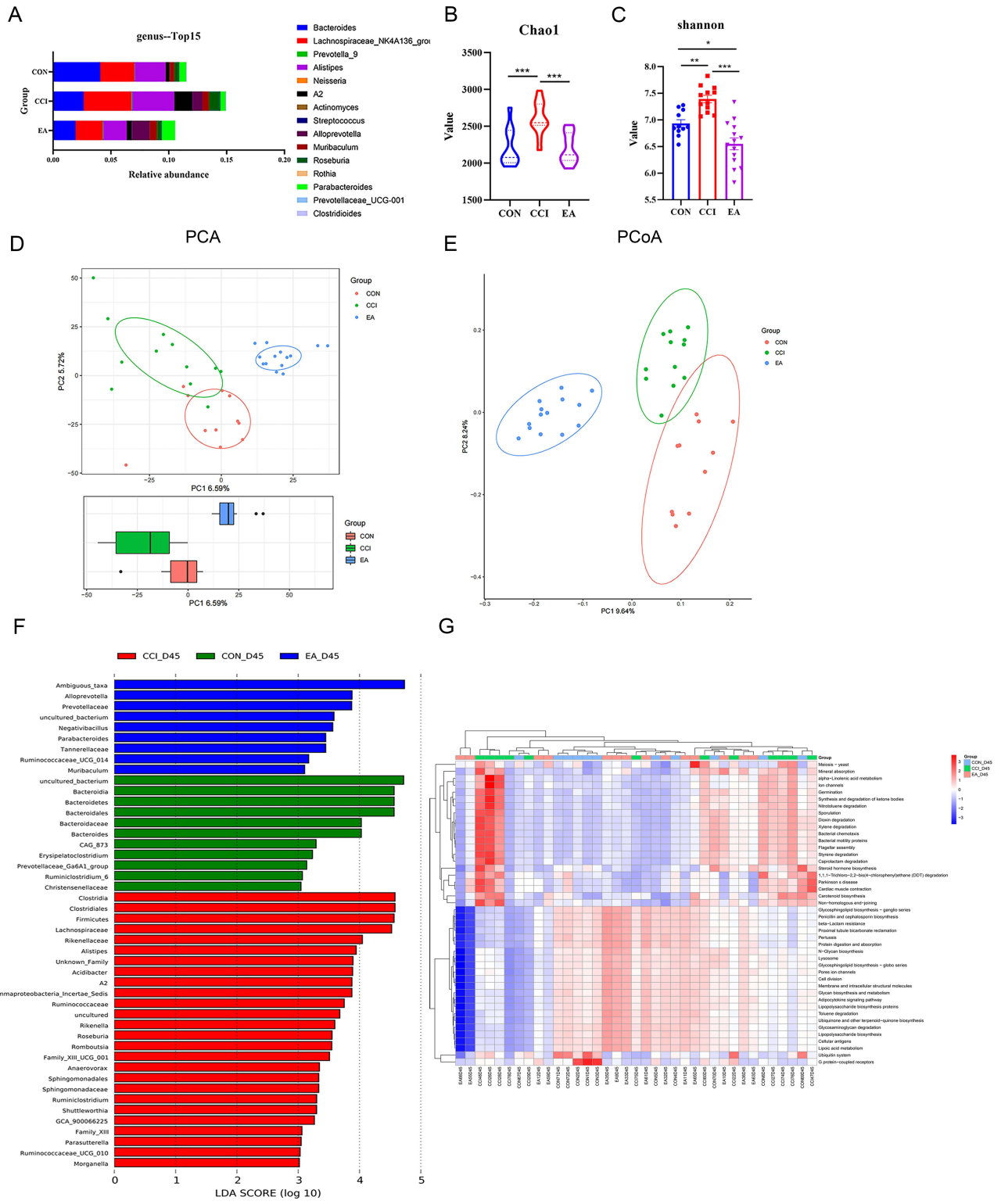

Figure 3 Fecal microbiota altered in the Con, CCI and EA mice. (**A**) Gut microbiota of three group taxa summaries (presented at the genus level) of bacterial relative abundance; (**B** and **C**) α-Diversity measured by Chao1 (**B**), and Shannon (**C**) indices with OTU richness and diversity measured by Welch's *t*-test. An asterisk indicates index with significant differences; (**D** and **E**) Principal coordinate analysis of gut microbiota from three groups, (**D**) PCA, (**E**) PCoA of β-diversity, based on weighted UniFrac distances. Pairwise comparisons using the Wilcoxon rank sum test; (**F**)Mean relative abundance of the genera that showed significant differences between Con, CCI and EA mice by LDA coupled with effect size measurements; (**G**) Heatmap of the KEGG analysis results about the underlying mechanisms through which EA treatment mediate gut microbiota to improve pain.

Note: *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$.

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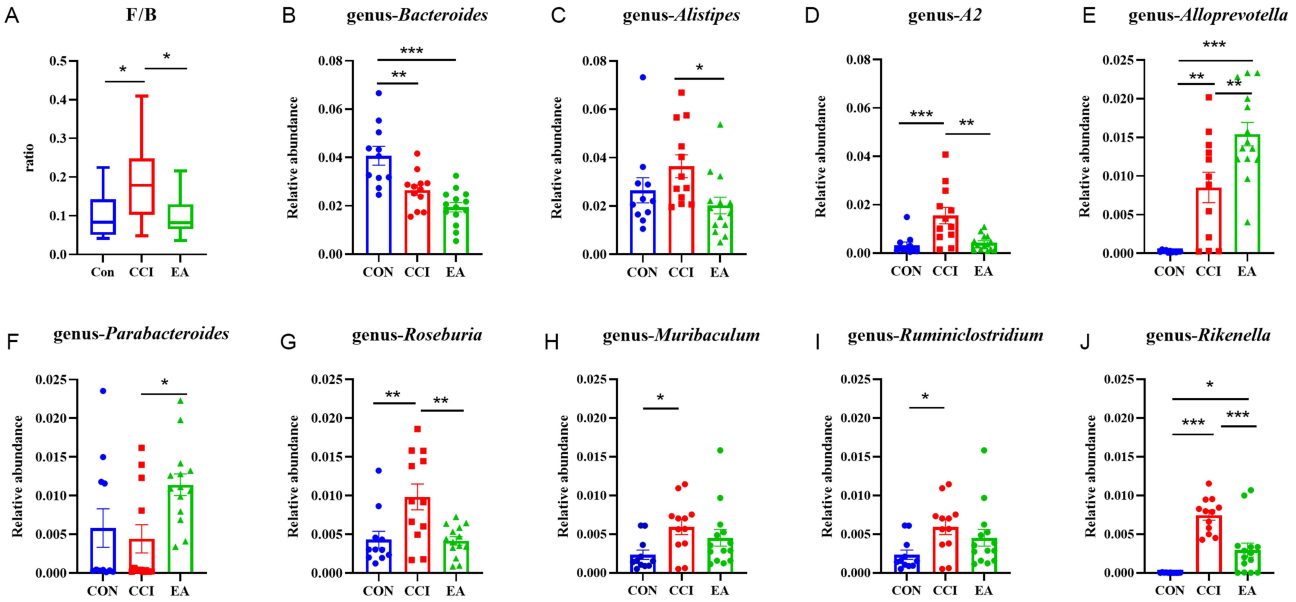

Figure 4 Comparison of relative abundance in the Con, CCI and EA mice of the *genus* that showed significant differences in Spearman correlation analysis. (**A**) *Firmicutes/Bacteroidetes* (F/B) ratio; (**B-J**) Genus-level differences in relative abundance of *Bacteroides, Alistipes, Alloprevotella, A2, Parabacteroides, Roseburia, Muribaculum, Ruminiclostridium,* and *Rikenella*.

Note: **P* < 0.05, ***P* < 0.01, ****P* < 0.001.

imbalanced status with the mice subjected to the CCI surgery, which corroborate the results of α-diversity we have acquired (Figure 4A). At the *genus* level, the abundance levels of *Bacteroides* decreased (Figure 4B), while those levels of *Alloprevotella, A2, Roseburia, Muribaculum, Ruminiclostridium*, and *Rikenella* increased in the CCI group when compared with the CON group (Figure 4D, E and G-J). Compared with the CCI group, the abundance levels of *Alistipes, A2, Roseburia*, and *Rikenella* were significantly decreased in the EA group (Figure 4C, E, G and J), while those levels of *Alloprevotella* and *Parabacteroides* were significantly increased (Figure 4D and F). We also conducted comparative analyses among the three groups at both the species and phylum levels. The results of these analyses were presented in Figure S1.

Discussion

In this study, we used 16s rDNA sequencing of fecal microbiota to investigate the changes of gut microbiota and underlying mechanisms of EA to mediate comorbid anxiety- and depression-like behaviors in chronic pain and demonstrated that EA has influenced on chronic pain and associated painful emotions, as well as changes in gut microbiota in CCI mice, suggesting that EA contributes to the improvement of chronic pain, and gut microbiota could be a potential therapeutic target.

Accumulated evidence has suggested that EA is effective in ameliorating NP. Recently, a randomized controlled study showed that EA can alleviate the pain intensity in trigeminal neuralgia and had a lower rate of adverse events compared with the carbamazepine treatment group.[37] The present study showed that EA treatment relieved the mechanical hyperalgesia and anxiety- and depressive-like behaviors induced by CCI of the sciatic nerve. The results are consistent with the previous studies showing that EA has analgesic, antianxiety and antidepressant-like effects on NP.[38,39]

The current studies showed that gut microbiota perform multiple functions and play an important role in somatic chronic pain.[40,41] A systematic review and meta-analysis indicated the presence of gut microbiota dysbiosis in patients with chronic pain.[42] For example, the abundance levels of *Escherichia-Shigella, Streptococcus, Ligilactobacillus*, and *Clostridia_UCG-014_unclassified* were elevated in patients with postherpetic neuralgia, while *Eubacterium_hallii, Butyricicoccus, Tyzzerella, Dorea, Parasutterella, Romboutsia, Megamonas*, and *Agathobacter* genera were reduced in comparison to healthy controls.[43] A clinical study has confirmed that fecal microbiota transplantation (FMT) from one healthy lean subject into fibromyalgia patients improved the clinical symptoms, including pain, sleep, anxiety and

depression.[44] It is now believed that acupuncture influences multiple diseases by regulating gut microbiota.[45] The α-diversity analysis is an assessment of species richness and evenness of distribution within a sample by calculating different alpha diversity indices.[23] In this study, we found that the diversity of gut microbiota in CCI group was significantly increased compared with the CON group. Meanwhile, EA treatment can inhibit the increase of the diversity of gut microbiota in CCI mice. Nevertheless, some studies reported the opposite results, which found that spared nerve injury (SNI) can significantly increase the diversity of gut microbiota in rats.[23,46] The result is, however, supported by a previous study by Ma et al who showed that gut microbiota depletion by antibiotics cocktail treatment ameliorated the thermal hyperalgesia and mechanical allodynia in three NP models induced by nerve injury, chemotherapy, and diabetes in mice.[47] These conflicting results indicated that more studies are still needed to explore the relationship between microbial diversity and NP.

It was found in our experiment that intestinal microbiota changed in CCI mice. For example, the relative abundance of *Clostridiales* was significantly increased in CCI and chronic visceral pain mice.[48,49] Meanwhile, the abundance of *Clostridiales* was also positively associated with anxiety.[50] The results are compatible with our current results.

On the other hand, EA treatment can regulate the abundance of other flora in NP mice. The abundance levels of genus *A2, Roseburia*, and *Rikenella* were increased in the CCI group, and were markedly downregulated following EA treatment. *Roseburia* was most significantly associated with an elevated risk of trigeminal neuralgia.[51] Another study found that *Roseburia* had a higher relative abundance in patients of persistent post-operative pain following breast cancer surgery.[52] However, a study found that *Roseburia* was negatively correlated with chronic unpredictable mild stress-induced anxiety-like and depression-like behavior of rats.[53] *Rikenella*, as a kind of pro-inflammatory bacteria, marked increased in mice models of NP, anxiety, and depression.[54–57] *Alloprevotella* is a benign bacterium, which was negatively associated with peak pain at movement during 24 h after surgery and was significantly lower in patients with depression.[58,59] *Parabacteroides* could interact directly with nociceptors by reducing activation level on capsaicin, inflammatory soup, and bradykinin stimulations to alleviate chronic abdominal pain.[60] Another study found that *Parabacteroides* might prove anxiety-like behavior by alleviating colon- and neuro-inflammation and improving gut and brain-blood barrier function.[61] This study found that the abundances of *Alloprevotella* and *Prevotellaceae* were significantly enriched in NP mice after EA treatment.

While our study has identified specific microbial communities that appear to influence the efficacy of EA in treating chronic neuropathic pain and its associated negative emotions, the precise mechanisms by which these microbial communities exert their effects remain elusive. Our future work will be directed towards elucidating these mechanisms, which is crucial for a comprehensive understanding of the interplay between the microbiome and pain management. Future work should delve into elucidating the roles and specific mechanisms of these differential microbes in pain regulation and the analgesic effects of EA.

Conclusion

In summary, these findings in the study suggested that alleviation of pain and the emotional dysfunction in CCI mice through EA treatment may be closely related to the diversity and abundance of gut microbiota.

Data Sharing Statement

The datasets generated and analyzed during the current study are available in the Genome Sequence Archive (https://www.ncbi.nlm.nih.gov/bioproject/PRJNA1169938/) repository (BioProject: PRJNA1169938). The other data generated in this study are contained within the article or supplementary material file.

Ethical and ARRIVE Statement

The experiments adhered to the Animal Protection and Use Committee of Yue Yang Hospital for Integrative Medicine, Shanghai University of Traditional Chinese Medicine, China (Ethics No. YYLAC-2019-047). The study is reported in accordance with ARRIVE guidelines.

Author Contributions

All authors made a significant contribution to the work reported, whether that is in the conception, study design, execution, acquisition of data, analysis and interpretation, or in all these areas; took part in drafting, revising or critically reviewing the article; gave final approval of the version to be published; have agreed on the journal to which the article has been submitted; and agree to be accountable for all aspects of the work.

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Disclosure

The authors declare no competing interests in this work.

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