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Chronic low-dose pharmacological amoxicillin exposure promotes depressive and anxious behavior in mice: Alterations in gut microbiota and local field potentials within the brain.

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Abstract:
Mood and anxiety disorders are prevalent in contemporary society. Recent studies suggest a potential link between exposure to antibiotics and these disorders, with antibiotics potentially adversely affecting the gut microbiome, which in turn affects physiological and neurological processes, including mood and cognition. However, the underlying mechanisms have not been extensively studied. Two-month-old male ICR mice were randomized into three groups: (1) control group; (2) 2 mg/kg amoxicillin-treated group and (3) 4 mg/kg amoxicillin-treated group. After seven days of treatment with 2 mg/kg or 4 mg/kg amoxicillin or saline, mice were subjected to tests of anxiety- and depression-like behaviors, fear memory, and spatial recognition memory. At the end of the behavioral assessment, colon histopathology and gut microbiota composition were examined in each group of mice. In addition, local field potentials (LFPs) and auditory evoked potentials (AEPs) in the hippocampus (HPC), amygdala (AMY), and ventral tegmental area (VTA) of mice in different groups were recorded. The results showed that chronic administration of amoxicillin increased anxiety- and depressive- like behaviors, enhanced cue-induced fear memory, and slightly impaired spatial recognition memory in mice. The LFP study showed an increase in gamma oscillations in VTA and AMY. Late components of the AEP in these two brain regions were also affected by amoxicillin. After long-term administration of amoxicillin, the colon of mice in the 4 mg/kg group exhibited mucosal damage and reduced crypt structures with severe inflammatory cell infiltration. Amoxicillin greatly reduced the diversity of the microbiota and also altered the composition of the gut microbiota and its predicted function. Amoxicillin treatment significantly alters gut microbiota, leading to an increase in gamma oscillations and changes in auditory evoked potential (AEP) waves in the ventral tegmental area (VTA) and amygdala (AMY), which are indicative of altered neuronal activity in brain regions associated with emotional and reward processing. These alterations may underlie the observed increase in anxiety and depressive-like behaviors, indicating that greater attention should be paid to the impact of antibiotics on psychiatric disorders.

Key words:

Amoxicillin; Anxiety; Gut microbiota; Local field potentials (LFPs); Auditory evoked potential (AEP)

Introduction

Neuropsychiatric disorders, including anxiety and depression, are one of the most common mental illnesses in the world, affecting more than 350 million people globally. These disorders significantly impair personal health and social functioning, driving up global healthcare expenditures and economic burden (Baxter et al., 2014). Recently, a growing number of studies have shown the correlation between gut flora and psychiatric disorders such as anxiety and depression (Sherwin et al., 2018, Li et al., 2026). However, the underlying mechanisms are not clear.

The gut microbiome, a complex community of various microbial cells colonizing the gut, plays crucial roles in regulating nutrient absorption, fat distribution, intestinal barrier homeostasis, and intestinal motility. These microbial communities also have far-reaching effects beyond the gastrointestinal tract. Dysbiosis of the intestinal flora is associated with the occurrence and development of a variety of diseases, such as obesity, allergies, type-2 diabetes, and cancer. Recent evidence increasingly suggests that the gut microbiome is related to brain function. Extensive evidence supports the notion that the intestinal flora plays an essential role in the pathophysiology of depression and anxiety (Sherwin et al., 2018). Changes in the gut microbiota have been reported in patients with depressive disorders and anxiety (Chen et al., 2018), and similar results have been observed in rodent models with these conditions (Fan et al., 2022, Shen et al., 2023). The microbiome-gut-brain axis theory posits that the gut microbiota can influence brain function and behavior through microbial-derived metabolites, cytokines, immunity, vagus nerve pathways, and other mechanisms (Zhang, 2024). However, there is a relative scarcity of studies that delve into the underlying mechanisms, and further research is needed to better understand these complex interactions.

Antibacterial antibiotics are among the most used drugs in current medical practice because of their therapeutic effectiveness. While they are beneficial for their antimicrobial properties, they also have significant side effects. Recent research has highlighted the connection between antibiotics and mental health. For example, chloramphenicol exacerbates depressive states, some quinolones and azithromycin is associated with increased risk of suicide, vancomycin induces cognitive disorders and gentamicin can provoke anxiety (for a review, see (Nechifor et al., 2023)). A nested case-control study using a large population-based medical record database from the United Kingdom during the years 1995-2013 found that treatment with a single course of antibiotics was associated with a mild increase in the risk of depression and anxiety, and this risk increased with recurrent

antibiotic exposures (Lurie et al., 2015). Penicillin, due to its β-lactam ring structure, inhibits the γ-aminobutyric acid transmission of cortical pyramidal cells, thereby causing extensive neurotoxic manifestations in humans, including epileptic seizures. It has been suggested that the overuse of antibiotics can alter the gut microbiota, which may play a role in the development of neurological and psychiatric disorders (Hao et al., 2021). On the other hand, probiotic treatment has been shown to improve clinical symptoms in patients with depression and anxiety (Slykerman et al., 2017), indicating that gut microbes are involved in the development of these conditions and could be a potential target for drug development in their management. However, the specific mechanisms by which antibiotics increase the risk of depression and anxiety, as well as the underlying neurobiological processes, remain unclear and require further investigation.

Amoxicillin is the most common used semi-synthetic penicillin broad-spectrum β-lactam antibiotic with strong bactericidal and cell membrane penetrating ability. As a broad-spectrum antibiotic, it has a relatively high rate of use in hospitals, especially in emergency and pediatric outpatient settings. In the present study, we want to investigate whether chronic amoxicillin administration could affect anxiety-like behavior or learning and memory in mice. Mice were chronic injected with amoxicillin (2 mg/kg, or 4 mg/kg) for 7 days, and then they were tested for anxiety-like behavior in the open field, recognition memory in the Y- maze and fear memory in the fear conditioning, during which amoxicillin were injected once daily until the end of the behavioral tests. Rodent studies have investigated the roles of the amygdala and hippocampus in anxiety-related behaviors (Bannerman et al., 2003, Babaev et al., 2018). Thus, we also investigated whether chronic amoxicillin affect local field potential (LFP) powers and coherence in the VTA, HPC and AMY. Auditory invoked potentials in these three groups were also tested.

Material and Method

2. Materials and methods

2.1. Animals

Adult male ICR mice (28–33 g body weight, at age of 8 weeks) from Hunan Slack Jingda Experimental Animal Co., Ltd (Hunan, China) were used. Animals were housed on a 12-h light/dark cycle with light on from 07:00 to 19:00 h. They had food and water available ad lib and were allowed to familiarize with the experimenter and adapt to the laboratory conditions for 1 week before the experiment started. The experiments were carried out during the light phase of the cycle. All experiments were conducted in accordance with the guidelines for the Care and Use of Lab Animals and the study was approved by the Medical Animal Care &

Welfare Committee of Kunming University of Science and Technology (Approval No. KUST202602035).

2.2 Drugs

Mice were divided into three groups: the control group, 2 mg/kg amoxicillin group and the 4 mg/kg amoxicillin group. Amoxicillin (Chongqing Pioneer Animal Co. China) was dissolved in sterile saline. Mice received intraperitoneal injection (i.p.) of saline, 2 mg/kg amoxicillin or 4 mg/kg amoxicillin daily for 7 days. After that, the behavioral tests begin, as shown in Fig.1. Amoxicillin were administrated once daily until the behavioral tests or electrophysiological experiments ended.

2.3 Behavioral experiments

2.3.1 Open Field Test

The open field is a square arena (60 cm × 60 cm ×40 cm, width × length × height), with an open top, dark walls and a dark floor (MED Associates Inc., Georgia, Vermont, USA), as described in our previous work (Chen et al., 2019). The arena was subdivided in 'periphery' (15 cm from the wall), and 'center' (30 ×30 cm) zones. A camera was installed above the center of the field. Immediately after a mouse was placed in the corner of the open field, the movements and position of the animals were recorded and the time (s) spent in the center of the open field and the total distance moved (cm) during the 5-min test were analyzed by the software (Smart 2.0, Panlab). The floor of the open field was cleaned with 70% ethanol solution between tests to prevent transmission of olfactory cues.

2.3.2. Light/dark box

The light/dark box was a rectangular box (45 cm × 30 cm × 35 cm, width × length × height) composed of two compartments, one third for the dark compartment and two thirds for the light compartment, as described in our previous study (Chen et al., 2014). This procedure is based on the natural avoidance of mice for light places. Mice were placed individually in the light box, facing to the dark compartment. Then the mice were allowed to explore the light/dark box for 5 min. All trials were recorded on a VCR by using a ceiling-mounted CCD camera. Video recordings were later analyzed and the latency to the dark box, total time spent in the white box and the numbers of transfers from one compartment to the other were analyzed. A mouse that has entered its four paws in the new box was considered as having changed boxes. The floor of the apparatus was cleaned with 70% ethanol solution between trials to prevent transmission of olfactory cues.

2.3.3 The recognition Y-maze test

The behavioral apparatus used in the recognition Y-maze test was the same as we used before (Ma et al., 2007). Three arms were randomly designated: start arm, in which the mouse started to explore (always open), novel arm, which was blocked at the 1st trial, but opened at the 2nd trial, and other arm (always open). The start arm and other arm were designed randomly to avoid the spatial memory error. The floor of the maze was covered with sawdust, which was mixed after each individual trial in order to eliminate olfactory stimuli. The Y-maze test consisted of 2 trials separated by an inter-trial interval (ITI). The first trial (training) was 10 min duration and allowed the mouse to explore 2 arms (start arm and other arm) of the maze, with the 3rd arm (novel arm) being blocked. After a 1 h ITI, the 2nd trial (retention) was conducted. For the second trial (memory), the mouse was placed back into the maze from the same starting arm, with free access to all the 3 arms for 5min. n = 7–8 for each group. A ceilingmounted camera was used to record all trials in the experiment. Video recordings were later analyzed and the number of entries and the time spent in each arm were analyzed. The percentage of time spent in and entries into the novel arms score the spatial recognition memory.

2.3.4 Fear conditioning

Two experimental chambers (Med Associates Inc., Georgia, Vermont, USA) with different patterns on the wall (context A or context B) were used in fear conditioning as in our previous studies (An et al., 2020). The behavioral experiment includes three sessions: the habituation, conditioning and testing. On the first day of habituation, mice were placed in context A and context B for 10 minutes each. During the second day, mice were placed in either context A or context B, in which after the appearance of a 19 s tone (CS: 80–85 db, 2 kHz), it received an electrical foot shock (US: 0.7 mA, 1.0 s) with the tone on, this CS-US pairing was repeated for five times with an inter trial interval of two minutes. 24 h after the training, mice were re-exposed to the original training chamber for a 10 min context test session in which no tones or shocks were presented. 24 h later, the tone test session was carried out by placing the mice in the other chamber and presenting the tone five times (the same as in the conditioning) beginning 2 min after the mouse was placed in the chamber. The chamber floor bars and waste trays were cleaned with ethanol cleaning solution between each session, and the animals were tested at approximately the same time of day on each day. Behavior was recorded via digital cameras in the chamber and freezing behavior was analyzed by software (Video Freeze, Med Associates Inc.).

Freezing was defined as the absence of all movements except those associated with respiration. Percentage of freezing (freezing ratio) during the baseline (2 min) and after each of the 5 training trials (2 min) was calculated to investigate the effect of chronic amoxicillin on learning. Percentage of freezing during the first 6 min in the contextual test was calculated to investigate the effect of chronic amoxicillin on context-dependent

memory. Percentage of freezing after the first three tones, which was also totally 6 min was calculated to investigate the effect of chronic amoxicillin on cue-dependent memory. In training and tone dependent memory test, the freezing time and total time did not include the 20 s tone.

2.3.5. Forced swim test

Mice were forced to swim individually in a vertical glass cylinder (height 25 cm, diameter 10 cm) filled with water maintained at 22–23◦C to a depth of 19 cm , as described in our previous work (Chen et al., 2019). 15 min later, mice were removed and allowed to dry in a heated enclosure. 24 hours after the swim, they were forced to swim individually in the cylinder again for 5 min. Duration of immobility (making only minimal movements to keep the head above water or floating) was measured from videotapes by a trained blind observer.

2.4 HE Staining of Colon Tissues

Mice were euthanized by cervical dislocation performed by trained personnel. Death was confirmed by the absence of respiration, heartbeat, and pedal reflex. The colon of each mouse was excised, and the attached adipose tissue was removed. The colon specimens were fixed in 4% paraformaldehyde, embedded in paraffin sections (5 μm), and stained with hematoxylin and eosin (HE). The sections were then photographed and recorded at magnifications of ×200 and ×400 (Nikon DS-U3, Tokyo, Japan). Afterwards, the sections were photographed and recorded at magnifications of ×200 and ×400 (Nikon DS-U3, Tokyo, Japan), the basic pathological changes such as congestion, bruising, hemorrhage, edema, degeneration, necrosis, proliferation, fibrosis, mechanization, granulation tissue, inflammatory changes, etc. were observed in detail.

2.5 16S rRNA sequencing of fecal microbiota

Contents of colon were collected directly from the mice in each group were euthanized. Fecal samples were then stored at − 80 °C for further use. The genomic DNA from the total 27 samples was extracted using a QIAamp Fast DNA Stool Mini kit (Qiagen, Germany) according to the manufacturer's instructions. The quality and quantity of genomic DNA were then determined by 1% agarose gel electrophoresis and using a NanoDrop 8000 instrument (Thermo, USA). Total genome DNA from samples was extracted using CTAB method. DNA concentration and purity was monitored on 1% agarose gels. According to the concentration, DNA was diluted to 1ng/μL using sterile water. The V4–V5 region of the bacterial 16 s rRNA gene was amplified by polymerase chain reaction (PCR) using the primers: 5′-GTGCCAGCMGCCGCGGTAA-3′ and 5′-CCGTCAATTCCTTTGAGTTT-3′. The PCR protocol involved an initial denaturation at 98 °C for 1 min; 30 cycles of denaturation at 98 °C for 10 s, annealing at 50 °C for 30 s and extension at 75 °C for 30 s; and then a

final extension at 72 °C for 5 min. PCR products was then purified with Qiagen Gel Extraction Kit (Qiagen, Germany). The library quality was assessed on the Qubit@ 2.0 Fluorometer (Thermo Scientific) and Agilent Bioanalyzer 2100 system. At last, the library was sequenced on an Illumina NovaSeq platform and 250 bp paired-end reads were generated.

2.6. Electrophysiology experiment

2.6. 1. Electrodes implantation

As described in our previous work (Zhou et al., 2024), mice were anesthetized with pentobarbital sodium salt (60 mg/kg, Merck) and atropine sulfate (0.4 mg/kg). Then, they were placed in a stereotactic apparatus (RWD Life Science). Electrodes were made by Teflon coated stainless steel wires (50 μm diameter, Stainless Steel (HH), 790600, A-M Systems Co.). Electrodes were implanted into the target brain areas at the following coordinates (VTA: 3.4 mm posterior, 0.4 mm lateral, 4.3 mm ventral to bregma; AMY: 0.94 mm posterior, 2.75 mm lateral, 4.75 mm ventral to bregma; HPC: 1.94 mm posterior, 1.75 mm lateral, 1.5 mm ventral to bregma). Two stainless steel screws (one served as a ground electrode, and the other served as an anchor) were inserted into the skull through drilled holes without piercing the dura. The whole assembly was sealed and fixed to the skull using dental acrylic. All mice were housed individually for at least one week of recovery following the surgery, during which mice were injected with amoxicillin (4 mg/kg, 2 mg/kg) or saline daily.

2.6.2. Local field potential recording and Data Analyses

Four days after surgery, mice were adapted to the LFP recording procedures and recording chamber (a custom-made Faraday cage) for three days, 15 min each day. Experiments began on 7 days after the amoxicillin treatment. LFP in each group were collected for 15 min. Tests were conducted from 10:00 to 18:00, which ensured a relatively stable physiological state. During testing, the rats were awake and moving freely. Signals recorded from all electrodes were amplified (1000 ×), low-pass filtered (<300 Hz), and then digitized (sampling frequency of 1000 kHz) by using an acquisition board (ME32- FAI-uPA-System, Germany) controlled by MC-Rack software (Germany). Such original signals were stored in a computer for offline analyses.

As described in our previous work (Zhou et al., 2024), all the spectral and coherence analysis were performed using custom-made scripts in Matlab (Version 2013a, The Mathworks, Inc). LFPs were firstly imported into MATLAB, opened by EEGLAB (Version 12.0.2.6b). Data with high amplitude caused by body movement were rejected from further analysis. Power spectra were calculated using the Chronux signal processing toolbox (version 2.0). EEG data were first segmented into 10 s epochs for each calculation, which

were then averaged to get the final results. Spectral analysis of absolute and relative power was carried out for the six frequency bands: delta (1–4 Hz), theta (4–8 Hz), alpha (8–12 Hz), beta (12–30 Hz), low gamma (30–60 Hz) and gamma (60–90 Hz).

2.6.3 Auditory-evoked potential (FEP) Procedure and Data Analyses

One hour after the LFP recording, AEPs were recorded from electrodes in the VTA, HPC and AMY. AEP recordings were made in a shielded recording room with dim background illumination, as described in our previous work (Chen et al., 2021). The animals were freely-moving in a white Plexiglas testing box (40 cm × 30 cm × 50 cm, length × width × height). The sound signals are generated by MATLAB software, which controls a loudspeaker located in the recorder box. For AEP recording, a train of 500 click stimuli (2000 Hz, 85 dB SPL) were generated, each stimulus lasted 30 ms. The inter-trial interval of click was 1 s to 4 s (random). The background noise of the recording room was about 45 dB. Evoked potentials were amplified by UEA-BZ preamplifiers (SYMTOP, Beijing, China) with a 1-kHz sample rate, band-pass filtered at 2–60 Hz. Data were processed using EEGLAB 13.4 (MatLab toolbox). LFPs recorded in each brain area were filtered with a digital finite impulse response filter (band-pass 2–60 Hz). MATLAB controls the sound from the stereo while using the other channel of the stereo to send a current signal to the EEG amplifier, thus marking the time the sound is given (time zero). Waveforms were averaged in 500 ms epochs, 100 ms before and 400 ms after the onset of a click.

2.7. Statistical analyses

Data was analyzed using Prism 7 or SPSS 13.0. One-way ANOVA followed by Turkey's post hoc tests were used for pair-wise comparisons among groups. Two-tailed paired t tests were used to compare differences between the saline and 4 mg/kg amoxicillin treated groups. Data were expressed as the mean ± SEM. Significance was accepted at $p < 0.05$.

3. Results

3.1. Chronic Amoxicillin increased anxiety- and depressive- like behavior in mice.

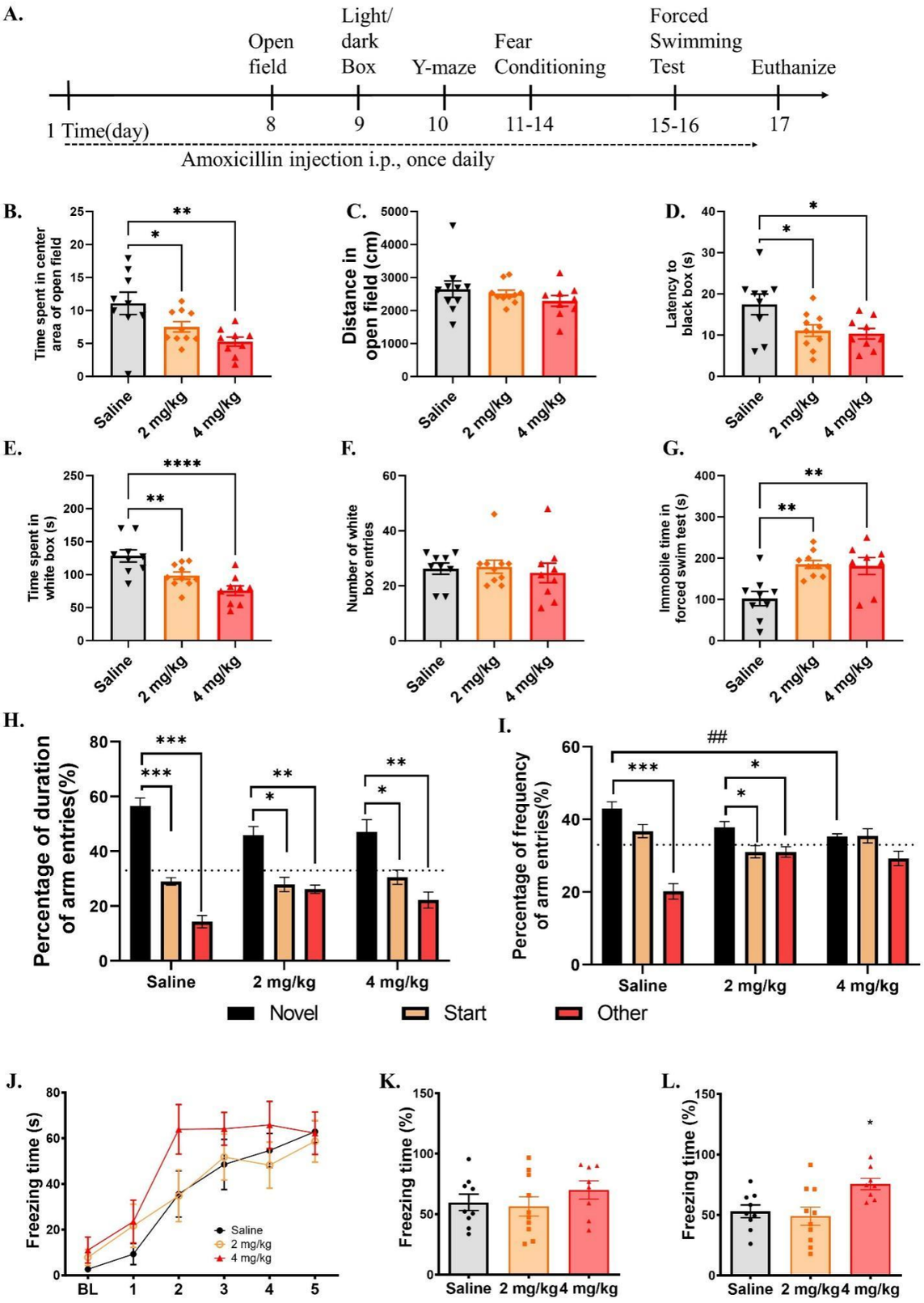

A.
Open field
Light/ dark Box
Y-maze
Fear Conditioning
Forced Swimming Test
Euthanize
1 Time(day)
8
9
10
11-14
15-16
17
Amoxicillin injection i.p., once daily
B.
Time spent in center area of open field
C.
Distance in open field (cm)
D.
Latency to black box (s)
E.
Time spent in white box (s)
F.
Number of white box entries
G.
Immobile time in forced swim test (s)
Saline
2 mg/kg
4 mg/kg
H.
Percentage of duration of arm entries(%)
I.
Percentage of frequency of arm entries(%)
Novel
Start
Other
J.
Freezing time (s)
BL
K.
Freezing time (%)
L.
Freezing time (%)

Fig. 1. Behavioral tests in chronic saline and amoxicillin treated mice.

(A) Timeline for amoxicillin administration and behavioral testing. (B)Time spent in the center of the open field during the 5 min test; (C) Total distance moved in the open field. (D) The latency of mice went into the black box in the black-white box test. (E) Total time spent in the black-white box. (F) The number of white box entries; (G) Duration of immobility in forced swim test. (H) Percentage of duration of arm visits for mice visiting the novel, start and other arms in Y -maze with a 1 h ITI. (I) Percentage of number of arm visits during the Y-maze test. (J) The freezing times during the five CS-US training trials in fear conditioning. (K) The freezing ratio during the contextual fear memory test. (L) The freezing ratio during the cue-induced fear memory test. Data were expressed as the mean ± S.E.M. n = 8-10 for each group. *$p < 0.05$, **$P < 0.01$, ***$P < 0.001$ for difference between saline treated mice and amoxicillin treated mice. For the Y-maze test, Open bar: the novel arm, hatched bar: the start arm, and solid bar: the other arm. *$p < 0.05$, **$p < 0.01$, ***$p < 0.01$ for difference between the novel arm vs. other arms.

Seven days after the chronic administration of saline or amoxicillin, anxiety-like behavior in mice were tested using the open field. The results showed that amoxicillin treated mice spent less time exploring center area of the open field than the controls (Fig. 1 B, F (2,25) = 6.484, p =0.005; Turkey: saline vs. 2 mg/kg: p =0.034, saline vs. 4 mg/kg: p =0.002). Chronic amoxicillin did not change the distance traveled in the open field (Fig. 1 C).

In the light/dark box test (Fig. 1 D-F), after the mice were placed in the light box, the latency to enter the dark box was decreased by amoxicillin treatment (F (2,25) = 3.358, p =0.05; Turkey: saline vs. 2 mg/kg: p =0.038, saline vs. 4 mg/kg: p=0.03). The time spent in the white box during the 5 min test was also decreased in amoxicillin treated mice when compared with the control mice (F (2,25) = 12.23, $p < 0.001$; Turkey: saline vs. 2 mg/kg: p =0.008, saline vs. 4 mg/kg: p<0.001). The number of white box entry was not changed by amoxicillin administration.

In the forced swim test (Fig. 1 G), the duration of immobility of amoxicillin mice was greater than that of the control mice during the 5 min test (F (2,24) = 8.955, p =0.001; Turkey: saline vs. 2 mg/kg: p =0.002, saline vs. 4 mg/kg: p =0.006).

When testing spatial recognition memory in Y-maze, the percentage of duration of arm visits in the novel arm was significantly increased compared with the start and other arms after 1 h ITI in Y-maze (Fig. 1 H, main effect of arm: F (2,54) = 57.396, $p < 0.001$). Combined ANOVA showed a significant amoxicillin × arm interaction (F (2,54) = 3.127, p = 0.022). When the arm differences in each group were analyzed, percentage of

duration of arm visits in the novel arm was significantly increased than in the start and other arm in both saline treated and amoxicillin treated mice (saline: $F(2,18) = 61.02$, $p < 0.001$, novel vs. start: $p < 0.001$, novel vs. other: $p < 0.001$; 2 mg/kg: $F(2,18) = 12.32$, $p < 0.001$, novel vs. start: $p = 0.011$, novel vs. other: $p = 0.001$, 4 mg/kg: ($F(2,18) = 9.07$, $p = 0.002$, novel vs. start: $p = 0.037$, novel vs. other: $p = 0.006$). The percentage of number of arm visits in the novel arm was also significantly increased compared with the start and other arms after 1 h ITI in Y-maze (main effect of arm: $F(2,54) = 23.402$, $p < 0.001$), and there was a significant amoxicillin × arm interaction ($F(2,54) = 6.293$, $p < 0.001$). When the arm differences in each group were analyzed, percentage of number of arm visits in the novel arm was significantly increased than in the start and other arm in saline treated and 2 mg/kg amoxicillin treated mice (saline: $F(2,18) = 23.70$, $p < 0.001$, novel vs. other: $p < 0.001$; Low: $F(2,18) = 4.04$, $p = 0.035$, novel vs. start: $p = 0.046$, novel vs. other: $p = 0.027$), but not in the 4 mg/kg amoxicillin treated mice ($F(2,18) = 2.92$, $p = 0.080$). The percentage of novel arm visits in the high amoxicillin group is lower than that of in the control mice ($F(2,27) = 6.865$, $p = 0.004$, saline vs. 4 mg/kg: $p = 0.003$, One-way ANOVA with Turkey's test). Thus, 4 mg/kg amoxicillin slightly impaired spatial recognition memory in mice.

We then examined the role of chronic amoxicillin on fear conditioning. During the five CS–US pairing trials, the average freezing time increased significantly over the 5 trials (main effects of trial: $F(5,115) = 44.49$, $p < 0.001$). However, two-way ANOVA with repeated measure showed no effects of amoxicillin on the training curve. Context- dependent fear memory was tested 24 h after training, the freezing ratio was not changed by amoxicillin. 48 h after training, tone-dependent fear memory was tested. The freezing ratio after the first three tones in both 4mg/kg amoxicillin treated mice was increased significantly compared with the controls mice ($F(2,24) = 5.143$, $p = 0.014$, saline vs. 4 mg/kg: $p = 0.048$).

3.2. Chronic Amoxicillin altered LFP powers in VTA and AMY.

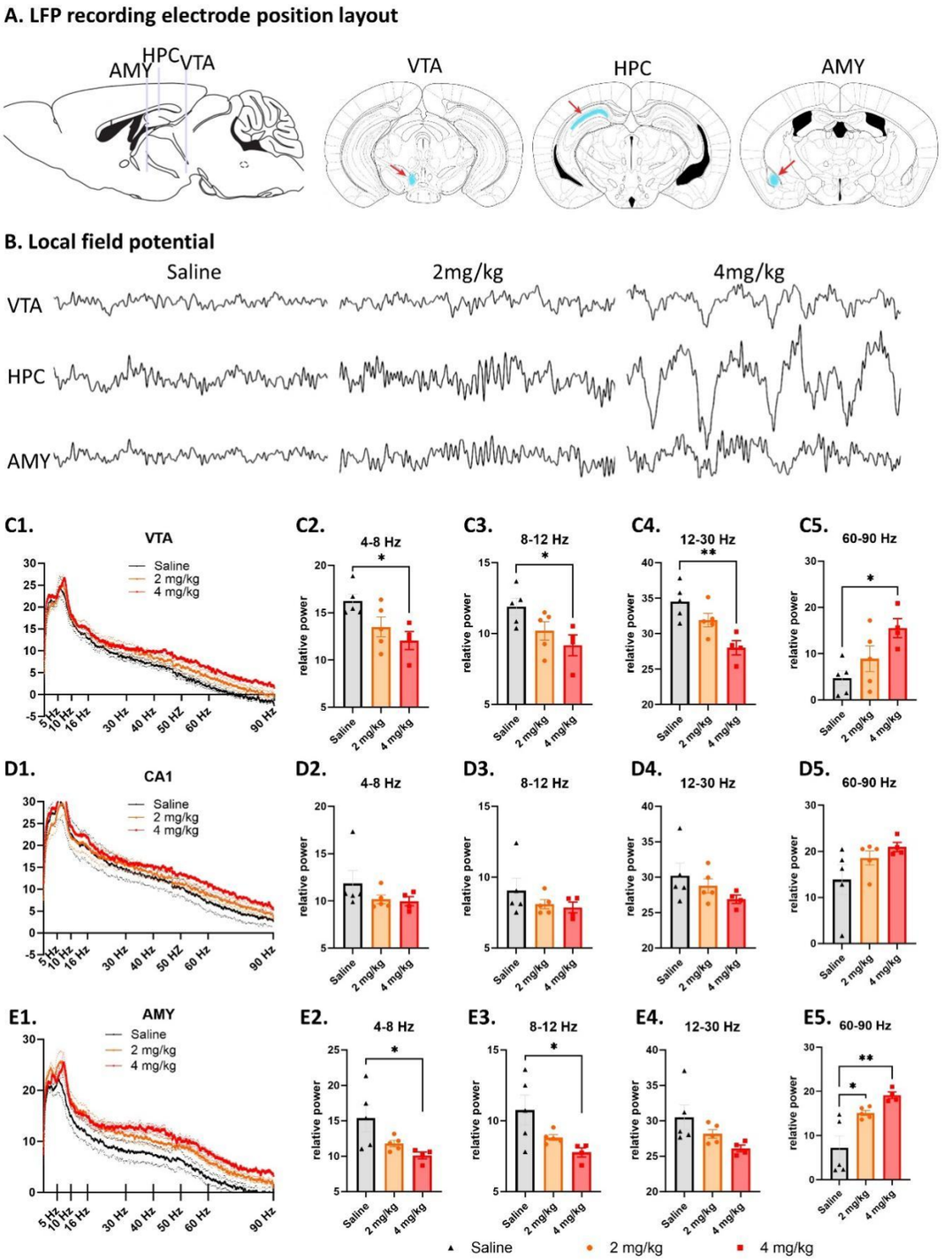


Fig. 2. Local field potentials recorded in VTA, HPC and AMY of the saline and amoxicillin treated mice.

(A) LFP recording electrode positions. (B) Representive LFP traces from VTA, HPC and AMY in saline and amoxicillin treated mice (n=4-5). (C1-C5) chronic amoxicillin decreased theta, alpha and beta oscillations, but increased gamma oscillations in VTA. (D1-D5) chronic amoxicillin did not alter LFP oscillations in hippocampus. (E1-E5) chronic amoxicillin decreased theta

and alpha oscillations, but increased gamma oscillations in amygdala. Data are expressed as mean ±SEM. *p <0.05, **p <0.01 for difference between saline and amoxicillin treated mice.

We then tested the effects of chronic amoxicillin exposure on local field potentials in the VTA, HPC and AMY. LFPs were recorded for 15 min in these areas after 7 days' saline or amoxicillin (2 mg/kg or 4 mg/kg) injections (once daily). Spectral powers in each channel were analyzed. As shown in Fig. 2 C-E, chronic amoxicillin (4 mg/kg) decreased theta and alpha powers in the VTA (theta: F (2,11) =5.204, p=0.026, Turkey: saline vs. 4 mg/kg: p=0.024; alpha: F (2,11) =4.294, p=0.042, Turkey: saline vs. 4 mg/kg: p=0.038) and AMY (theta: F (2,11) =4.587, p=0.036, Turkey: saline vs. 4 mg/kg: p=0.035; alpha: F (2,11) =4.728, p=0.033, Turkey: saline vs. 4 mg/kg: p=0.031). However, no changes were found in the hippocampus. Chronic amoxicillin (4 mg/kg) also decreased beta oscillation (F (2,11) =9.485, p=0.004, Turkey: saline vs. 4 mg/kg: p=0.003) and increased high-gamma oscillation (60-90Hz) in the VTA (F (2,11) =6.926, p=0.011, Turkey: saline vs. 4mg/kg: p=0.009). In AMY, powers in both low-gamma (30-60Hz) and high-gamma bands (60-90Hz) were greatly increased by chronic amoxicillin treatment (30-60 Hz: F(2,11)=3.964, p=0.05, Turkey: saline vs. 4mg/kg: p=0.046; 60-90 Hz: F(2,11)=9.072, p=0.005, Turkey: saline vs. 2mg/kg: p=0.043, saline vs. 4mg/kg: p=0.004). No changes of LFP power were found in 2 mg/kg amoxicillin treated mice. These results suggested that LFP powers in the mice VTA and AMY could be altered by chronic amoxicillin administration in a dose-dependent manner.

3.3. Chronic Amoxicillin altered later cognitive waves of AEP in HPC, VTA and AMY.

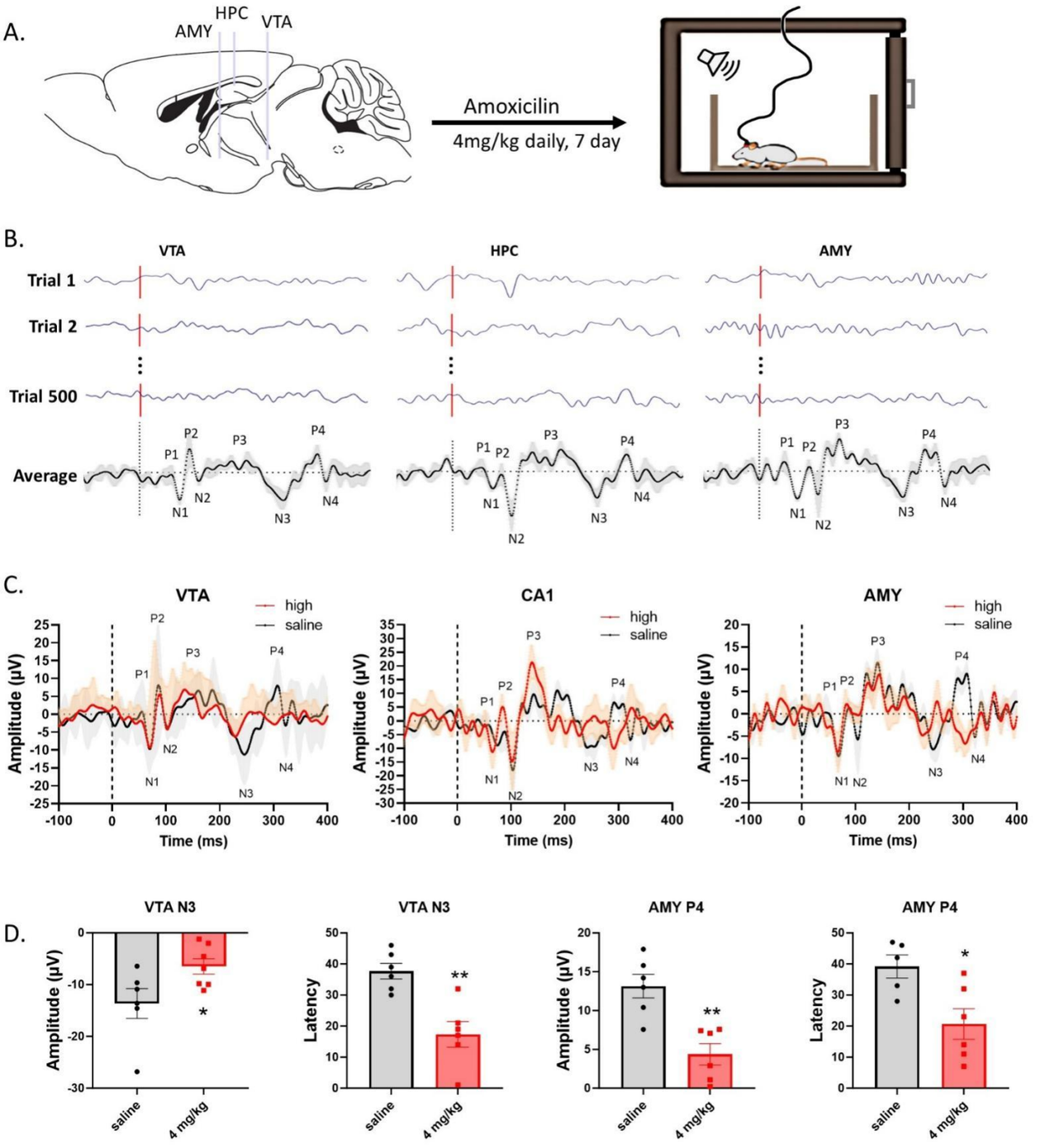


Fig. 3. Auditory-evoked potential (AEP) in VTA, HPC and AMY of mice.

(A) schematic of AEP recording in freely moving mice. (B) waveform of AEPs averaged from 500 trials (vertical lines, flash onset) of each mouse, and then averaged from all the saline mice. AEP in mice has four positive peaks, P1, P2, P3 and P4, and four corresponding negative N1, N2, N3 and N4 peaks. (C) Differences in AEP between saline treated and amoxicillin (4mg/kg) treated

mice. (D) chronic amoxicillin altered AEP in VTA and AMY in the late phase. Error bars represent SEM. N= 5-6 for each group. *$p < 0.05$, **$p < 0.01$ for differences compared with saline treated mice.

After one week of amoxicillin treatment, we measured auditory-evoked potential in VTA, HPC and AMY of mice. Figure 3 B shows an example of AEP recorded from VTA, HPC and AMY in one of the control mice. The AEP recorded from mice usually appeared as four positive peaks, P1, P2, P3 and P4, and four corresponding negative N1, N2, N3 and N4 peaks. Like AEP recorded from humans, both early sensory peaks (P1, N1, and P2) and later cognitive waves (P3 and N3) were visible in the recording. Chronic amoxicillin did not alter early sensory peaks in mice. However, the later cognitive waves in VTA and AMY were altered. The latency of N3 peak in VTA was advanced ($t(10) = 4.2$, $p=0.002$) by amoxicillin, amoxicillin also decreased N3 amplitude in VTA ($t(10) = 2.294$, $p=0.04$) and. In AMY, Amoxicillin decreased P4 amplitude ($t(10) = 4.25$, $p=0.002$) and advanced P4 latency ($t(10) = 2.89$, $p=0.018$) in mice (Fig.3 D).

3.4. Main alterations in gut microbiota α- and β-diversity at genus and species levels after amoxicillin treatment.

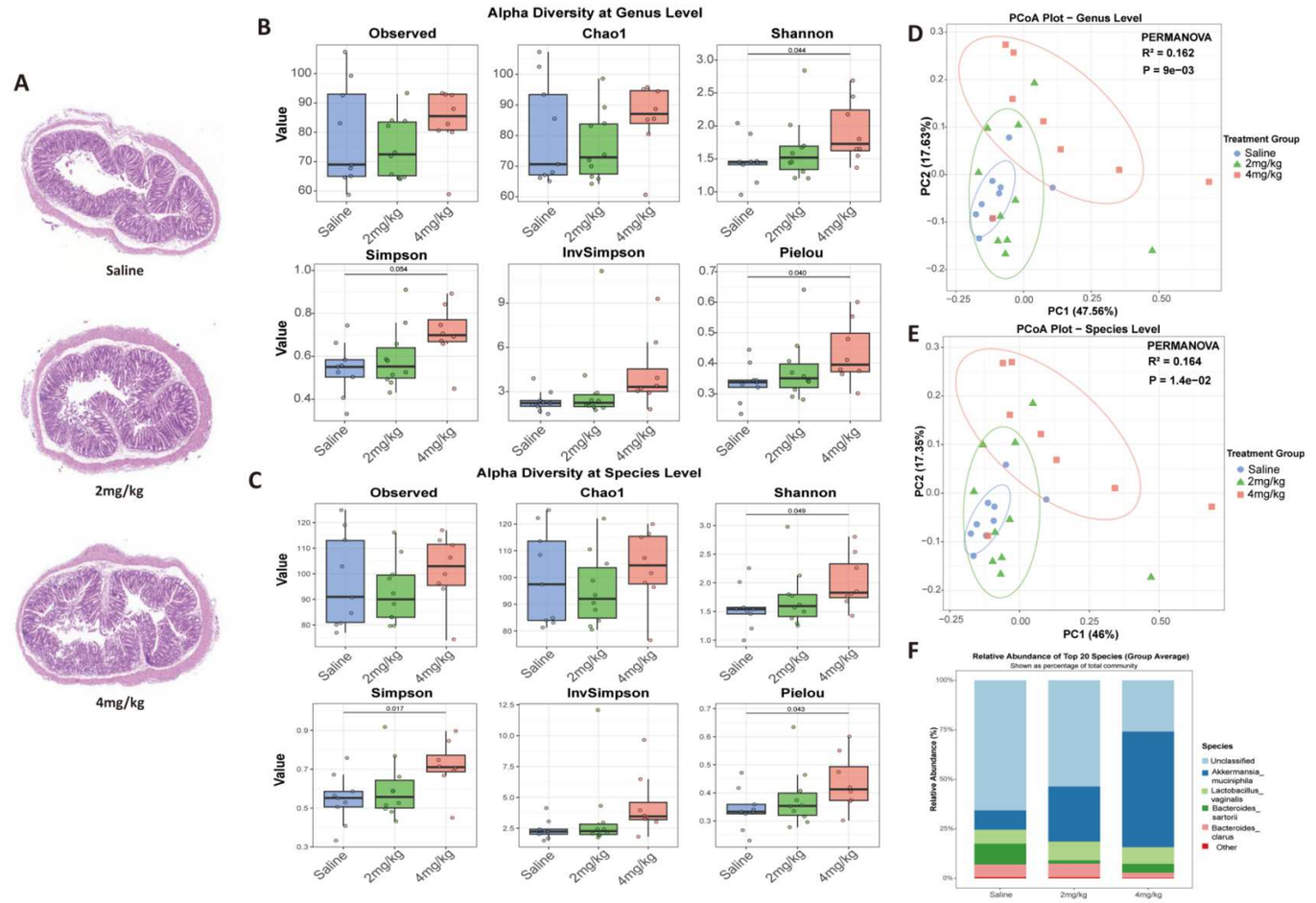


Fig. 4. Diversity and composition analysis of gut microbiota from the three groups.

(A) Typical HE staining of colon tissues from the three groups. (B) Alpha diversity indices at the genus level: Observed, Simpson (p = 0.054), InvSimpson, Shannon (p = 0.044), and Pielou (p = 0.040). (C) Alpha diversity indices at the species level: Observed, Simpson (p = 0.017), InvSimpson, Shannon (*p = 0.049), and Pielou (p = 0.043). (Only significant p-values are indicated.) (D) Principal Coordinate Analysis (PCoA) plot at the genus level with PERMANOVA results (R^2 = 0.162, $P = 9 \times 10^{-3}$). (E) PCoA plot at the species level with PERMANOVA results (R^2 = 0.164, $P = 1.4 \times 10^{-2}$). (F) Relative abundance of the top 20 species averaged by treatment group. Statistical results are based on 10 mice per group.

Colons in 4 mg/kg amoxicillin groups exhibited mucosal damage and crypt architecture reduction of the tissue, accompanied by severe inflammatory cell infiltration, and the symptoms are not obvious in 2 mg/kg group (Figure 4 A). Then, significant alterations in the gut microbiota diversity and composition were observed following amoxicillin treatment. Alpha diversity analysis at the genus level revealed a dose-dependent increase across all indices in the amoxicillin treatment groups compared to the Saline control. Specifically, the Shannon index was significantly elevated in the 4 mg/kg amoxicillin group (p = 0.044 versus Saline), indicating an overall

increase in community diversity. Furthermore, a significant increase in the Pielou evenness index was noted in the amoxicillin treatment groups (p = 0.040 for 4 mg/kg), suggesting a more uniform distribution of taxa. The Simpson index showed a non-significant but trending increase (p = 0.054) (Figure 4 B). Similarly, at the species level, alpha diversity was significantly enhanced, with the Simpson (p = 0.017), Shannon (p = 0.049), and Pielou (p = 0.043) indices all showing statistically significant increases in the 4 mg/kg amoxicillin group (Figure 4 C), confirming a robust, dose-dependent enrichment of microbial richness and evenness.

Beta diversity analysis, assessed via Principal Coordinate Analysis (PCoA), demonstrated a significant separation of microbial communities based on amoxicillin treatment. At the genus level, PERMANOVA revealed a significant difference among groups (R^2 = 0.162, P = 9e-03), indicating that the treatment explained 16.2% of the observed variance in community structure (Figure 4 D). This significant separation was also confirmed at the species level (R^2 = 0.164, P = 1.4e-02)(Figure 4 E). These P-values ($P < 0.05$ for both levels) provide statistical evidence that the overall compositional profiles of the gut microbiota were distinctly altered by the amoxicillin treatment, rather than due to random variation.

Finally, at the species level, analysis of relative abundance revealed a specific and dose-dependent shift in key bacterial taxa. Most notably, the abundance of *Akkermansia muciniphila* exhibited a dose-dependent increase correlating with the rising amoxicillin dosage. In contrast, the levels of other specific species, such as *Bacteroides sartorii and Lactobacillus vaginalis*, remained largely stable across all treatment groups and showed no marked alterations compared to the Saline control, aligning with the observed diversity and structural changes (Figure 4 F).

3.5. The changes in microbial composition and function after amoxicillin treatment.

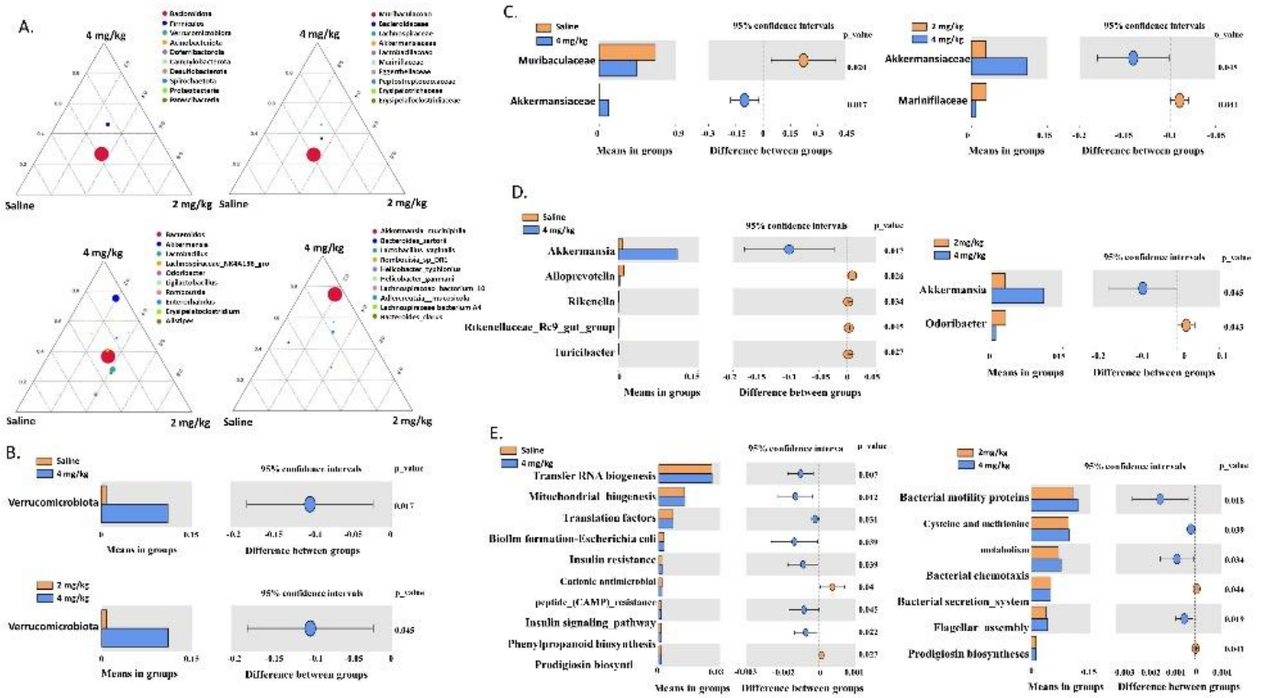


Fig. 5. Differential microbiota analysis in three groups.

(A) The three vertices represent phylum, family, genus and species, and the circles represent the different microbiota at the three levels. The size of the circle is proportional to the relative abundance; the closer the circle is to the vertex, the higher the content of this species in this group. (B) Differential microbiota in the Phylum level. (C) Differential microbiota in the Family level. (D) Differential microbiota in the Genus level. (E) Function prediction analysis of intestinal microbiota in the three groups.

In order to identify the differences in microbiota in the three groups, we performed a ternary plot analysis （Fig. 5 A）. The results showed that the relative abundance of *Bacteroidetes* was highest in the three groups. *Verrucomicrobia* (p = 0.017) are the most abundant phyla in the 4 mg/kg group compared to saline group （Fig. 5 B）. The relative abundance of *Muribaculaceae* was highest in the three groups. *Akkermansiaceae* (p = 0.017) and *Muribaculaceae* (p = 0.02) are the most abundant phyla in the 4 mg/kg group compared to the control group (Fig. 5 C). The relative abundance of *Akkermansiaceae* (p = 0.045) significantly increased in 4 mg/kg group compared with the 2 mg/kg group. On the contrary, the relative abundance of *Marinifilaceae* (p =0.044) significantly increased in 2 mg/kg group compared to 4 mg/kg group. The relative abundance of Bacteroides was highest in the three groups. The relative of abundance of *Akkermansia* (p =0.017) significantly increased in 4 mg/kg group compared 2 mg/kg group and Saline group. *Alloprevotella* (p =0.026), *Rikenella* (p =0.034) and *Turicibacter* (p=0.027) significantly enriched in Saline group. Odoribacter (p =0.043) significantly

enriched in 2 mg/kg group. *Akkormnansia muciriphila* was significantly enriched in 4mg/kg group. No differential microbiota was found between 2mg/kg group and Saline group. We then used Tax4Fun for the function prediction analysis. Mitcchondrial biogenesis, Pheryiproparcid biosynthesis and Bacterial chemotaxis were correspondingly increased in the 4 mg/kg amoxicillin group. Cationic antimicrobial peptide (cAMP) resistance was correspondingly decreased in amoxicillin group. Prodigiosin biosynthesis and Bacteria secretion system were increased in 2 mg/kg group.

3.6 Body weights during the chronic saline and amoxicillin treatment.

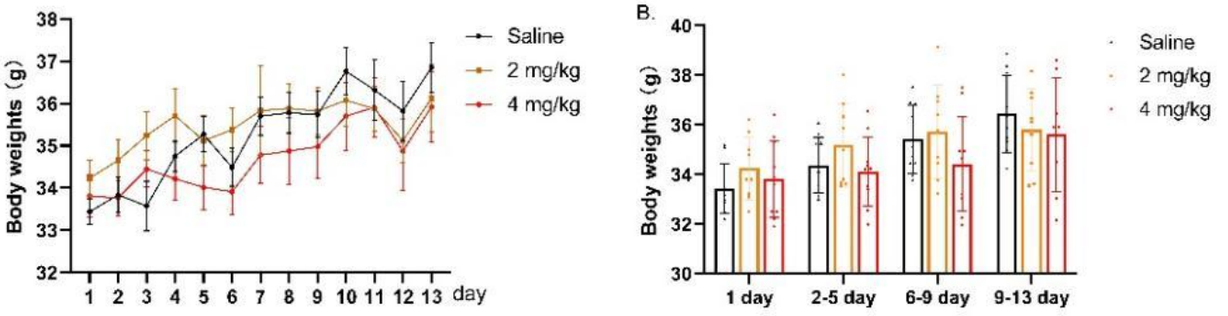


Fig. 6. Effects of chronic amoxicillin treatment on body weight in mice.

(A) the body weight of mice during the experiment. (B) Histogram of body weight. No difference was found in the body weight between each group. Data were expressed as mean ± S.E.M, n=8-10 for each group.

The mean body weight of saline or amoxicillin treated mice during the experiment was shown in Fig. 6. Mice were weighted daily. Body weights increased during the tests (overall effect of age: $F(12,300) = 12.95$, $p < 0.001$). Although amoxicillin treated mice gained weights differently from the controls (interaction of amoxicillin × age: $F(24,300) = 2.203$, $p < 0.001$). No group effects were found when analyzed using One-Way ANOVA, and there were no pair-wise difference between each two groups. These results indicate amoxicillin did not affect body weights during the chronic administration.

Discussion

In this study, mice were intraperitoneal injected with amoxicillin daily (2mg/kg or 4mg/kg) for more than 7 days. After that, they were tested for anxiety- and depressive-like behavior in the open field, light/dark box and forced swim test. The results showed that chronic amoxicillin increased both anxiety- and depressive- like behavior in mice. Amoxicillin treated mice also showed increased fear memory in fear conditioning and slightly

impaired spatial recognition memory in the Y-maze. LFP studies showed that chronic amoxicillin increased gamma oscillations in the VTA and AMY. Late component of auditory evoked potential which related to sensory gating in VTA and AMY were also altered by amoxicillin. The colon in the amoxicillin group showed mucosal damage and shrinkage of crypt organization with severe inflammatory cell infiltration. The relative abundance of *Muribaculaceae*, which was highest in both saline and amoxicillin treated mice, was decreased in amoxicillin groups than in the saline group. *Alloprevotella*, *Turicibacterales*, *Akkermansia muciniphila (A. muciniphila)* were also changed by amoxicillin. These results indicate that amoxicillin not only altered gut flora, but also altered brain functions related to reward and emotional processing in mice.

Various antibiotics have been reported to cause adverse effects on the digestive system, allergic reactions, hematologic responses, and cognitive functions. Amoxicillin is one of the most commonly prescribed antibiotics. It has been found to be a potentially epileptogenic drug that can be damaging to the nervous system (Raposo et al., 2016). However, how gut microbiota would be changed after chronic amoxicillin treatment, and what is the mechanism between gut microbiota changes and depression is not yet very clear. In this study, we found increased anxiety-and depressive-like behavior in mice treated with chronic amoxicillin. This result proves once again that amoxicillin lead to neurological adverse effects in mice. It was reported that chronic amoxicillin administration significantly reduced the levels of serotonin in rat brain tissues, which is thought to be related to depression (Atli et al., 2016). Some studies suggested that antibiotics, particularly macrolides, can enhance the activity of certain neurons in the brain through the inhibition of GABAergic signaling in a dose-dependent manner which has been associated with psychiatric adverse effects (Xiao and Huang, 2021). Prenatal amoxicillin exposure was also found to be developmentally toxic to the hippocampus thus to induce developmental damage to various hippocampal cells (Qin et al., 2023). All of these studies underscores the complex relationship between antibiotics and neurological health.

In this study, LFPs were significantly changed by chronic amoxicillin treatment. High-gamma (60-90 Hz) oscillations in VTA and amygdala were significantly increased, while theta and alpha oscillations in these areas were decreased by amoxicillin (Fig.2). As an aspect of neural activity in the brain, LFP provide a uniquely observable window into the process of excitatory and inhibitory synaptic integration of neural population activity, different frequency-band oscillations reflect diverse neural processing pathways and contributions of transmembrane current from cellular activities (Buzsáki et al., 2012). LFP has been widely used to explore the electrophysiological mechanism of psychiatric disorders, and LFPs can provide stable signals for long-term chronic experiments or clinical applications. For example, the presence of abnormal LFP beta bursts is

significantly correlated with the degree of motor impairment and the severity of rigidity/ bradykinesia in Parkinson's disease (Lofredi et al., 2023). Gamma oscillations have been implicated in attention, memory, and emotional regulation (Headley and Paré, 2013). Emotional negative stimuli can induce enhanced gamma activity (Keil et al., 2001). Individuals with high anxiety show stronger gamma band activity when anticipating arithmetic problems or processing emotional faces (Luo et al., 2009). These findings corroborate our results linking chronic amoxicillin exposure to increased gamma oscillations alongside anxiety- and depressive-like behaviors in mice.

Auditory-evoked potentials (AEPs) are a series of electrical changes that occur in the peripheral and central nervous system in response to auditory processing. They are event-related potentials that are evoked by auditory stimulation and can reflect the neural activity in the auditory cortex and related brain areas. Early waveforms of AEP largely depending on the stimulus and activities in the auditory cortex, while later waveforms of AEP reflects cognitive processing (Rigolli et al., 2022). AEPs can serve as a quick and noninvasive readout of brain activity and are meaningfully related to cognitive and attentional deficits often seen in conditions like autism (Jeste and Nelson, 2009), Alzheimer's Disease (Mei et al., 2021), and drug addiction (Bakhtadze et al., 2021). In this study, chromic amoxicillin advanced the latency of N3, a later component of AEP, in VTA, while decreased its amplitude. Amoxicillin also has an effect on the AEP in AMY, specifically on the P4 component. The VTA is a critical component of the brain's reward system and is involved in regulating both depression and anxiety. The VTA dopamine circuit projecting to the basolateral amygdala selectively controls anxiety-like behaviors in mice (Tong et al., 2023). Our results indicate that chromic amoxicillin altered cognitive and emotional processing in VTA and AMY.

When exploring gut microbes of chronic amoxicillin treated mice by using the 16S rRNA, we found that the alpha diversity significantly decreased in amoxicillin group compared to the saline group, which is consistent with previous studies (Deprey and Uno, 2016). It was showed that amoxicillin decreased intestinal microbial diversity and increased stress-associated behaviors in zebrafish (Deprey and Uno, 2016). The microbial composition of 4 mg/kg amoxicillin group is quite different compared to the 2 mg/kg and saline group. In both the saline and amoxicillin groups, the relative abundance of *Muribaculaceae* was the highest in the colons of the mice. But *Muribaculaceae* was decreased in 4mg/kg amoxicillin groups than in the saline group. In multiple depression models, the alterations of family *Muribaculaceae* were gut microbiota and fecal metabolome features (Liu et al., 2022). The family taxon *Muribaculaceae* exerted anti-inflammatory effects by synergistically producing short-chain fatty acids (SCFAs) (Smith et al., 2019). SCFAs and SCFA-producing

bacteria were beneficial to the stabilization of blood-brain barrier. They are important for maintaining the gut epithelial barrier, increasing thickness of the protective mucus layer, and modulating the secretion of cytokine and antibodies. SCFAs also stimulated enteroendocrine cells to secreta neurotransmitter by exciting nerve pathways, activating G protein-coupled receptors, and inhibiting histone deacetylases. It was found that an aged microbiome alone is sufficient to decrease SCFAs in the host and to produce cognitive decline (Lee et al., 2020). In another study, *norank_f_Muribaculaceae* was found to negative correlated with serum CORT, CRF and ACTH, which are HPA axis-related hormones, and it was positive correlated with 5-HT and BDNF in the hippocampus (Fan et al., 2021). All these study indicate that the decreased abundance of *Muribaculaceae* in amoxicillin treated mice is related with their anxiety and depressive-like behavior.

Besides the *Muribaculaceae*, the *Alloprevotella*, *Rikenella*, *Rikenellaceae_Rc9_gut_group* and *Turicibacter* were also decreased in 4 mg/kg amoxicillin treated mice than the controls (Fig.5). *Alloprevotella*, which produces short-chain fatty acids and indole derivatives, is more abundant in healthy patients compared to those with depression, that may be involved in depression (Zheng et al., 2021). A study on postpartum depression found that *Alloprevotella* was associated with depressive phenotypes (Tian et al., 2021). Depression-like symptoms in mice subjected to unpredictable chronic mild stress were relieved after CD36 knockout, and analysis of gut microbiota in CD36 KO mice revealed a significant increase in the abundance of *Alloprevotella* (Bai et al., 2021). Decreased abundance of *Alloprevotella* were also found in Sigma-1 receptor knockout mice, which showed depression-like behaviors (Li et al., 2023). *Turicibacterales*, *Turicibacteraceae*, and *Turicibacter* were related to the increased production of bile acids, the bile acids levels were negatively correlated with the severity of depressive symptoms. And this taxa has also been reported to reduce in depression state (Lin et al., 2024). All these finding supports the idea that alterations in the gut microbiota play an essential role in the etiology of anxiety and depression. Gut flora was found to affect brain metabolites such as dopamine and tryptophan (precursors to 5-HT). Disturbances in gut flora lead to morphological rearrangements of astrocytes and microglia in brain regions controlling emotional behavior. Thus, intestinal flora dysbiosis might be largely associated with the adverse effects of amoxicillin.

A. muciniphila is a mucin-degrading bacterium of the intestinal niche, exerting beneficial effects on the host metabolic profile. In this study, the relative abundance of *Akkermansiaceae* significantly increased in 4 mg/kg group compared with the saline group ($p = 0.017$) and the 2 mg/kg group ($p = 0.045$). *A. muciniphila* is widely recognized as a promising therapeutic probiotic against metabolic disorders such as obesity, type 2 diabetes and cardiovascular diseases. *A. muciniphila* was found to be decreased in depressive patients. However,

in specific intestinal microenvironments, its excessive enrichment may be not beneficial. *A. muciniphila* was reported to participate in the development of neuropsychiatric disorders by aggravating inflammation and influencing mucus production. For example, the gut microbiota of patients suffering from Parkinson's disease (PD) or multiple sclerosis exhibits a characteristic signature of *A. muciniphila* abundance (for a review, see (Chiantera et al., 2023)). Many studies have revealed an increase in *A. muciniphila* abundance in PD patients. Individuals with rapid eye movement sleep behavior disorder, which is considered a pre-motor symptom of PD, also exhibited elevated intestinal *Akkermansia* levels. Although speculative, some authors hypothesized that the activation of the Toll-like Receptor 2 (TLR2) or the modulation of glucose and cholesterol homeostasis [9,25], induced by *Akkermansia*, may determine unexpected deleterious consequences for neurological health in certain individuals. Our results suggested that chronic amoxicillin might increase the susceptibility of PD by increasing *Akkermansia* abundance in mice, which needs further researches.

Tax4Fun can predict the functional capabilities of microbial communities based on 16S rRNA data samples. It offers a rapid method to estimate the functional spectrum of microbial communities by linking the taxonomic information of microbial communities with pre-calculated metabolic reference profiles, enabling a quick analysis of metabolic profiles. In this study, mitcchondrial biogenesis, pheryiproparcid biosynthesis, bacterial chemotaxis, insulin resistance, insulin signaling-pathway of microbial communities were correspondingly increased in the 4 mg/kg amoxicillin group, while the cationic antimicrobial peptide (cAMP) resistance was correspondingly decreased by amoxicillin, suggests that changes in microbial communities also lead to changes in their functioning. Although the relationship between these functional alterations in gut microbes and anxiety and depression is unknown, similar alterations have been implicated in studies of brain cell functioning. For example, there is a complex association between insulin resistance and anxiety and depression, and this relationship is primarily mediated through effects on insulin signaling pathways. Insulin resistance in the brain may lead to hypometabolism, vascular alterations, dysregulated neuroplasticity, and structural changes, which in turn trigger or amplify depressive symptoms (Alberry and Silveira, 2023). In insulin-resistant states, dopamine release may be directly affected, leading to anxiety- and depression-like behaviors. In addition, abnormalities in the insulin signaling pathway may further influence mood regulation by affecting neurotransmitter function, neurogenesis, and mitochondrial function (Gruber et al., 2023). Thus, insulin resistance and abnormalities in the insulin signaling pathway may be important molecular mechanisms in the development of anxiety and depression. On the other hand, cAMP signaling in unmedicated depressed patients' brain is decreased, and treatment with selective serotonin reuptake inhibitors (SSRIs), which are

commonly used antidepressants, increases cAMP signaling (Fujita et al., 2017).

There are several limitations of this study should be acknowledged. First, the amoxicillin doses used in this study (2 and 4 mg/kg, i.p.) represent a low-dose intervention model rather than a maximal microbiota-depleting regimen. This precludes direct comparison with studies utilizing broad-spectrum antibiotic cocktails or germ-free models. Second, while our data reveal concurrent changes in gut microbiota composition, LFP oscillatory patterns, and behavioral outputs, the lack of restoration experiments—such as fecal microbiota transplantation or specific strain supplementation—prevents us from strictly concluding that the observed neural and behavioral alterations are directly mediated by the specific microbial shifts identified. Thus, our findings should be interpreted as strong associative evidence warranting further mechanistic validation. However, even chronic, sub-therapeutic exposure to amoxicillin—administered intraperitoneally—was sufficient to elicit anxiety- and depressive-like behaviors and to enhance gamma oscillations, underscoring that greater attention should be paid to the psychiatric consequences of low-level antibiotic exposure.

In conclusion, we found that long-term administration of amoxicillin resulted in significant changes in the composition and function of the gut microbiota. These mice had increased anxiety- and depressive- like behaviors, enhanced cue-induced fear memory, and mild impairment of spatial recognition memory. As an aspect of neural activity in the brain, LFP studies showed increased gamma oscillations in VTA and AMY, which are brain areas critical for reward and emotional processing. Auditory-evoked potential in these two brain area were also affected by amoxicillin. Amoxicillin is widely used in the treatment of respiratory tract infections, skin and parenchyma infections, gonorrhea and other infectious diseases. It is also one of the most commonly used prophylactic medications following surgical treatment or injury (Cuevas-Gonzalez et al., 2023). Like other antibacterial antibiotics, improper use of amoxicillin leads to drug side effects such as drug resistance and other problems. Our study indicate again that gut microbes are involved in the development of anxiety and depression, and could be a potential target for drug development in the management of these disorders. We should pay more attention to the effects of antibiotic use on psychiatric disorders.

Conflict of Interest:

The authors declare no competing financial interest.

Funding Declaration:

This work was supported by the National Natural Science Foundation of China (82201597, 32460211) and the Natural Science Foundation of Yunnan Province (202401AT070332).

Data Availability

The 16S rRNA gene amplicon sequencing data generated in this study have been deposited in the NCBI Sequence Read Archive (SRA) under BioProject accession number PRJNA1548237 and are publicly available at https://www.ncbi.nlm.nih.gov/bioproject/PRJNA1548237. All other datasets generated and/or analysed during the current study, including the behavioral, electrophysiological and histological data, are available from the corresponding author on reasonable request

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