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ORIGINAL RESEARCH

Impact of Altered Gut Microbiota on Ketamine-Induced Conditioned Place Preference in Mice

Chan Li[1,2,*], Chen Zhu[3,*], Genghong Tu[4,*], Zhijie Chen[1], Zhixian Mo[1,5], Chaohua Luo[1,5]

[1]School of Traditional Chinese Medicine, Southern Medical University, Guangzhou, People's Republic of China; [2]School of Life Sciences, Guangzhou University, Guangzhou, People's Republic of China; [3]The Second Affiliated Hospital of Guangzhou University of Chinese Medicine, Guangzhou, Guangdong, People's Republic of China; [4]Department of Sports Medicine, Guangzhou Sport University, Guangzhou, Guangdong, People's Republic of China; [5]Guangdong Provincial Key Laboratory of Chinese Medicine Pharmaceutics, Southern Medical University, Guangzhou, People's Republic of China

*These authors contributed equally to this work

Correspondence: Zhixian Mo, Mo; Chaohua, Luo, School of Traditional Chinese Medicine, Southern Medical University, 1023-1063 Shatai South Road, Guangzhou, 510515, People's Republic of China, Tel +020-61648261; +020-61648244, Email cherrymo@fimmu.com; 120557369@qq.com

Objects: Ketamine is a drug of abuse worldwide and current treatments for ketamine abuse are inadequate. It is an urgent need to develop novel anti-addictive strategy. Since gut microbiota plays a crucial role in drug abuse, the present study investigates the impact and mechanisms of the gut microbiota in addictive behaviors induced by ketamine addiction.

Methods: Conditioned place preference (CPP) was employed to assess addiction, followed by 16S rRNA gene sequencing to elucidate alterations in the gut microbiota. Furthermore, qRT-PCR, ELISA, and immunohistochemistry were conducted to evaluate the expression levels of crucial genes and proteins associated with the gut-brain axis. Additionally, we investigated whether ketamine addiction is regulated through the gut microbiota by orally administering antibiotics to establish pseudo-germ-free mice.

Results: We found that repeated ketamine administration (20 mg/kg) induced CPP and significantly altered gut microbiota diversity and composition, as revealed by 16S rRNA gene sequencing. Compared to the control group, ketamine exposure exhibited differences in the relative abundance of 5 microbial families, with 4 (*Lachnospiraceae, Ruminococcaceae, Desulfovibrionaceae* and *Family-XIII*) showing increases, while one (*Prevotellaceae*) displayed a decrease. At the genus level, five genera were upregulated, while one was downregulated. Furthermore, COG analysis revealed significant differences in protein functionality between the two groups. Additionally, axis series studies showed that ketamine dependence reduced levels of tight junction proteins, GABA and GABRA1, while increasing BDNF and 5-HT. Moreover, an oral antibiotic cocktail simulating pseudo germ-free conditions in mice did not enhance the addictive behavior induced by ketamine.

Conclusion: Our study supports the hypothesis that ketamine-induced CPP is mediated through the gut microbiota. The present study provides new insights into improvement of efficient strategy for addiction treatment.

Keywords: ketamine abuse, BDNF, GABRA1, gut microbiota, gut-brain axis

Introduction

Repeated use of ketamine, an N-methyl-D-aspartic (NMDA) receptor antagonist, could lead to abuse and trigger central excitatory, hallucinogenic, euphoric, and analgesic effects.[1] Numerous studies have shown that ketamine abuse is widespread across China, particularly among adolescents. It has resulted in negative impacts on the physical and mental health of young individuals.[2] Furthermore, it is worth mentioning that a single dose of ketamine (10 mg/kg) produces rapid antidepressant effects. However, its addictive and neurotoxic properties have significantly restricted its utilization.[3] Given currently available treatments for ketamine abuse are far from ideal, it is urgent need to develop novel treatment methods.

Emerging studies indicate that gut microbiota, a vast community of microorganisms inhabiting the gastrointestinal tract, profoundly influences brain function and behavioral changes associated with drug abuse. Accumulating clinical

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evidences show the compositions of gut microbiota were changed in the patients with the drugs used disorder.[4,5] In turn, fecal microbiota from alcohol abusers transplanted to the germ free (GF) mice show increased intestinal permeability and alcohol consumption.[4] Furthermore, the rats treating with oral antibiotics enhanced the sensitivity to the rewarding effects of cocaine.[5] These findings highlight the bidirectional relationship between the gut microbiota and the central nervous system, mediated by the microbiota-gut-brain axis (MGBA).[6–8] Additionally, the specific bacterial specie *Bacteroidetes* was altered exposed to the morphine, while giving *Bacteroidetes* to the mice reversed the conditioned place preference (CPP) caused by morphine.[9] Notably, ketamine abusers frequently seek medical attention due to gastrointestinal issues. Approximately one-quarter (168, 27.5%) of 611 ketamine abusers reporting gastrointestinal symptom.[10] These findings provide solid evidences that gut microbiota is a novel target for addiction treatment. However, the effect of intestinal microbiota on ketamine addiction and its mechanisms of action have not been characterized, and better understanding will improve ketamine addiction.

In this study, we performed bioinformatics analysis on the gut microbiota during development of antibiotic treatment and ketamine dependence. We also determined mechanisms of action, and treatment methods for ketamine addiction. Finally, we characterized the effects of gut microbiota on ketamine addiction and the mechanisms through the gut-brain axis. Our study provided a basis for development of novel strategies for treatment of ketamine addiction.

Materials and Methods

Animals

Kunming mice (male, 18–22 g) were provided by the Experimental Animal Center from Southern Medical University (Guangzhou, China). The mice were housed at room temperature (20 ± 2) °C in a room with a relative humidity of 60 ± 5% with a 12 h light/dark cycle. The animals were provided food and water *ad libitum*. The mice were allowed to acclimatize for a minimum of 3 days prior to commencement of experiments. All animal procedures were approved by the Institutional Animal Care and Use Committee of Southern Medical University (permission number: L2018173) and conducted in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals (NIH Publication No. 85–23, revised 1985).

Drug Administration

Ketamine was purchased from the China National Narcotics Laboratory and was intraperitoneally injected (i.p). with a dose of 20 mg/kg as our previous study.[11] For oral antibiotic experiment, the antibiotic cocktail consisted of Bacitracin (0.5 mg/mL), Neomycin (2 mg/mL), Vancomycin (0.2 mg/mL), Pimaricin (1.2 μg/mL), which have good antibacterial effect on gut. Based on the administered dose, weighed the appropriate amount of antibiotics, then added them to 1000 mL of 0.9% sterile water. After fully dissolving, prepare the antibiotic mixture for drinking water. Each group received 200 mL, and the solution was replaced every two days. For injection antibiotic experiment, the antibiotic cocktail consisted of Bacitracin (293 U/kg), Neomycin (20 mg/kg), Vancomycin (1.67 mg/kg) in saline. This mixture was administered daily at a dosage of 0.1 mL per 10 g of body weight for seven days prior to behavioral testing. Both drugs and antibiotic were diluted in 0.9% sterile saline.

Conditioned Place Preference Testing

CPP testing was conducted as previously described, with slight modifications[9,12] (Figure 1A). The test consisted of three stages over a 19-day period. Mice were assessed for ketamine place preference using a CPP box and Smart 3.0 software. In the first stage (days 1 to 3), preferences were determined at baseline, and mice with a preference for the white boxes were excluded. In the second stage (days 4 to 18), the mice were randomized into the following 2 groups (n = 8 per group): control group and ketamine group. Place preference conditioning was performed by training the mice for 45 min in a white box with an injection of ketamine (20 mg/kg) or saline at 9:00 am. A second injection of saline was paired with the black box at 17:00 pm. This procedure was repeated for 14 consecutive days. In the third stage (Day 19), CPP testing was performed by allowing each mouse to explore the two boxes freely, and time spent in the white box was recorded (Figure 1A).

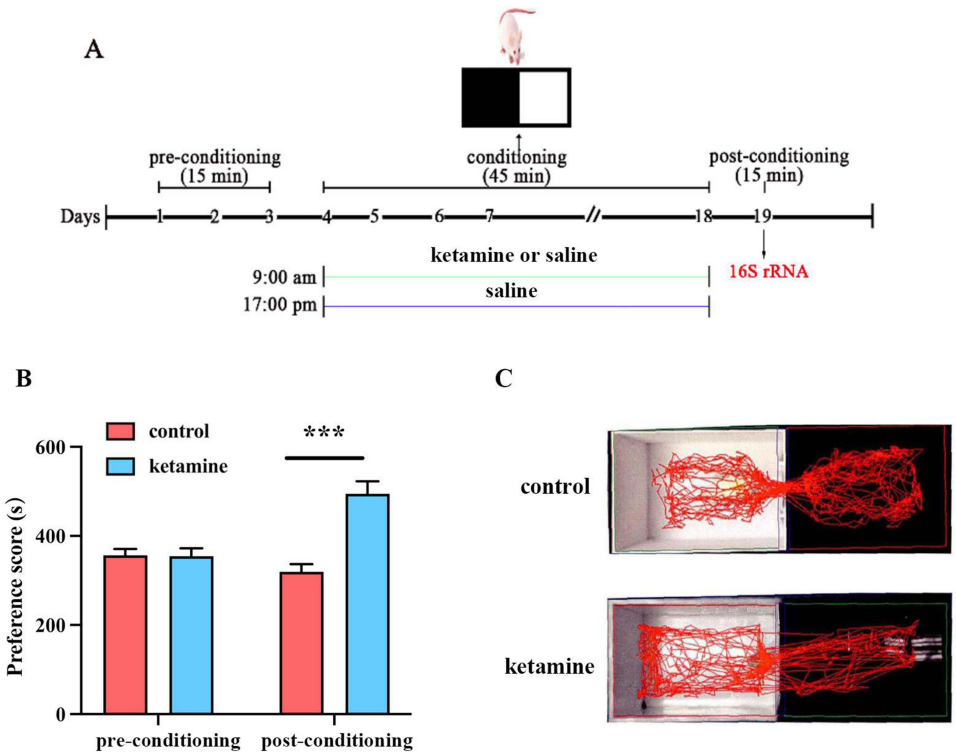

Figure 1 Ketamine induced CPP in mice. (**A**) The experimental design for CPP testing. (**B**) Time spent in the white box by mice was measured, showing no differences before establishing the ketamine-induced CPP model. (**C**) Representative trajectories of mice in the white box during post-conditioning phase with or without ketamine administration. Each bar represents the mean ± SEM. ***$P < 0.001$ by unpaired two-tailed Student's *t*-test.

For oral antibiotic experiment, the mice were randomized into the following 3 groups (n = 8 per group): (1) control group, (2) oral antibiotic-treated (Abx) group, (3) Abx + ketamine group. Briefly, the mice were treated with sterile water or antibiotic cocktail for 21 days and ketamine or saline were injected on day 12. The experimental protocols of the pre-conditioning, training and post-conditioning phases were the same as those for the CPP test. To measure the behavioral effects of parenterally administered antibiotics, we also injected the antibiotic cocktail in saline daily for seven days prior to behavioral testing.

16S rRNA Gene Sequence Analysis of the Gut Microbiota in Fecal Samples

Sequencing was performed by Majorbio Bio-pharm Technology Co., Ltd, Shanghai, China. Fresh fecal samples from all mice were collected using a sterile fecal collector, kept on ice, and stored at −80°C. Microbial DNA was extracted from fecal samples using the E.Z.N.A.® soil DNA Kit (Omega Bio-tek, USA) following the manufacturer's protocols. The final DNA concentration and purity were determined using a NanoDrop 2000 UV–Vis spectrophotometer (Thermo Scientific, USA), and DNA quality was confirmed using 1% agarose gel electrophoresis. The V3-4 regions of the bacterial 16S rRNA gene was amplified by PCR using the forward primer (338F: 5'-ACTCCTACGGGAGGCAGCAG-'3) and the reverse primer (806R: 5'-GGACTACHVGGGTWTCTAAT-'3). The PCR products were separated by gel electrophoresis and purified using an AxyPrep DNA Gel Extraction Kit (Axygen Biosciences, USA). The 2×300 PE library was constructed using the purified amplified fragments, and bacterial communities were investigated using a MiSeq PE300 sequencing platform (Illumina, USA).

Bioinformatics Analysis

Data analysis was performed using the free online platform Majorbio I-Sanger Cloud Platform (http://www.i-sanger.com). Operational taxonomic units (OTUs) were clustered at a 97% similarity cutoff using UPARSE (version 7.1, http://drive5.com/uparse/), with chimeric sequences identified and removed via UCHIME. Taxonomic classification of each 16S rRNA gene sequence was conducted using the RDP Classifier algorithm (http://rdp.cme.msu.edu/) against the Silva

(SSU123) 16S rRNA database, applying a confidence threshold of 70%. The following statistics were performed using Mothur software (http://www.mothur.org/wiki/Calculators): Chao index was calculated for evaluation of α-diversity. Principal co-ordinates analysis (PCoA) was performed to estimate β-diversity. The Wilcoxon rank-sum test was performed using STAMP (version v.2.1.3) to identify phyla, family and genera that showed significant differences in abundance among the groups (confidence interval method) using the Stats package in R and the SciPy package in PYTHON. Phylogenetic Investigation of Communities by Reconstruction of Unobserved States (PICRUSt) was used to predict microbial functions.

Total RNA Extraction and Quantitative Real-Time PCR

Total RNA from the hippocampus and colon were isolated using TRIzol according to the manufacturer's instructions (Takara Bio, Japan). Five hundred nanograms of total RNA was reverse transcribed using PrimeScript™ RT reagent kit (Takara Bio, Japan). The reactions were incubated at 42 °C for 15 min, then at 85 °C for 5 s, and the resulting cDNA was stored at −20 °C. Quantitative real-time PCR (qRT-PCR) were performed using SYBR® Premix Ex Taq ™ (Takara Bio, Japan) and a LightCycler®96 Real-Time PCR system (Roche Diagnostics). Each well was loaded with 20 μL comprising 2 μL of cDNA, 10 μL of SYBR *Premix Ex* Taq II, 0.8 μL of target primers and 7.2 μL of water. The thermal program was as follows: precycling at 95 °C for 15s, then 40 cycles of denaturation at 95 °C for 5 s, and annealing at 60 °C for 30s. The $2^{-\Delta\Delta CT}$ method was used to determine the expression levels of brain derived neurotrophic factor (BDNF) and α1 subunit of γ-aminobutyric acid A receptor (GABRA1). In addition, Zonula occluden-1 (ZO-1) and occludin were quantitated relative to the control gene GAPDH.

Immunohistochemistry

Brain tissues were collected from mice (n = 3 in each group) after transcardial perfusion with 1 × PBS and 4% paraformaldehyde (PFA). The tissues were then postfixed in 4% PFA for 24 h. Brain slices were prepared as described previously.[11] For immunohistochemistry, primary antibodies (anti-GABRA1 or anti-BDNF antibody) were diluted with 10 mm PBS at a ratio of 1:200. Protein expression was quantified by Image J.

Neurotransmitter Quantitation

After the animals were sacrificed, serum was collected and stored at −80 °C. Serum levels of gamma-aminobutyric acid (GABA) and serotonin (5-HT) were measured using commercial ELISA kits (GABA: Meimian, China; 5-HT: Cusabio, China).

Statistics Analysis

Statistical analyses were performed using IBM SPSS 22.0 statistical software. Student's *t*-test and Kruskal–Wallis rank sum test were used to analyze 16S rRNA gene sequencing data. Student's *t*-test and one-way ANOVA were used for analysis of all other data. Differences between individual groups were assessed by Tukey's multiple comparison test. Sample sizes were determined based on prior experience with the tests used. Data are presented as the mean ± standard error of the mean (SEM). $P < 0.05$ was considered statistically significant. All histogram results were shown using the GraphPad prism v9 software.

Results

Ketamine Induced CPP in Mice

Figure 1A illustrates the timeline of our behavioral experiment. Prior to the start of training, there were no significant differences in the time spent in the white box among the groups (Figure 1B). However, after 14 consecutive days of twice-daily training sessions, intraperitoneal injection of ketamine led to a significant increase in both the time spent and the movement trajectories within the white box, indicating the successful induction of CPP by repeated ketamine administration (Figure 1B and C).

Ketamine Addiction Perturbed the Expression of Proteins and Neurotransmitters Associated with Drug Dependence

Accumulating researches has identified BDNF as a key biological marker involved in the neuropathology of substance use disorders.[13,14] It is also notable that GABRA1 closely related to learning memory, cognition and drug dependence[15] and further knockout of GABRA1 results in symptoms that resemble the severe drug dependence phenotype observed in human patients.[16] Then, we investigated whether the expression of BDNF and GABRA1 were changed after ketamine dependence. We found significant increase in BDNF mRNA expression (Figure 2A) and a decrease in GABRA1 mRNA expression (Figure 2B) in the hippocampus, consistent with previous observations regarding ketamine's effects on these genes.[15–17]

To further explore the impact of neurotransmitters on the neurological system, we evaluated the serum expression levels of GABA and 5-HT. Our results showed that ketamine significantly reduced serum GABA levels (Figure 2C), while serum 5-HT levels increased with ketamine-induced disorder (Figure 2D). Recently, it has been recognized that various neurotransmitters originating from the gut microbiota modulate both peripheral and central sensitization, thereby influencing learning memory. The systemic availability of neurotransmitters and neurotrophic factors heavily relies on the composition of gut microbiota and the integrity of the intestinal barrier. These findings suggest that changes in serum neurotransmitter levels may be closely associated with functional connectivity within the gut-brain axis.

Ketamine Addiction Impair the Intestinal Barrier

To determine whether the intestinal barrier permeability modulated the expression levels of adhesion molecules such as the tight junction (TJ) proteins ZO-1 and occludin, which are critical to intestinal barrier integrity.[18] Our results revealed that ketamine decreased the mRNA expression levels of both ZO-1 and occludin in the colon (Figure 3A and B), suggesting damage to intestinal barrier function.

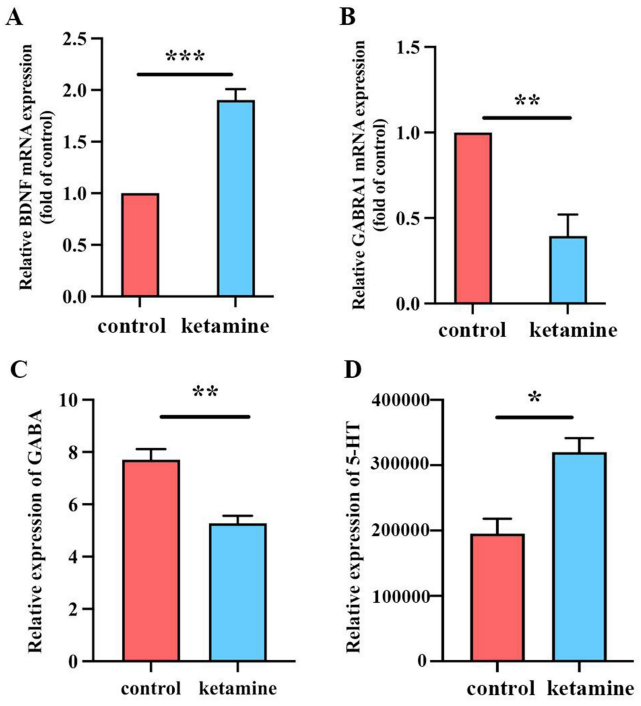

Figure 2 Ketamine aperturbed the expression of proteins and neurotransmitters associated with drug dependence. (**A** and **B**) The mRNA expression levels of BDNF (**A**) and GABRA1 (**B**) in mouse hippocampus samples (n = 3). (**C** and **D**) Serum levels of GABA and 5-HT were measured using ELISA (n = 3). Each bar represents the mean ± SEM. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ by unpaired two-tailed Student's *t*-test.

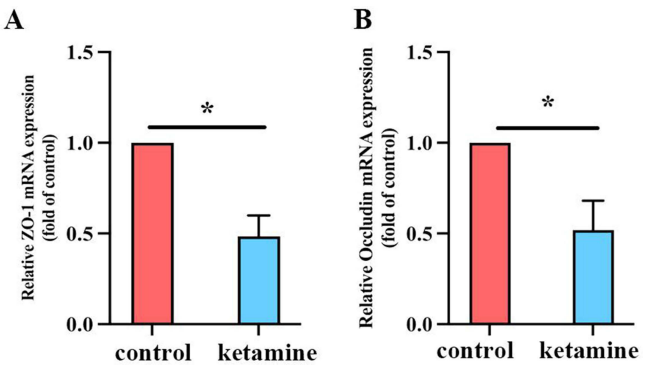

Figure 3 Ketamine addiction resulted in reduced intestinal endothelial TJ mRNA expression. (**A**) The mRNA expression levels of ZO-1 in colon samples from control and ketamine-treated mice (n = 3). (**B**) The mRNA expression levels of occludin in colon samples from control and ketamine-treated mice (n = 3). Each bar represents the mean ± SEM. $*P < 0.05$ by unpaired two-tailed Student's *t*-test.

Structural Changes in Fecal Microbiota Following Ketamine Use Disorder

It has been demonstrated that the gut microbiota plays a critical role in the gut-brain axis and in the pathophysiology of drug addiction.[19–21] To determine whether ketamine dependence alters the composition of the gut microbiota, we performed 16S rRNA gene sequencing analysis. Fecal samples from both the control and ketamine groups were analyzed using an Illumina MiSeq sequencing system. A total of 673 OTUs and 18812 sequences per sample were identified and clustered using a 97% similarity threshold. These OTUs were utilized for subsequent analysis.

Based on the data of OTUs, the Chao index is used to evaluate the α-diversity of gut microbiota. The results showed that the gut microbiota in the ketamine group exhibited a reduced richness trend with lower Chao, compared to that in the control group (Figure 4A). Additionally, β-diversity analysis was conducted to assess similarities and differences in the overall community structure, providing a clearer insight into compositional changes in the gut microbiota of mice treated with ketamine. The PCoA was employed to assess β-diversity. As shown in Figure 4B and C, the ketamine group exhibited a noticeable structural shift. Furthermore, a significant compositional difference in microbiota structure was observed between these two groups.

We further investigated the gut bacterial composition in each group at the phylum, family and genus levels between the two groups (Figure 4D–F). A total of 7 phyla were identified in the current study (Figure 4D). The composition of gut microbiota revealed 5 family of microbes with relative abundance different from those in the control group, of which 4 were increased and other one was decreased by ketamine treatment (Figure 4G). In detailed, *Lachnospiraceae, Ruminococcaceae, Desulfovibrionaceae* and *Family-XIII* levels in the ketamine were significantly up-regulation, while *Prevollaceade* was down-regulation. At a genus level, compared to the control group, ketamine exposure resulted in a significant increase in relative abundance of 5 genera of the fecal microbes, including *Desulfovibrio, Ruminococcaceae_UCG-004, Lachnospiraceade_UCG-006, Ruminiclostridium_9, norank_f_Ruminococcaceae*. Conversely, *Rikenella* was down-regulation after ketamine addiction (Figure 4H). Taken together, these results indicated that the composition of gut microbiota were remarkable alterations in mice and further support that changes in gut microbiota are involved in the formation of addictive behaviors induced by ketamine.

Changes in Microbiota Function Induced by Ketamine

To further explored ketamine-induced alterations in the microbiota on protein function. We performed PICRUSt and each OTU was then evaluated using the Clusters of Orthologous Groups of proteins (COG) database. The results showed that compared to the control group, relative abundance of translation and signal transduction mechanisms are significantly increased; however, ribosomal structure and biogenesis, cell wall/membrane/envelope biogenesis, transcription, coenzyme transport and metabolism, nucleotide transport and metabolism, posttranslational modification, protein turnover, and chaperones were decreased in ketamine group (Figure 5).

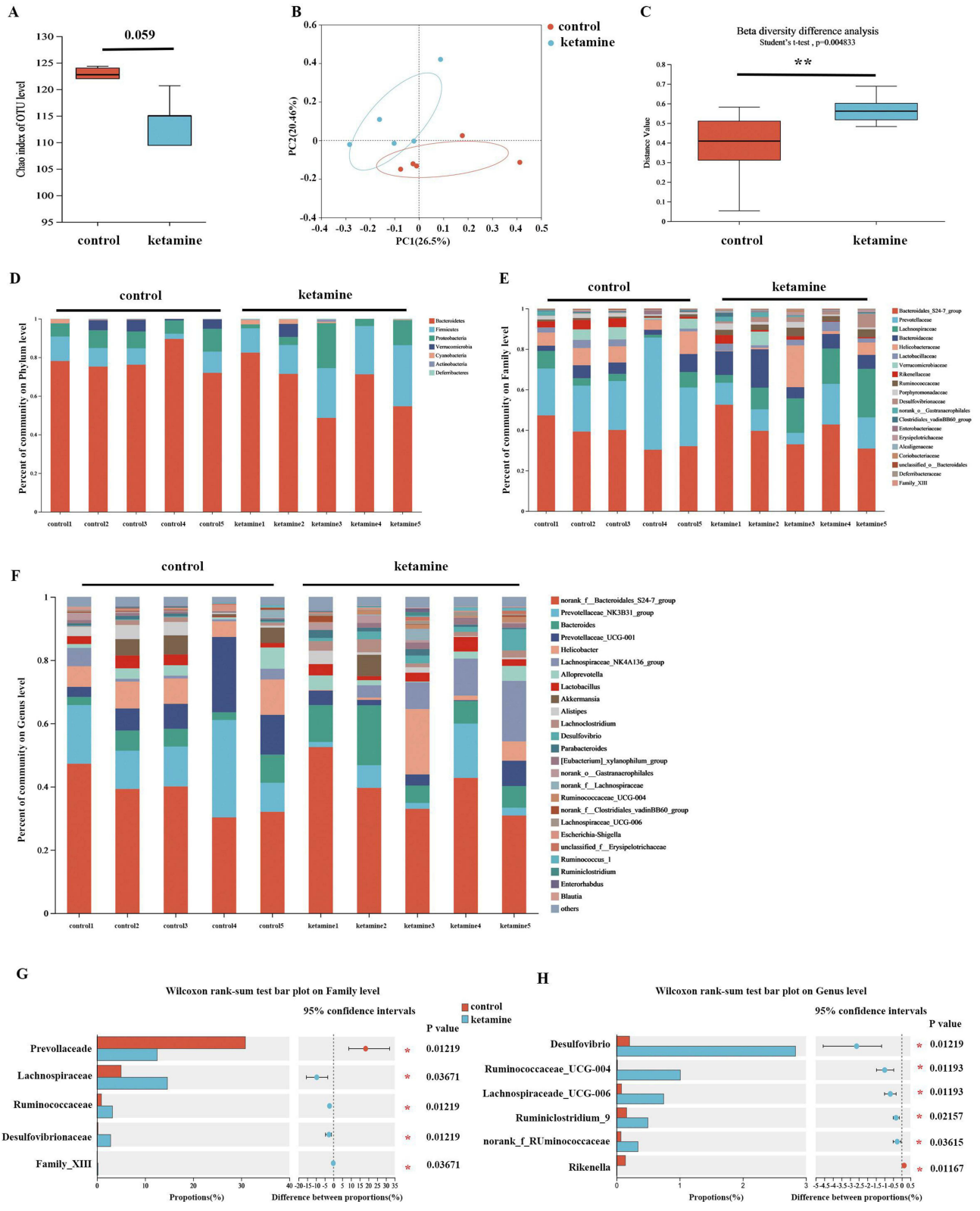

Figure 4 Changes in the composition of intestinal microbiota during ketamine exposure. (**A**) α-diversity of microbial communities in each group, measured using Chao index (n = 5). (**B**) Principal Coordinates Analysis (PCoA) analysis based on the weighted UniFrac distance, showing β-diversity differences among groups (**C**) β-diversity difference analysis of the microbial communities in each group. (**D**–**F**) Characteristics of gut microbiota composition at the phylum level (**D**), family level (**E**) and genus level (**F**) after treatment with ketamine. (**G**–**H**) Significant differences in the relative abundances of microbes at the family level (**G**) and genus level (**H**) between the control and ketamine groups.Each bar represents the mean ± SEM. $*P < 0.05$, $**P < 0.01$ by Wilcoxon rank-sum test.

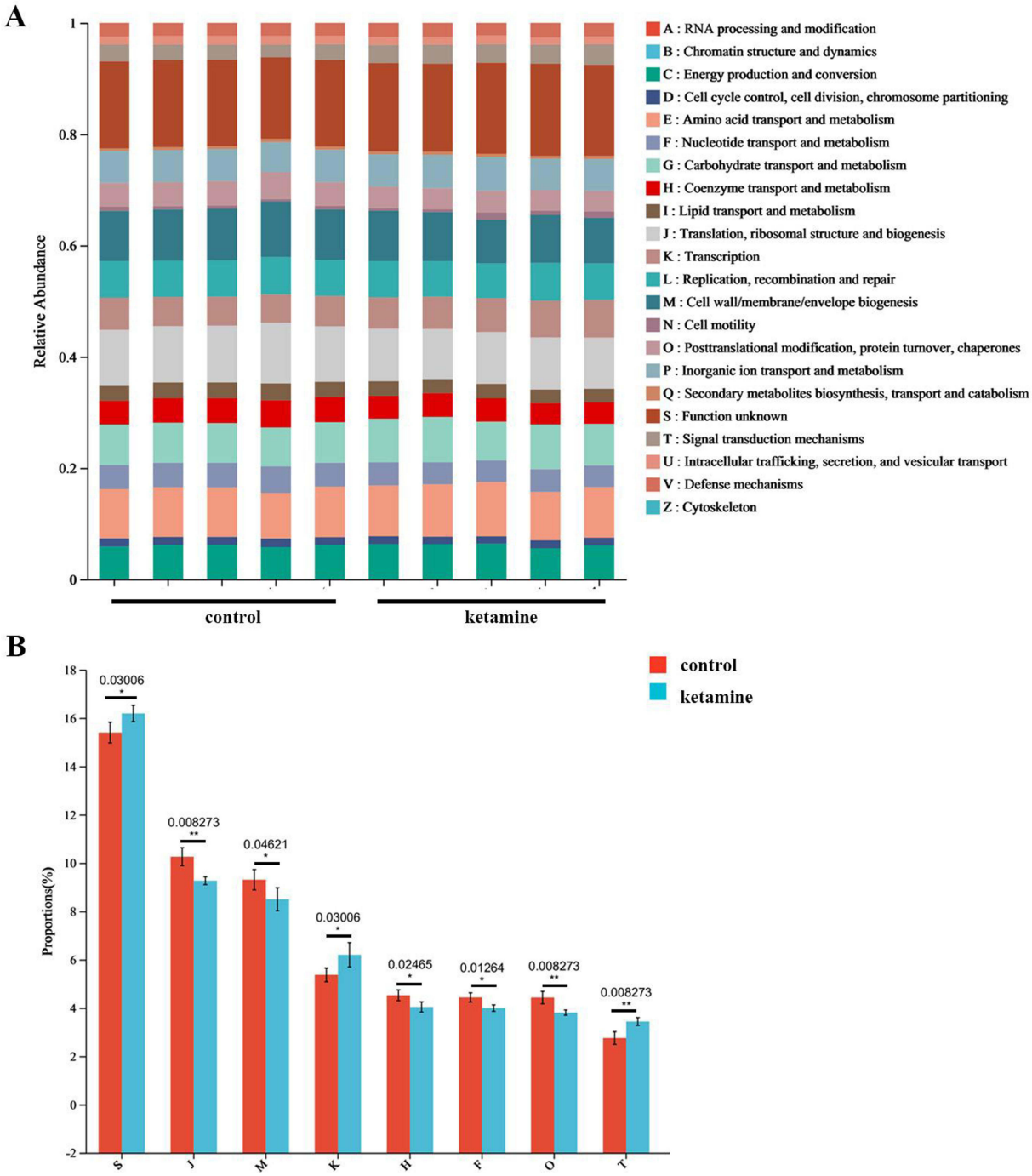

Figure 5 Microbiota function prediction according to COG. (**A**) The relative abundance of COG function classification in the control and ketamine group (n =5). (**B**) The Student's t-test box bloy of COG between two groups. Each bar represents the mean ± SEM. $*P < 0.05$, $**P < 0.01$ by Student's *t*-test.

Alteration in Gut Bacteria with Oral Antibiotics Affects the Behavioral Responses to Ketamine Abuse

To further explore the impact of gut microbiota on ketamine abuse, we administered a mixture of four non-absorbable antibiotics (Abx) for 21 days to deplete the gut microbiota (Figure 6A). Compared to the control group, the microbiota α-diversity and β-diversity in the mice gavaging with antibiotics cocktail for 21 consecutive days were significantly decreased (Figure 6B–D). Furthermore, PCoA indicated significant changes in the fecal microbial community composition between the Abx group and the Abx + ketamine group compared to the control

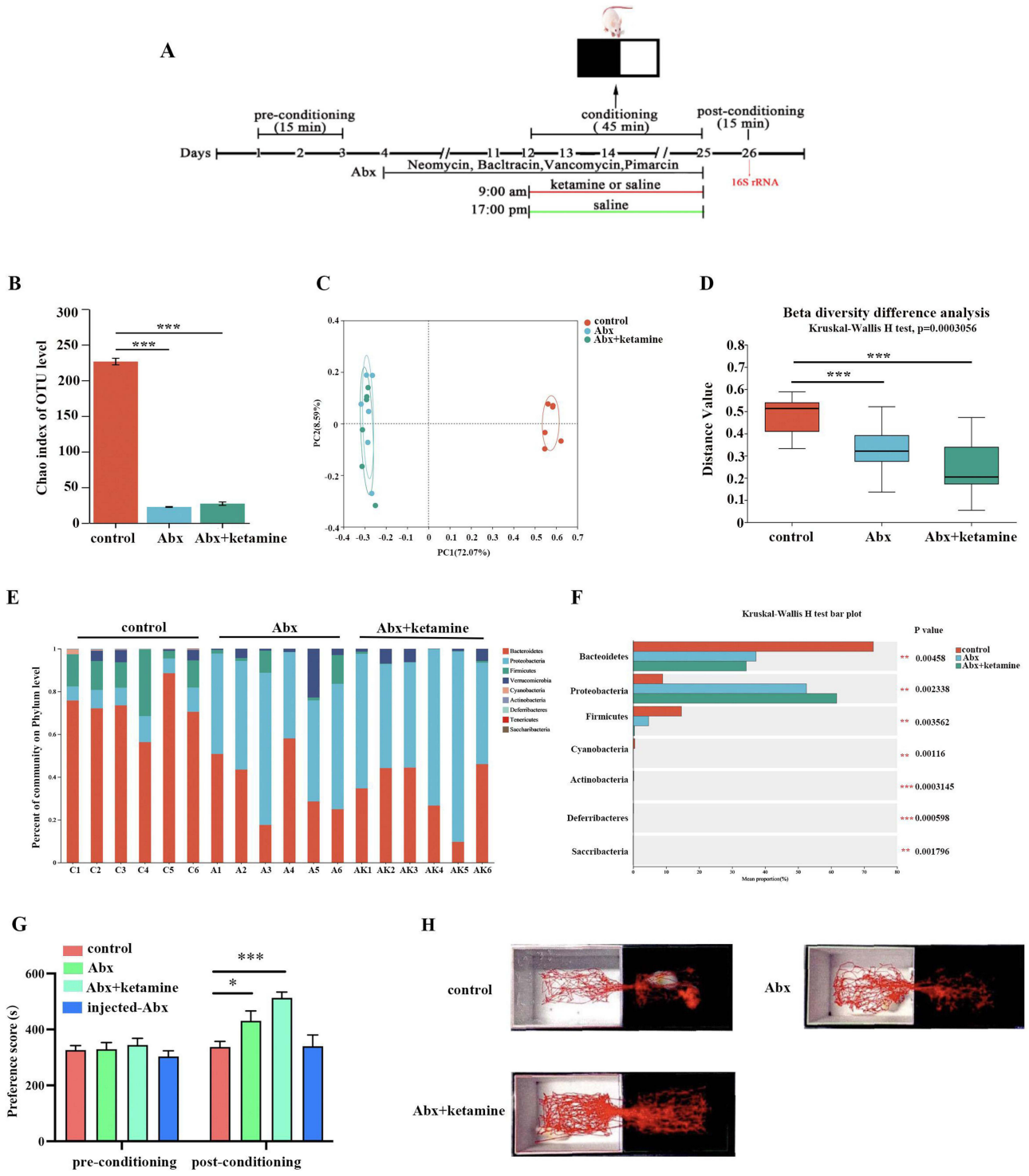

Figure 6 The effect of antibiotic on ketamine addiction. (**A**) Experimental timeline for the oral antibiotic treatment and CPP training. (**B**) Chao index for each group (n = 6). (**C**) PCoA analysis of the microbial communities in each group (n = 6). (**D**) β-diversity analysis of the microbial communities among groups. (**E**) Composition plot at the phylum level in control, Abx, Abx+ketamine group. (**F**) Significant differences in the relative abundances of microbes at the phylum level among the groups. (**G**) The mice were assessed for the time spent in the white box before and after treatment with or without an oral antibiotic cocktail and ketamine (n = 8). (**H**) Representative trajectories of mice in the white box during the post-conditioning phase. Each bar represents the mean ± SEM.**P* < 0.05, ***P* < 0.01, ****P* < 0.001 by Kruskal–Wallis *H*-test and one-way ANOVA.

group (Figure 6C). At the phylum level, we identified 9 phyla, with significant alterations observed in the abundance of 7 phyla (Figure 6E and F). Above all, these results show that oral antibiotic cocktail effectively depletes gut microbiota.

To further determine the addictive behaviors underlying the effect of gut microbiota imbalance, the CPP experiment showed oral antibiotics resulted in significantly increased time spent in the white box, which indicated that Abx can affect the CPP test. Moreover, compared to the Abx group, the time spent in the white box by the Abx + ketamine group showed minimal impact on CPP behavior. Meanwhile, we studied the effect of parenteral antibiotics in this assay and found it has little effect on CPP (Figure 6G and H). These findings support our explanation that the behavioral effects observed with oral antibiotic treatment are due to the depletion of gut microbiota rather than the systemic effects of antibiotic cocktail.

Morphological and Barrier Disruptions in the Intestine Induced by Gut Microbiota Depletion and Ketamine Abuse

Here, we validated that the changes observed in the gross morphology of the cecum indeed served as indicators of the effectiveness of antibiotic treatment. Depletion of gut microbiota led to cecal enlargement and increased weights.[22,23] Consistently, mice administered antibiotics via gavage for 21 days exhibited significantly enlarged ceca with increased weights compared to the control group on day 25. However, when compared to the effects of antibiotics alone, the combination with ketamine had minimal impact on the morphology of the cecum (Figure 7A and B). As shown in Figure 7C and D, Abx exposure alone or administration of ketamine in conjunction with Abx led to a dramatical reduction in ZO-1 or occludin expression in the colonic tissues, suggesting that addictive behaviors induced by ketamine maybe through the gut microbiota.

Gut Microbiota Regulates Ketamine-Induced CPP via GABRA1 and BDNF

To determine whether gut microbiota imbalance and intestinal barrier impairment affect peripheral neurotransmitter levels, we next tested the level of GABA and 5-HT. ELISA analysis indicated that Abx or combined with ketamine treatment has little effect on the level of GABA; however, these treatments resulted in an increase in 5-HT levels (Figure 8A and B).

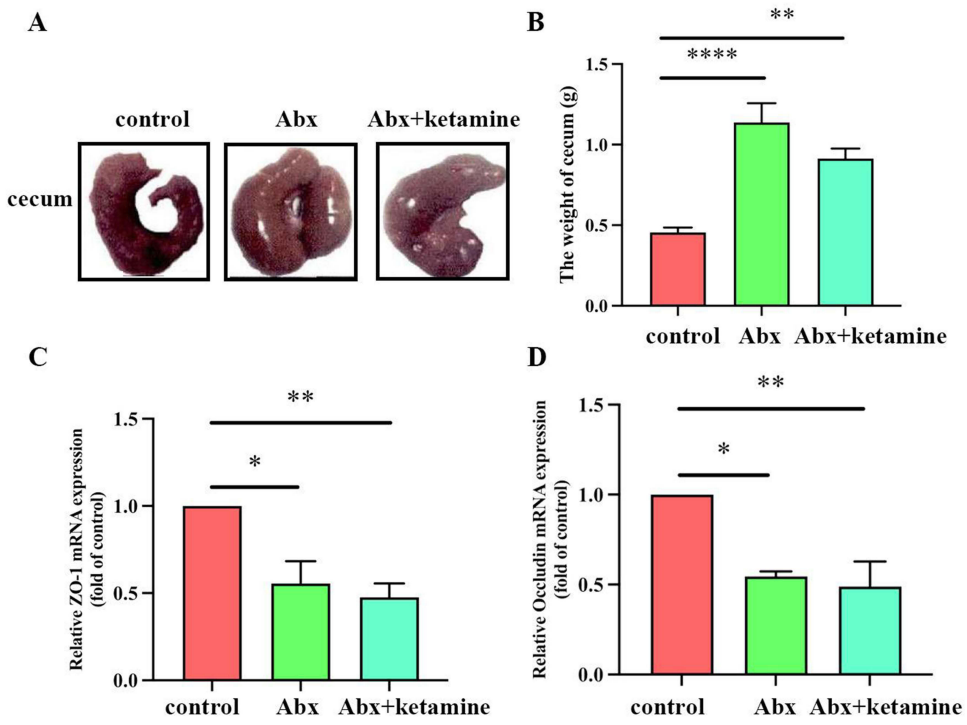

Figure 7 Morphological and barrier disruptions in the intestine induced by gut microbiota depletion. (**A**) Representative cecal tissues from mice in different groups (n = 8). (**B**) Analysis of cecum weight. (**C**) The mRNA expression levels of ZO-1 in colon samples (n = 3). (**D**) The mRNA expression levels of occludin in colon samples (n = 3). Each bar represents the mean ± SEM. $*P < 0.05$ by one-way ANOVA.

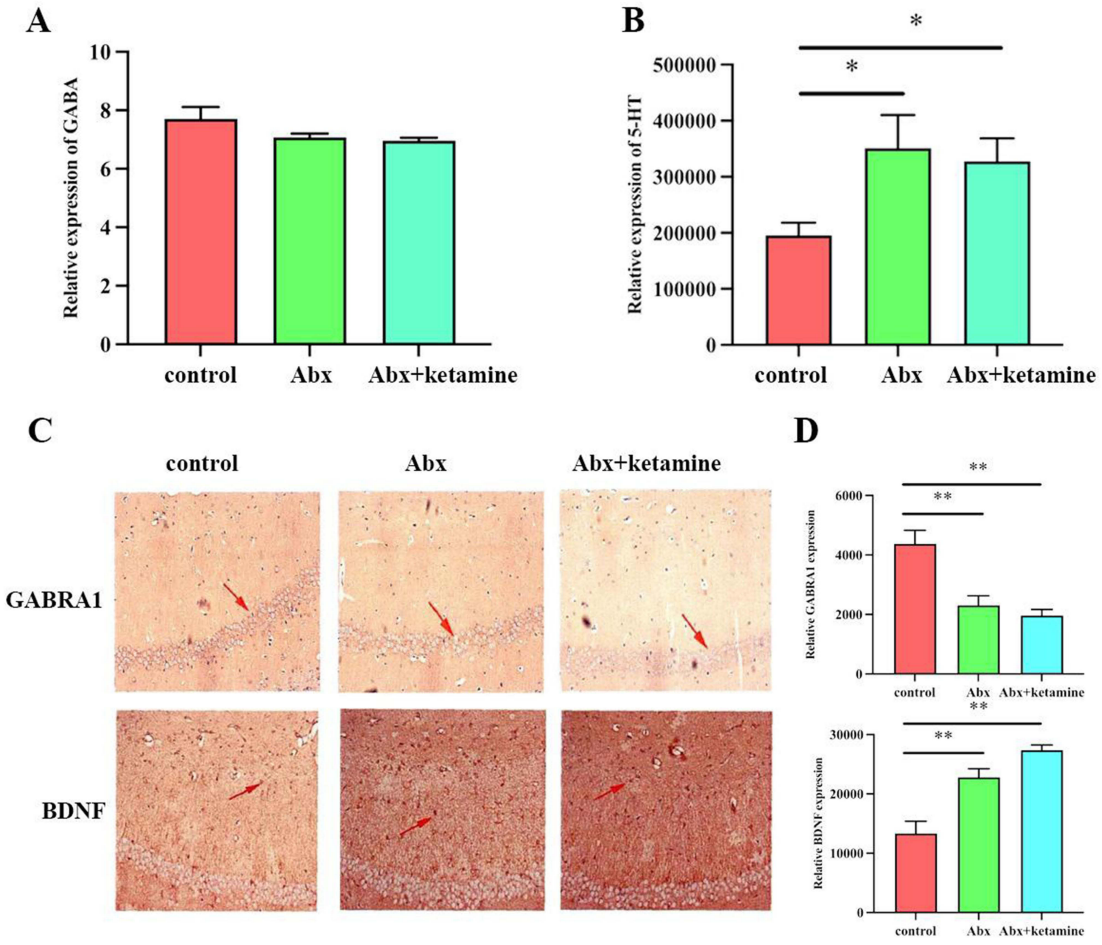

Figure 8 Gut microbiota depletion disturbed the expression of proteins and neurotransmitters associated with drug dependence. (**A** and **B**) The serum levels of GABA (**A**) and 5-HT (**B**) in mouse hippocampus samples (n = 3). (**C** and **D**) The expression levels of GABRA1 and BDNF were measured by immunohistochemistry (n = 3). The red arrows in the figure indicate the proteins. Each bar represents the mean ± SEM. $*P < 0.05$, $**P < 0.01$ by one-way ANOVA.

To investigate the potential mechanisms underlying the behavioral effects of Abx treatment, we evaluated the GABRA1 and BDNF expression in the hippocampus in response to Abx or Abx with ketamine. We found Abx treatment decreased the expression of GABRA1 and increased the expression of BDNF. What is more, compared to the control group, a combinative administration of ketamine with Abx markedly changes the expression of these proteins, suggesting ketamine abuse mediated the expression of GABRA1 and BDNF through gut microbiota (Figure 8C and D).

Discussion

There is an urgent need to identify novel targets for combating ketamine addiction. Our results support the hypothesis that repeated administration of ketamine (20 mg/kg) induces addictive effects in a CPP behavioral model, associated with changes in BDNF and GABRA1 expression in the hippocampus, and alterations in serum levels of GABA and 5-HT. These effects are likely mediated by disruptions in the intestinal barrier and gut microbiota composition. Moreover, oral antibiotic treatment, mimicking pseudo-germ-free conditions, induced addictive-like behaviors similar to those seen with ketamine. Notably, oral antibiotics, whether alone or combined with ketamine, further exacerbated gut microbiota dysfunction, compromised intestinal barrier integrity, and altered neurotransmitter levels and key gut-brain axis proteins. This study is, to our knowledge, the first to provide evidence of the microbiota's role in ketamine addiction. Our findings emphasize the complex interaction between gut microbiota and brain function in drug addiction, suggesting that targeting the gut-brain axis could be a promising new approach for treating ketamine addiction.

In this study, a mouse model of ketamine dependence was established using the CPP method. The recreational dose of ketamine is typically about 15–20% lower than the standard anesthetic dose.[24] At subanesthetic doses, ketamine repeated use can induce behavioral and neurochemical changes, and may produce symptoms resembling those of schizophrenia in humans.[25–27] Our results show that treatment with ketamine (20 mg/kg) for 14 consecutive days resulted in a significant increase in residence time in the white box compared to the control group, indicating successful establishment of the ketamine-induced CPP model.

In the present study, the diversity in the ketamine group was significantly different from that in the control group, which indicated that ketamine dependence caused changes in the composition of gut microbiota. To further explore how ketamine exposure alters the biological components of the intestinal tract within the gut-brain axis, we evaluated the gut microbiota at both the family and genus levels. As seen in Figure 4, the relative abundances of *Ruminococcaceae* (the genera *Ruminococcaceae_UCG-004*, norank_f_*Ruminococcaceae* and *Ruminiclostridium_9* are the members of *Ruminococcaceae* family), *Lachnospiraceae* (the genera *Lachnospiraceade_UCG-006* belongs to *Lachnospiraceae), Desulfovibrionaceae* (*Desulfovibrio* genus is classified within the *Desulfovibrionaceae* family) and *Family_XIII* in the ketamine group were significantly higher than those in the control group. Consistent with our results, extensive previous studies reported the abundance of *Lachnospiraceae* and *Ruminococcaceae* were altered in methamphetamine[28–30] and morphine[31] addiction. It was reported *Lachnospiraceae* and *Ruminococcaceae* exhibit macronutrient-specific negative correlations with cognitive function,[32] and reward learning.[33] Moreover, the GT/ST phenotype, used to assess addiction-related behaviors, was found to be positive correlated with the bacterial families *Ruminococcaceae* and *Lachnospiraceae*, indicating sex-dependent associations between addiction-related behaviors and the microbiota.[33] However, it is interesting to note that changes in *Ruminococcaceae* and *Lachnospiraceae* have been identified in various health conditions. Importantly, alterations in these two bacterial families have been associated with reduced intestinal permeability and inflammation in conditions such as psychological stress,[34,35] and depression.[36,37] These conflicting results suggest the need for further investigation into the role of *Ruminococcaceae* and *Lachnospiraceae* in drug addiction. Specifically, future studies should explore the effects of administering *Ruminococcaceae* and *Lachnospiraceae* separately on ketamine-reduced CPP formation and investigate the underlying mechanisms involved. Additionally, consistent with our results, a higher abundance of the *Desulfovibrio* genera has been shown to influence host lipid metabolism and is distinctly increased in the colon of rodent model with chronic baijiu or ethanol feeding.[38] To date, no previous studies reported an association between *Family_XIII* or *Prevollaceade* and addiction. Our study showed that *Family_XIII* level was higher and *Prevollaceade* was lower in the ketamine group than those in the control group, but the effects of this microbes on ketamine addiction need further characterization.

Clinical researches on methamphetamine addiction reveal differences in gut microbiota composition based on the duration of abstinence and suggest a strong connection between cognitive models and gut microbiota changes.[39–41] While clinical studies on ketamine addiction are limited, research on methamphetamine addiction highlights the critical role of gut microbiota in drug dependence. These findings suggest that gut microbiota could also play a key role in ketamine addiction, despite the distinct mechanisms of addiction between the two substances.

To bridge the "composition" and "function" of the gut microbiota, PICRUSt function prediction was conducted. Based on the functional analysis of core genes from significantly different expressed microbes, our data show common core genes were assigned to 26 COG types, primarily associated with increased translation and signal transduction mechanisms, and decreased ribosomal structure and biogenesis, cell wall/membrane/envelope biogenesis, transcription, coenzyme transport and metabolism, nucleotide transport and metabolism, posttranslational modification, protein turnover, and chaperones in the clusters of COGs functional category. However, a majority of genes were classified as 'function unknown. To our knowledge, there have been few studies analyzing the COGs of the gut microbiota in relation to drug addiction.

To determine how changes in the gut microbiota contribute to ketamine addiction, we evaluated the expression of TJ proteins in the intestine. Reduced expression of ZO-1 and occludin result in increased intestinal permeability, and damage to this physical barrier allows microbes and other molecules to diffuse from the intestinal lumen into the lamina propria and systemic circulation.[42] In this study, qPCR was used to determine the expression levels of ZO-1 and occludin in the colon. Our results showed that colonic ZO-1 and occludin levels were significantly decreased in the ketamine group,

which indicated that the intestinal barrier was damaged. Gut microbiota abolish, furthermore, also reduce the expression of these two key gene in gut barrier, supporting the crucial role of the microbiota in MGBA.

Neurotransmitters play a crucial role in the interaction between the gut microbiota and CNS. Specifically, the gut microbiota contributes significantly to the synthesis of GABA and 5-HT. Studies have shown that GABA can be produced by intestinal bacteria,[43] and probiotics can affect depressive behavior in mice by regulating the expression of GABA.[44] Serotonin is another important neurotransmitter in the MGBA.[45,46] Although 5-HT plays a crucial role in regulating brain function and behavior, approximately 95% of 5-HT is synthesized in peripheral organs, particularly in intestinal epithelial cells.[47] Notably, increased serum or colonic 5-HT have been implicated in various inflammatory and diarrheal disorders.[48,49] Evidence suggests that GABA or 5-HT produced in the gut or serum cannot directly cross the blood–brain barrier, but it can impact the permeability of the blood–brain barrier or via vagus nerve, potentially leading to brain dysfunction or inflammation.[50] Particular microbial metabolites, namely short chain fatty acids, have been shown to be elevated by spore-forming microbiota and subsequently promoted 5-HT levels in endochromaffin cells in the epithelia.[51] As shown in Figure 2, GABA levels were significantly decreased, and 5-HT levels were significantly increased in the serum of the ketamine group compared to those in the control group. These results are consistent with our previous study on morphine addiction.[9] Specifically, the up-regulation of *Ruminiclostridium_9* in the ketamine group inversely correlated with changes in serum GABA levels. This finding aligns with the results of a previous study where changes in serum GABA levels correlated negatively with *Ruminiclostridium_9*.[52] Similar to our study, in the 5-HT transporter knockout mice, which indicated increased extracellular 5-HT availability, up-regulated the abundance of *Desulfovibrionaceae*.[49]

BDNF, the most abundant neurotrophic factor, and GABRA1, a subtype of $GABA_A$ receptor, regulates spatial learning, memory, and cognition.[53–55] Evidence has shown that dysbiosis of the gut microbiota during chronic alcohol exposure is closely associated with alcohol-induced neurobehavioral changes and the expression of BDNF/GABRA1 axis.[15] In current study, we found ketamine significantly up-regulated the expression of BDNF and decreased GABRA1level in the mouse hippocampus. Taken together, these solid data again highlights the role of gut microbiota in ketamine addiction could not be ignored.

To further identify the effect of gut microbiota imbalance on ketamine addiction, we continue to use oral intestinal non-absorbable antibiotic cocktail, including Bacitracin, Neomycin, Vancomycin and Pimaricin, to change and imbalance the composition of gut microbiota. These antibiotics are difficult to be absorbed by gastrointestinal tract and then enter the blood circulation.[5] This can exclude the direct impact of antibiotics on the brain through the cerebrospinal fluid, which is ideal model for studying the effect of changes in the composition of the gut microbiota on the CNS system. We found the diversity and richness of the gut microbiota of the two groups of mice that were given an oral antibiotic cocktail were significantly reduced and robust changes in composition, resulting in imbalance of the gut microbiota of the mice. It is noteworthy that mice in the Abx group spent significantly more time in the white box. However, no synergistic effect was observed in the CPP when Abx was used in conjunction with ketamine, indicating that the formation of ketamine addiction is mediated by the gut microbiota. Ning and his colleagues also found rats pretreated by antibiotics showed a significantly stronger methamphetamine-induced CPP than did the controls.[30] It also emphasizes the importance of the gut-brain axis in the systems pathophysiology of addiction in biochemical experiments, including neurotransmitters, key proteins and gut barrier.

The present study also had several limitations. First, female mice were not included. Previous studies demonstrated that gender differences in the preferred method of drug use[56] and the composition and diversity of gut microbiota suffering from drug abuse differ greatly by sex.[33] Hence, future studies on ketamine addiction may address this issue with consideration for sex differences. Second, given that intestinal metabolites act as mediators in the MGBA, it is crucial to investigate the specific metabolites and their regulatory mechanisms in ketamine addiction. Therefore, subsequent research should examine this.

Conclusion

In summary, our study demonstrates ketamine-induced CPP is mediated by the gut microbiota. Key findings include increased abundance of *Lachnospiraceae, Ruminococcaceae, Desulfovibrionaceae*, and *Family-XIII*, with a decrease in *Prevotellaceae*. These microbial changes were accompanied by reduced levels of tight junction proteins, GABA and GABRA1, along with increased BDNF and 5-HT. The pseudo-germ-free model using an oral antibiotic cocktail did not

exacerbate ketamine-induced addictive behaviors. These results highlight the impact of gut microbiota on ketamine dependence and suggest potential therapeutic targets. Future research should focus on the specific mechanisms underlying these interactions to develop targeted interventions.

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Disclosure

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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