(R)-ketamine ameliorates demyelination and facilitates remyelination in cuprizone-treated mice: A role of gut–microbiota–brain axis

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ABSTRACT

Multiple sclerosis (MS) is the most common demyelinating disease that attacks the central nervous system. We recently reported that the new antidepressant (R)-ketamine could ameliorate the disease progression in experimental autoimmune encephalomyelitis model of MS. Cuprizone (CPZ) has been used to produce demyelination which resembles demyelination in MS patients. This study was undertaken to investigate whether (R)-ketamine could affect demyelination in CPZ-treated mice and remyelination after CPZ withdrawal. Repeated treatment with (R)-ketamine (10 mg/kg/day, twice weekly, for 6 weeks) significantly ameliorated demyelination and activated microglia in the brain compared with saline-treated mice. Furthermore, pretreatment with ANA-12 (TrkB antagonist) significantly blocked the beneficial effects of (R)-ketamine on the demyelination and activated microglia in the brain of CPZ-treated mice. The 16S rRNA analysis showed that (R)-ketamine significantly improved abnormal composition of gut microbiota and decreased levels of lactic acid of CPZ-treated mice. In addition, there were significant correlations between demyelination (or microglial activation) in the brain and the relative abundance of several microbiome, suggesting a link between gut microbiota and brain. Interestingly, (R)-ketamine could facilitate remyelination in the brain after CPZ withdrawal. In conclusion, the study suggests that (R)-ketamine could ameliorate demyelination in the brain of CPZ-treated mice through TrkB activation, and that gut-microbiota–microglia crosstalk may play a role in the demyelination of CPZ-treated mice. Therefore, it is likely that (R)-ketamine could be a new therapeutic drug for MS.

1. Introduction

Multiple sclerosis (MS) is the most common inflammatory demyelinating disease on the central nervous system (CNS). Symptoms of MS patients cause a major economic burden on the patients, their families and caregivers, employers, and the healthcare system (Dahham et al., 2021; Nicholas et al., 2021). Therefore, the development of new drugs to attenuate demyelination and to facilitate remyelination in MS patients is an unmet medical need.

Increasing preclinical findings demonstrated that the new antidepressant (R)-ketamine, (R)-enantiomer of (R,S)-ketamine, has potent anti-inflammatory effects in several animal models (Fujita and Hashimoto, 2020; Fujita et al., 2020; Fujita et al., 2021; Qu et al., 2021; Xiong et al., 2015; Yang et al., 2015; Yao et al., 2018; Yao et al., 2021; Zhang et al., 2014; Zhang et al., 2018; Zhang et al., 2020; Zhang et al., 2021a; Zhang et al., 2021b; Zhang et al., 2021c). Interestingly, side effects of (R)-ketamine in animals and humans were lower than those of (R,S)-ketamine and (S)-ketamine (Bonaventura et al., 2021; Chang et al., 2019; Hashimoto et al., 2017; Leal et al., 2021; Tan and Hashimoto, 2020; Tian et al., 2018; Yang et al., 2015; Yang et al., 2016b). Collectively, (R)-ketamine could be a novel anti-inflammatory drug without ketamine-like side effects (Hashimoto, 2019; Hashimoto, 2020; Yang et al., 2019; Wei et al., 2020; Wei et al., 2021a; Zhang and Hashimoto, 2019).

It is well recognized that MS patients have high rates of depression (Jones et al., 2021; Skokou et al., 2012). Given high incidence of depression in MS patients, we recently reported that (R)-ketamine could ameliorate the disease progression in experimental autoimmune

Abbreviations: ANOVA, analysis of variance; ANOSIM, analysis of similarities; BDNF, brain-derived neurotrophic factor; CNS, central nervous system; CPZ, cuprizone; CSF1R, colony stimulating factor-1 receptor; LDA, linear discriminant analysis; LEfSe, LDA effect size; MBP, myelin basic protein; MS, multiple sclerosis; PCA, principal component analysis; PCoA, principal coordinates analysis.

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encephalomyelitis model of MS (Wang et al., 2021a). Cuprizone (CPZ), a selective and sensitive copper-chelating agent, has been used to produce the toxic demyelination that resembles demyelination in MS patients (Procaccini et al., 2015; Torkildsen et al., 2008; Zhan et al., 2020).

Interestingly, spontaneous remyelination can be observed as early as 4 days after CPZ withdrawal, indicating that CPZ model could be excellent to discover potential therapeutic drugs which can prevent demyelination and stimulate remyelination (Franklin and Ffrench-Constant, 2017; Torkildsen et al., 2008).

The present study was undertaken to investigate whether (R)-ketamine could affect demyelination and remyelination in CPZ-treated mice. Furthermore, we examined the role of TrkB in the effects of (R)-ketamine in CPZ-treated mice since brain-derived neurotrophic factor (BDNF) and its receptor TrkB plays a role in the beneficial effects of (R)-ketamine in other animal models such as depression, Parkinson’s disease, osteoporosis, and ulcer colitis (Fujita et al., 2020; Fujita et al., 2021; Tan et al., 2020; Tan et al., 2022; Yang et al., 2015). Accumulating evidence shows the role of gut microbiota in pathogenesis of MS (Can- tarel et al., 2015; Cekanaviciute et al., 2017; Farshbafnadi et al., 2021; Gherzi et al., 2021; Maghzi and Weiner, 2020; Parodi and Kerlero de Rosbo, 2021; The iMSMS Consortium, 2020). Finally, we examined the role of gut microbiota in the effects of (R)-ketamine in CPZ model since gut-microbiota–brain axis may play a role in the antidepressant-like effects of (R)-ketamine in rodents (Qu et al., 2017; Yang et al., 2017).

2. Materials and methods

2.1. Animals

Adult male C57BL/6J mice (8–10 weeks old, body weight 19–22 g, Japan SLC, Inc., Hamamatsu, Japan) were acclimated housed under controlled temperatures and 12 h light/dark cycles (lights on between 07:00 and 19:00 h) with food (CE-2; CLEA Japan, Inc., Tokyo, Japan) and water ad libitum. The experimental protocol was approved by the Chiba University Institutional Animal Care and Use Committee (Permission number: 1–466). This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health, USA. Animals were deeply anesthetized with isoflurane before being killed by cervical dislocation. All efforts were made to minimize suffering.

2.2. Cuprizone (CPZ) model

CPZ model was induced by feeding mice with 0.2% w/w cuprizone (bis-cyclohexanone oxalidihydrazone; catalog number: B0476, Tokyo Chemical Industry Co., Ltd. Tokyo, Japan,) in powder chow. Mice received 0.2% CPZ food pellets for demyelination (6 weeks) and remyelination (6 weeks followed by 2 weeks normal chow) paradigms. Chow was replaced three times a week. Age-matched untreated controls were fed powder chow without CPZ.

2.3. Reagents

(R)-ketamine hydrochloride was prepared as described previously (Zhang et al., 2014). The dose (10 mg/kg as hydrochloride salt, I.P.) of (R)-ketamine was dissolved in the saline, as described previously (Qu et al., 2021; Yang et al., 2015; Yang et al., 2018; Zhang et al., 2021c). ANA-12, N2-2-(2-[(2-oxoazepan-3-yl) amino] carbonyl) phenyl benzo[b]thiophene-2-carboxamide (0.5 mg/kg, I.P., Sigma–Aldrich Co., Ltd., Tokyo, Japan), was dissolved in 17% dimethylsulfoxide (DMSO) in saline (Fujita et al., 2020; Ren et al., 2015; Tan et al., 2020; Tan et al., 2022; Zhang et al., 2015).

2.4. Collection of fecal samples from mice

Fresh mouse fecal samples were collected at around 9:00 a.m. and then placed into sterilized screw-cap microtubes. Then the microtube containing mouse fecal samples was immediately placed into liquid nitrogen and stored under –80 °C until use.

2.5. 16S rRNA analysis of feces

Extraction of DNA from fecal samples and 16S rRNA analysis were done at MyMetagenome Co, Ltd. (Tokyo, Japan), as previously described (Kim et al., 2013; Pu et al., 2021; Shinho-Hashimoto et al., 2021; Wang et al., 2020; Wang et al., 2021b). Briefly, the common primer ZFpmod (5′-AGRGTGGTATGMMCTCAG-3′) and 338R (5′-TGCTGCTTCGCTAGGAT-3′) were used to amplify the V1–V2 region of the bacterial 16S rRNA gene by polymerase chain reaction (PCR).

α-diversity was analyzed by Shannon, the observed OTUs. β-diversity was measured by the principal component analysis (PCA) and principal coordinates analysis (PCoA) and statistical significance was done by analysis of similarities (ANOSIM). Linear discriminant analysis (LDA) effect size (LEfSe) (Segata et al., 2011) was based on the bacterial abundance to explore significant differential biomarkers between groups with different taxonomic levels (http://huttenhower.sph. harvard.edu/galaxy/). Only taxa with LDA scores >4.0 and p value <0.05 were considered significantly enriched. The results with taxonomic bar charts and cladograms were visualized.

2.6. Determination of short-chain fatty acids (SCFAs)

The levels of short chain fatty acids (SCFAs) (i.e., succinic acid, lactic acid, acetic acid, malic acid, butyric acid) in stool samples were determined at TechnoSuruga Laboratory, Co., Ltd. (Shizuoka, Japan), as reported previously (Pu et al., 2021; Shinho-Hashimoto et al., 2021; Wang et al., 2020; Wei et al., 2021; Zhang et al., 2019). The results of SCFAs were recorded as milligrams per gram of excrement.

2.7. Histopathology and immunofluorescence

The mice were anesthetized with 5% isoflurane and sodium pentobarbital (50 mg/kg), and perfused transcardially with isotonic saline and ice-cold 4% paraformaldehyde in 0.1 mM phosphate buffer (30 ml per mouse, pH 7.4). Then the brain was collected, post-fixed overnight at 4 °C, and cut into 30 μm slice by vibratome (VT1000S, Leica Microsystems AG, Wetzlar, Germany). The slices were selected from bregma 1.10 to –0.58 in mice brain for immunofluorescence. The immunofluorescence is performed as reported previously (Wang et al., 2021a). Briefly, the slices were washed with 0.1 mM phosphate buffer three times for 15 mins and blocked with 3% BSA with 0.3% TritonX-100 for 2 h. Incubation with primary antibody [mouse, anti-MBP (myelin basic protein), Catalog number: sc-271,524, Santa Cruz Biotechnology, Inc., USA, 1:100; rabbit, anti-Iba1, Catalog number: 019–19,741, FujiFilm Wako Pure Chemical Corporation, Tokyo, Japan, 1:250] was conducted overnight at 4 °C, then the slices were incubated by secondary antibody (Alexa Fluor 546 goat anti-mouse IgG, 1:1000; Alexa Fluor 488 donkey anti-rabbit IgG, 1:1000) for 2 h at room temperature. Then the slices were washed by 0.1 mM phosphate buffer with 0.1% tween-20 for three times for 15 mins, and were coverslipped with mounting medium with DAPI (4′,6′- diamino-2-phenylindole, Catalog number: H-1200, Vector laboratories, Inc., USA) and analyzed by Keyence BZ-900 microscope (Tokyo, Japan) and Image J software.

2.8. Statistical analysis

Data represent the mean ± standard error of the mean (S.E.M.) The demyelination area in MBP immunofluorescence, Iba1-positive area and SCFA levels were analyzed by one-way analysis of variance (ANOVA), followed by Fisher’s least significant difference (LSD) test. The Kruskal-Wallis test with Dunn’s post-hoc test was utilized to analyze the α-diversity data of the gut microbiota and the relative bacterial abundance
at different level. For β-diversity of the gut microbiota, principal component analysis (PCA) of OUT level, Principal Coordinates Analysis (PCoA) and the unweighted UniFrac phylogenetic distance were performed using analysis of similarities (ANOSIM) by R package vegan (2.5.4) (Xia and Sun, 2017; Wei et al., 2021b). Correlation between demyelination and Iba1 expression was analyzed using Spearman correlation analysis. Correlations among SCFA levels, demyelination area, Iba1-positive area or the relative abundance of bacteria were analyzed using Spearman rank test. Integrative network of associations between the relative abundance of microbiota and SCFAs, Iba1-positive area, or demyelination area were tested with Spearman’s analysis and visualized by Cytoscape (Version 3.8.1). The level of significance was set as P < 0.05 for all analyses.

3. Results

3.1. Effects of (R)-ketamine on the demyelination and microglia activation of CPZ-treated mice

(R)-ketamine or saline was injected to mice twice per week for 6 weeks (Fig. 1A). To examine the role of TrkB signaling, vehicle or TrkB antagonist ANA-12 was injected to mice 30 mins before (R)-ketamine (or saline) administration (Fig. 1A). The demyelination area in the brain was examined using MBP immunofluorescence. The feeding of CPZ for 6 weeks caused complete demyelination in the corpus callosum of mice (Fig. 1B, C, E). Treatment of (R)-ketamine significantly ameliorated the demyelination area in the corpus callosum of CPZ-treated mice. Furthermore, effects of (R)-ketamine were significantly blocked by ANA-12 (Fig. 1B, C, E). Moreover, ANA-12 alone did not affect the demyelination area in the corpus callosum of CPZ-treated mice (Fig. 1B, C, E). Collectively, (R)-ketamine could ameliorate the demyelination in the brain of CPZ-treated mice through TrkB activation.

It is reported that microglial activation plays a key role in MS development (Deng and Sriram, 2005; Kalafatakis and Karagogeos, 2021; Mayrhofer et al., 2021). In this study, we performed immunofluorescence of the microglial marker Iba1 in the corpus callosum of CPZ-treated mice. In the control + saline group, Iba1-positive cells were broadly and evenly distributed in the corpus callosum of mice, indicating that these cells were small and ramified, a morphology typical of resting microglia (Fig. 1B, D, F). In the corpus callosum of CPZ + saline group, there was a robust increase in the area of Iba1-positive staining compared with control + saline group. Treatment of (R)-ketamine significantly attenuated the Iba1-positive area in the corpus callosum of CPZ-treated mice. Furthermore, ANA-12 significantly blocked the effects of (R)-ketamine on Iba1-positive staining in the corpus callosum of CPZ-treated mice (Fig. 1B, D, F). Furthermore, ANA-12 alone did not affect the demyelination area in the CPZ-treated mice (Fig. 1B, D, F). There was a significant positive correlation (r = 0.6995, P < 0.001) between demyelination area and Iba1-positive area (Fig. 1G), suggesting a link between demyelination and microglial activation. Collectively, (R)-ketamine could ameliorate the microglia activation in the brain of CPZ-treated mice through TrkB activation.

3.2. Composition of gut microbiota

The composition of the gut microbiota among control + saline group, CPZ + saline group, CPZ + (R)-ketamine group was analyzed using α- and β-diversity. For α-diversity, there were no differences in the Shannon and observed OTUs among the three groups (Fig. 2A and B). Regarding β-diversity, PCA and PCoA were applied to analyze the bacterial community composition of gut microbiota among the three groups. PCA revealed significant separation in the community composition evaluated by ANOSIM (R = 0.6231, P = 0.001) (Fig. 2C) based on the OTU level. The ordination of unweighted UniFrac distance by Wilcox rank tests showed that the β-diversity among the three groups were significant difference (R = 0.7128, P = 0.001) (Fig. 2D). Importantly, boxplot of Unweighted Unifrac distance by Wilcox rank tests showed that the β-diversity among the three groups were significant difference (R = 0.7128, P = 0.001) (Fig. 2E). Collectively, (R)-ketamine could ameliorate abnormal β-diversity of gut microbiota in the CPZ-treated mice.

3.3. Altered composition in the gut microbiota at different levels

At the phylum level, the relative abundance of Proteobacteria in the CPZ + (R)-ketamine group was significantly lower than that of the CPZ + saline group (Fig. 3A and B). At genus level, there were significant differences in the relative abundance of Eisenbergiella, Barnesiella, Prevotella, Butyrvibrio, Faecalibaculum, Dorea, Parabacteroides, Turicibacter, Mailhella, Parvibacter, and Mordavella among the three groups (Fig. 3C, D, Table S1). Furthermore, at species level, the relative abundances of Eisenbergiella massiliensis, Barnesiella viscerciola, Lactobacillus murinus, Lactobacillus intestinalis, Clostridium bolteae, Butyrvibrio proteoclasticus, Faecalibaculum rodentium, Prevotella loescheii, Bacteroides sartorii, and Escherichia ramulus were significant different among the three groups (Fig. 3E, F, Table S2).

3.4. LEfSe analysis

The gut microbiota changes of the abundant taxa among Control + saline group, CPZ + saline group, CPZ + (R)-ketamine group were analyzed using the LEFSe algorithm, which permits the identification of microbial markers that are more important in one group than in another group. The color differences illustrated differences in the abundant taxa among the groups (Kwak et al., 2020). LEFSe analysis showed that (R)-ketamine produced significant different effects on gut microbiota (Fig. 4A). The species level phylotype including Lactobacillus murinus, Clostridium bolteae, E. ramulus, and Clostridium aminophilum were identified as potential gut microbial markers for control + saline group (Fig. 4B). Two species level phylotypes including the Eisenbergiella massiliensis, B. proteoclasticus were identified as potential gut microbial markers for CPZ + saline group (Fig. 4B). Four species level phylotypes including Lactobacillus intestinalis, Barnesiella viscerciola, Faecalibaculum rodentium, and Prevotella loescheii were identified as potential gut microbial markers for CPZ + (R)-ketamine group in mice (Fig. 4B and Table S3).

3.5. Levels of SCFAs in the fecal samples and correlations among bacterial relative abundance, SCFAs, demyelination area, or Iba1-positive area

The concentration of lactic acid was significantly lower in the CPZ + saline group than that in control + saline group and CPZ + (R)-ketamine group (Fig. 5A). In contrast, there were no significant differences in the other SCFAs (succinic acid, acetic acid, malic acid, and butyric acid) among the three groups (Fig. 5A).

The heat map shown the correlations between lactic acid, demyelination, Iba1-positive area and the relative bacterial abundance that differ significantly at species level (Fig. 5B). Furthermore, the correlations (Spearman analysis r > 0.5, P < 0.05) among lactic acid, demyelination area, Iba1-positive area, the bacterial relative abundance that differ significantly with any other group were also shown in correlation network (Fig. 5C).

The concentration of lactic acid was significantly and negatively correlated with the relative abundance of Eisenbergiella massiliensis (Fig. 5B and C).

There were significant positive correlations between demyelination area (or Iba1-positive area) and the relative abundance of Eisenbergiella massiliensis or B. proteoclasticus. Furthermore, there were significant negative correlations between demyelination area and the relative abundance of L. murinus, (Clostridium) bolteae, or E. ramulus (Fig. 5B).

The Iba1-positive area was positively correlated with the relative abundance of Eisenbergiella massiliensis, B. proteoclasticus. Faecalibaculum rodentium, or B. sartorii. The Iba1-positive area was negatively correlated
Fig. 1. Effects of (R)-ketamine on the demyelination of CPZ-treated mice.
A: The protocol of the experiment. B: The MBP (myelin basic protein) staining images were taken of the area outlined by the red line box. The Iba1 staining images were taken of the area outlined by the green line box. C: The representative photos of MBP and DAPI (4′,6′-diamino-2-phenylindole) in the corpus callosum of brain from Control + saline, CPZ + saline, CPZ + (R)-ketamine, CPZ + ANA-12, and CPZ + ANA-12/(R)-ketamine groups. D: The representative photos of Iba1 and DAPI in the corpus callosum of brain from Control + saline, CPZ + saline, CPZ + (R)-ketamine, CPZ + ANA-12, and CPZ + ANA-12/(R)-ketamine groups. E: Quantitative data of demyelination area in the corpus callosum (one-way ANOVA: F_{4,35} = 9.402, P < 0.001). The percentage of demyelination area was determined by the calculation [(corpus callosum area - MBP-positive area)/corpus callosum area × 100%]. *P < 0.05, **P < 0.01. F: Quantitative data of Iba1-positive area in the corpus callosum (one-way ANOVA: F_{4,35} = 20.938, P < 0.001). The percentage of Iba1-positive area was determined by the calculation [(Iba1-positive area/corpus callosum area) × 100%]. G: A positive correlation (r = 0.6995, P < 0.001) between the demyelination area and Iba1-positive area from three groups. *P < 0.05, **P < 0.01, ***P < 0.001. N = 8/group. Scale bar for MBP immunostaining was 300 μm. Scale bar for Iba1 immunostaining was 100 μm. ND: no detectable. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)
with L. murinus, [Clostridium] bolteae, or E. ramulus (Fig. 5B).

There were also significant positive or negative correlations among the relative abundance of B. sartorii, Eisenbergiella massiliensis, B. proteoclasticus, L. murinus, Faecalibaculum rodentium, P. loescheii, B. viscericola, E. ramulus, [Clostridium] bolteae (Fig. 5C).

3.6. Effects of (R)-ketamine on remyelination and microglia activation in the CPZ-treated mice

During the last 2 weeks, CPZ food pellet was withdrawn. All mice were fed with normal chow (Fig. 6A). (R)-ketamine or saline was injected i.p. to mice twice per week for the last 2 weeks (Fig. 6A). After CPZ withdrawal, spontaneous remyelination was shown in CPZ-treated mice (Franklin and Ffrench-Constant, 2017; Torkildsen et al., 2008). Treatment with (R)-ketamine (10 mg/kg/day, twice weekly for 2 weeks) significantly reduced the demyelination area in the corpus callosum of CPZ-treated mice, suggesting that (R)-ketamine can facilitate the remyelination in CPZ-treated mice (Fig. 6B, C, E). Furthermore, (R)-ketamine ameliorated the microglial activation in the corpus callosum following CPZ withdrawal (Fig. 6B, D, F). Collectively, (R)-ketamine could stimulate the remyelination in the brain after CPZ withdrawal through anti-inflammation.

4. Discussion

The main findings of this study are as follows: First, (R)-ketamine ameliorated demyelination and microglial activation in the corpus callosum of CPZ-treated mice through TrkB activation compared to saline-treated mice. There was a positive correlation between demyelination and microglial activation in the corpus callosum. Second, (R)-ketamine partially normalized abnormal beta-diversity of gut microbiota of CPZ-treated mice. The LEfSe algorithm identified the species Lactobacillus intestinalis, Barnesiella viscericola, Faecalibaculum rodentium, and Prevotella loescheii as specific microbial biomarkers for CPZ + (R)-ketamine.
(R)-ketamine improved the decreased levels of lactic acid in the feces from CPZ-treated mice. Interestingly, there was a positive correlation between the relative abundance of the species *Eisenbergiella massiliensis* and lactic acid in the feces. Fourth, there were positive (or negative) correlations between the relative abundance of the species bacteria and demyelination (or microglial activation) in the brain. Finally, (R)-ketamine significantly facilitated remyelination in the corpus callosum after CPZ withdrawal. Collectively, (R)-ketamine could attenuate demyelination of CPZ-treated mice through TrkB activation, and (R)-ketamine could facilitate remyelination in the brain after CPZ withdrawal.

![Altered composition in the gut microbiota at different levels.](image_url)

Fig. 3. Altered composition in the gut microbiota at different levels. A, B: Phylum. Relative abundance distribution and the significantly changed bacteria among Control + saline, CPZ + saline, CPZ + (R)-ketamine group (Kruskal-Wallis test, $H = 9.215, P = 0.01$). C, D: Genus. Relative abundance distribution and the significantly changed bacteria among the three groups. The statistical results were in the Table S1. E, F: Species. Relative abundance distribution and the significantly changed bacteria among the three groups. The statistical results were in the Table S2. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$. 
withdrawal. Therefore, it is likely that (R)-ketamine would be a potential therapeutic drug for MS.

Multiple lines of evidence suggest a key role of microglia in the pathology of MS (Chu et al., 2018; Deng and Sriram, 2005; Gao and Tsirka, 2011; Guerrero and Sicotte, 2020; Voet et al., 2019). It is reported that microglial activation in the brain plays a role in the pathological changes of CPZ-treated mice and MS patients (Clarner et al., 2012). Depletion of microglia in the brain by BLZ945 [colony-stimulating factor-1 receptor (CSF1R) inhibitor] attenuated demyelination in the cortex and external capsule of CPZ-treated mice (Wies Mancini et al., 2019), suggesting a role of microglia in the demyelination. In contrast, BLZ945 failed to protect myelin or foster remyelination in myelin-rich areas such as corpus callosum (Wies Mancini et al., 2019), indicating regional differences. Recently, we reported that depletion of microglia in the brain by another CSF1R inhibitor PLX5622 caused abnormalities in gut microbiota in rodents (Yang et al., 2021), suggesting that repeated treatment of CSF1R inhibitor may affect behavioral and biological functions in animals. Therefore, it is possible that depletion of microglia in the brain by CSF1R inhibitor and subsequent abnormalities in the composition of gut microbiota may affect brain functions.

In this study, we found a positive correlation between demyelination and microglial activation in the brain of CPZ-treated mice, supporting a link between demyelination and microglial activation. Thus, the current data suggest that attenuation of demyelination in the corpus callosum by (R)-ketamine might come along with reductions of microglial activation in the same area. We reported that (R)-ketamine could produce beneficial effects in several animal models of inflammation through TrkB activation (Fujita and Hashimoto, 2020; Fujita et al., 2021; Tan et al., 2020; Tan et al., 2022). Furthermore, (R)-ketamine significantly attenuated the increases in microglial activation in the brain after a single administration of lipopolysaccharide or cecum ligation and puncture (Zhang et al., 2021a; Zhang et al., 2021b). Collectively, it is likely that (R)-ketamine can produce potent anti-inflammatory actions in a variety of models of inflammation (Wei et al., 2021a). Given the role of microglial activation in the brain of MS patients, it is possible that (R)-ketamine could ameliorate the demyelination of CPZ-treated mice through its potent anti-inflammatory actions.

Increasing evidence suggests that abnormalities in gut microbiota play a role in the pathogenesis of MS (Cantarel et al., 2015; Cekanaviciute et al., 2017; Farshbafnadi et al., 2021; Ghezzi et al., 2021; Maghzi and Weiner, 2020; Parodi and Kerlero de Rosbo, 2021; The iMSMS Consortium, 2020). In this study, we found that (R)-ketamine could attenuate the increased relative abundance of Eisenbergiella massiliensis.
in the CPZ-treated mice. Furthermore, we also found positive correlations between the relative abundance of *Eisenbergiella massiliensis* and demyelination (or microglial marker) in the brain. A recent study showed that the relative abundance of *Eisenbergiella massiliensis* was increased in the stools of mice with ketogenic diet, compared with normal diet (Ferrere et al., 2021). Although the precise functions of *Eisenbergiella massiliensis* remain unclear, it is possible that *Eisenbergiella massiliensis* or its produced metabolite(s) may play a role in the demyelination through neuroinflammation. Nonetheless, it is noteworthy that (R)-ketamine could attenuate the increased relative abundance of *Eisenbergiella massiliensis* in the CPZ-treated mice. Previously, we reported that (R)-ketamine could ameliorate abnormal composition of gut microbiota in mice with depression-like phenotypes (Qu et al., 2017; Yang et al., 2017). Collectively, it is possible that gut microbiota may play a role in the beneficial actions of (R)-ketamine (Hashimoto, 2019; Hashimoto, 2020; Wei et al., 2021a) although further study is needed.

Increasing evidence suggests that gut microbiota might regulate microglial functions in the brain (Abdel-Haq et al., 2019; Cryan et al., 2019; Lynch et al., 2021; Ma et al., 2019; Wang et al., 2018; Yang et al., 2021). A recent study shows that antibiotic-induced gut dysbiosis leads to microglial activation in the hippocampus, resulting in impairment of cholinergic gamma oscillation (Çalışkan et al., 2021). Furthermore, a recent study showed that microglia play a critical role in driving gut microbiome-mediated alterations of cerebral amyloid-β deposition (Dodiya et al., 2022). In this study, we found correlations between microglial marker in the brain and the relative abundance of several bacteria. For example, we found a positive correlation between relative abundance of several microbiome (*Eisenbergiella massiliensis*, *B. proteoclasticus*, *Faecalibaculum rodentium*, *B. sartorii*) and microglial activation, suggesting that these microbes may play a role in the

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**Fig. 5.** Levels of SCFAs in the fecal samples and correlations between bacterial relative abundance and SCFAs, demyelination area, and Iba1-positive area.

**A:** Levels of SCFAs in fecal samples from Control + saline, CPZ + saline, CPZ + (R)-ketamine groups. (one-way ANOVA, succinic acid: $F_{2,20} = 0.035$, $P = 0.966$; lactic acid: $F_{2,19} = 4.173$, $P = 0.031$; acetic acid: $F_{2,21} = 0.687$, $P = 0.514$; malic acid: $F_{2,21} = 0.702$, $P = 0.507$; butyric acid: $F_{2,21} = 0.291$, $P = 0.751$). **B:** The heat map displayed the correlation coefficient between bacterial abundance at species level and lactic acid, demyelination area, or Iba1-positive area. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$.* **C:** The integrative network showed the correlations (Spearman analysis $r > 0.5$, $P < 0.05$) among lactic acid, demyelination area, Iba1-positive area, the bacterial relative abundance that differ significantly at species level. N = 8/group. The succinic acid level of one mouse in CPZ + (R)-Ketamine group and the lactic acid levels of two mice in CPZ + saline group were no detectable.
*Shan*: Investigation, Writing – review & editing. *Yong Yang*: Investigation, Writing – review & editing. *Li Ma*: Investigation, Writing – review & editing. *Kenji Hashimoto*: Conceptualization, Funding acquisition, Writing – original draft, Writing – review & editing.

**Declaration of competing interest**

Dr. Hashimoto is the inventor of filed patent applications on “The use of R-Ketamine in the treatment of psychiatric diseases”, “(S)-norketamine and salt thereof as pharmaceutical”, “R-Ketamine and derivative thereof as prophylactic or therapeutic agent for neurodegeneration disease or recognition function disorder”, “Preventive or therapeutic agent and pharmaceutical composition for inflammatory diseases or bone diseases”, “R-Ketamine and its derivatives as a preventive or therapeutic agent for a neurodevelopmental disorder”, and “Preventive or therapeutic agent and pharmaceutical composition for inflammatory diseases” by the Chiba University. Dr. Hashimoto also declares that he has received research support and consultant from Dainippon Sumitomo, Otsuka, Taisho, Murakami Farm, and Perception Neuroscience. The other authors have no conflict of interest.

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Fig. 6. Effects of (R)-ketamine on remyelination and microglia activation in the CPZ-treated mice. A: The protocol of the experiment. B: The MBP (myelin basic protein) staining images were taken of the area outlined by the red line box. The Iba1 staining images were taken of the area outlined by the green line box. C: The representative photos of MBP and DAPI in the corpus callosum of brain. Scale bar: 300 μm. D: The representative photos of Iba1 and DAPI in the corpus callosum. Scale bar: 100 μm. E: Quantitative data of demyelination area in the corpus callosum (one-way ANOVA: F2,29 = 21.233, P < 0.001). The percentage of demyelination area was determined by the calculation [Corpus callosum area -MBP-positive area]/ corpus callosum area × 100%. F: Quantitative data of Iba1-positive area in the corpus callosum (one-way ANOVA: F2,28 = 57.598, P < 0.001). The percentage of Iba1-positive was determined by the calculation [Iba1-positive area/corpus callosum area] × 100%). N = 10 or 11/group. **P < 0.01. ***P < 0.001. ND: no detectable. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)
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