Esketamine prevents cognitive deficiency via intestinal flora/subdiaphragmatic vagus nerve/spleen axis after a secondary LPS exposure

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Abstract

After resolution of primary infection, patients are susceptible to more severe secondary infection with unclear underlying mechanisms. To investigate whether esketamine treatment immediately after primary LPS exposure could alleviate second-infection-induced cognitive impairment. In this study, mice were injected intraperitoneally (i.p.) with lipopolysaccharides (LPS; 5 mg/kg) 10 days apart. Esketamine (10, 15 or 30 mg/kg) was i.p. injected immediately after the primary LPS injection. Splenectomy or subdiaphragmatic vagotomy (SDV) was performed 7 days before secondary LPS exposure or administration of antibiotics. Esketamine at 30 mg/kg mitigated splenomegaly at 3 and 10 days post primary LPS injection. It countered cognitive dysfunction and proinflammatory cytokine increases from secondary LPS exposure. Mice with splenectomy or SDV showed reduced proinflammatory cytokines, heightened hippocampal BDNF, and improved cognition after secondary infection, without added esketamine effect. FMT from esketamine-treated endotoxic mice to pseudo germ-free (PGF) mice attenuated hippocampal BDNF downregulation and cognitive dysfunction in PGF mice without splenectomy. FMT failed to reverse PGF mice's SDV-induced splenomegaly. Blocking BDNF signaling negated esketamine's ameliorating effects on secondary LPS-induced cognitive dysfunction. In conclusion, the intestinal flora/subdiaphragmatic vagus nerve/spleen axis-mediated downregulation of hippocampal BDNF had a profound effect on secondary LPS-induced inflammation and cognitive dysfunction.

Introduction

Systemic injection of bacterial lipopolysaccharides (LPS) elicits an important effects on the initiation of the innate immune inflammatory response, leading to sickness in mice (1–4). An LPS injection animal model has been widely used as a means of replicate gram-negative bacterial infection to study the psychiatric and cognitive consequences of infection, systemic inflammation and neuroinflammation (5, 6). To address LPS-induced acute cognitive impairment, the systemic administration of interleukin (IL)-1 receptor antagonists (7, 8) or IL-17A-neutralizing antibodies (9) has been demonstrated to be effective. This suggests that LPS-induced systemic inflammation plays essential roles in cognitive dysfunction. Evidence has shown that critically ill patients or mice in the process of recovering from an initial infection face an elevated susceptibility to developing secondary infection (10, 11), which, in turn, lead to more severe inflammatory responses and tissue damage (11). However, the underlying mechanisms of secondary infection-induced inflammatory injury need to be fully elucidated.

A growing body of evidence suggests that (R,S)-ketamine, an antagonist of the N-methyl-D-aspartate (NMDA) receptor, has significant anti-inflammatory effects (12–16). (R,S)-Ketamine decreased cerebral immune response activation to transient hypoxia in foetal sheep, as demonstrated by a decreased number of microglia and macrophages in the hypothalamus and hippocampus (17). In traumatic brain injury (TBI), the combined use of (R,S)-ketamine could reduce the central and peripheral expression of inflammatory modulators (18). Moreover, (R,S)-Ketamine also reversed LPS-mediated upregulation of proinflammatory cytokines in vitro and in vivo (19, 20). (R,S)-Ketamine is an equal mixture of (R)-
ketamine and esketamine. \( (R) \)-Ketamine is well established to exert protective effects in a variety of inflammatory diseases, including LPS-induced depression-like behaviors (21), ischaemic stroke (22), Parkinson's disease (23), and ulcerative colitis (24). Complementarily, esketamine also possesses anti-inflammatory properties (25–27). Nonetheless, it remains unclear whether esketamine alleviates systemic inflammation and cognitive dysfunction in secondary LPS exposure-imitating secondary infection post-recovery from primary LPS injection-mediated endotoxaemia.

We and others have previously demonstrated that subdiaphragmatic vagus nerve-regulated splenic immunoinflammatory cells play important roles in splenomegaly and immunosuppression (28, 29). The spleen also plays essential roles in aggravating inflammatory injury. Splenectomy has been shown to attenuate systemic inflammation and enhance survival without impairment of bacterial clearance in mice with polymicrobial sepsis (30). The learning impairments and anxiety-like behaviors resulting from red light exposure after LPS-induced lethal sepsis could be reversed by splenectomy (31). Furthermore, splenectomy could nullify the augmentation in plasma proinflammatory cytokines and the diminution in pseudo germ-free (PGF) mice transplanted with faecal bacteria from LPS-injected mice subjected to sleep deprivation (32). It has been noted that splenic reservoir Ly-6C\(^{\text{high}}\) monocytes could migrate from the spleen to the circulation and ischaemic myocardium in response to surgically induced ischaemia of the myocardium (33). Additionally, previous studies have shown that repeated social defeat (RSD) amplifies monocytopoiesis in the bone marrow that causes selective accumulation of Ly-6C\(^{\text{high}}\) monocytes in the engorged spleen (34, 35), which can then traffic from the spleen to the circulation and brain and exaggerate immunological and behavioral responses following subsequent exposure to an acute subthreshold stress (36, 37). Preventive splenectomy prior to RSD could obstruct monocyte trafficking to the brain and anxiety-like behavior following subthreshold stress (36).

Based on these collective data, the objective of this study was to test the hypothesis that the enlarged spleen induced by the primary systemic administration of LPS could mediate systemic inflammation and cognitive dysfunction after secondary LPS exposure-mediated endotoxaemia. We also investigated whether esketamine treatment immediately after primary LPS exposure could alleviate second-infection-induced cognitive impairment by inhibiting the enlarged spleen-mediated downregulation of hippocampal brain-derived neurotrophic factor (BDNF).

**Results**

**Esketamine alleviated splenomegaly after the primary systemic LPS injection**

A previous study showed that systemic injection of LPS (5 mg/kg) could induce splenomegaly 24 hours after LPS injection (32). In this study, we further determined whether splenomegaly could be observed following recuperation from primary LPS injection-mediated endotoxaemia. We found that both spleen weight and spleen weight/body weight ratio witnessed a notable surge on Day 3 (Fig. 1B–D) and Day 10.
(Fig. 1E–G) after a single injection of LPS. The administration of esketamine at a dose of 30 mg/kg (not 10 or 15 mg/kg) immediately after primary LPS injection attenuated the increase in the spleen weight and spleen weight/body weight ratio on Day 3 (Fig. 1B–D) and Day 10 (Fig. 1E–G) after LPS injection. Thus, the dose of esketamine at 30 mg/kg was chosen for the following experiments.

**Esketamine prevented the secondary LPS exposure-induced inflammatory injury**

The plasma levels of the proinflammatory cytokines IL-6 (Fig. 2B), IL-17A (Fig. 2C), TNF-α (Fig. 2D) and IFN-γ (Fig. 2E) were significantly higher in secondary LPS-exposed mice compared to those in mice injected with saline after 24 hours. Secondary LPS-exposed mice had significantly lower protein levels of BDNF in the hippocampus compared with saline-treated mice (Fig. 2F). In the Y maze test, secondary LPS significantly reduced the number of entries (Fig. 2G) and duration (Fig. 2H) in the novel arm compared to the saline control. The latency of mice to eat the food was significantly extended in secondary LPS-exposed mice compared to that in saline-injected mice (Fig. 2I). In the NORT, the mice spent a comparable duration exploring each object in all groups during the training trial (Fig. 2J). During the retention trial performed 1 hour following training, the saline-injected mice spent a significantly longer duration exploring the novel object compared with the familiar object, whereas the secondary LPS-exposed mice explored the objects equally (Fig. 2K).

Importantly, esketamine treatment at a dose of 30 mg/kg (not 10 or 15 mg/kg) immediately after primary LPS injection attenuated the secondary LPS-induced increases in plasma IL-6 (Fig. 2B), IL-17A (Fig. 2C), TNF-α (Fig. 2D) and IFN-γ (Fig. 2E) and decreased hippocampal BDNF (Fig. 2F). Furthermore, compared with the secondary LPS-exposed mice, the number of entries (Fig. 2G) and duration (Fig. 2H) in the novel arm of the Y maze test were significantly increased, whereas the latency of mice to eat the food in the buried food test (Fig. 2I) was significantly decreased in esketamine-treated endotoxic mice. Notably, esketamine-treated endotoxic mice exhibited a significant preference for the novel object compared with the secondary LPS-exposed mice (Fig. 2K).

**Effects of splenectomy and subdiaphragmatic vagotomy on secondary LPS exposure-induced inflammatory injury**

To assess the roles of the spleen and subdiaphragmatic vagus nerve in the secondary LPS exposure-induced increase in plasma proinflammatory cytokines and cognitive dysfunction, splenectomy or SDV was performed 7 days prior the secondary LPS exposure. Notably, in secondary LPS-exposed mice subjected to splenectomy or SDV, the plasma IL-6 (Fig. 3B and 4B), IL-17A (Fig. 3C and 4C), TNF-α (Fig. 3D and 4D) and IFN-γ (Fig. 3E and 4E) were significantly decreased, whereas the hippocampal BDNF (Fig. 3F and 4F) was significantly increased compared to secondary LPS-exposed mice subjected to their respective sham operation. Moreover, the number of entries (Fig. 3G and 4G) and duration (Fig. 3H and 4H) in the novel arm of the Y maze test were significantly increased, whereas the latency of mice to eat the food in the buried food test (Fig. 3I and 4I) was significantly decreased in endotoxic mice subjected to
splenectomy or SDV compared to that in endotoxic mice subjected to sham operation. Furthermore, endotoxic mice subjected to either splenectomy or SDV exhibited a significant preference for the novel object compared with sham-operated endotoxic mice (Fig. 3K and 4K).

However, in endotoxic mice subjected to splenectomy or SDV, the preventive effects of esketamine on the secondary LPS exposure-induced levation in plasma proinflammatory cytokines (Fig. 3B–E and 4B–E), downregulation of hippocampal BDNF (Fig. 3F and 4F) and cognitive dysfunction (Fig. 3G–K and 4G–K) were eradicated.

The roles of the spleen in intestinal flora-mediated cognitive function after secondary LPS exposure

Given the previously reported key roles of the spleen in intestinal flora dysfunction-mediated inflammatory injury (28, 32), we sought to ascertain whether the spleen played important roles in mediating the detrimental effects of intestinal flora on the cognitive function induced by secondary LPS exposure. It was observed that PGF mice with sham-operation, but not those subjected to splenectomy, who received FMT with faecal suspensions from secondary LPS-exposed mice had significantly lower protein levels of BDNF in the hippocampus than sham-operated PGF mice that received FMT with faecal suspensions from saline-injected mice (Fig. 5B). Moreover, the number of entries (Fig. 5C) and duration (Fig. 5D) in the novel arm of the Y maze test were significantly decreased, whereas the latency of mice to eat the food in the buried food test (Fig. 5E) was significantly increased in sham-operated (but not splenectomized) PGF mice that received FMT with faecal suspensions from secondary LPS-exposed mice compared to sham-operated PGF mice that received FMT with faecal suspensions from saline-injected mice. In the NORT, the mice spent a comparable duration exploring each object in all groups during the training trial (Fig. 5F). However, during the retention trial performed 1 hour following training, the sham-operated PGF mice that received FMT with faecal suspensions from saline-injected mice spent a significantly longer duration exploring the novel object compared with the familiar object (Fig. 5G). Conversely, the sham-operated (but not splenectomized) PGF mice that received FMT with faecal suspensions from secondary LPS-exposed mice explored the objects equally.

However, FMT with faecal suspensions from secondary LPS-exposed mice treated with esketamine attenuated the downregulation of hippocampal BDNF (Fig. 5B) and cognitive dysfunction (Fig. 5C–G) in PGF mice without splenectomy, but not in PGF mice with splenectomy.

Effects of subdiaphragmatic vagotomy on the modulation of intestinal flora in the spleen after secondary LPS exposure

In view of the important roles of the subdiaphragmatic vagus nerve in the mediated effects of intestinal flora on the spleen (28, 32), we then explored whether the subdiaphragmatic vagus nerve could mediate the communication of intestinal flora and the spleen after secondary LPS exposure. Sham-operated (but not SDV) PGF mice that received FMT with faecal suspensions from secondary LPS-exposed mice had
significantly increased spleen weight (Fig. 6B and C) and spleen weight/body weight ratio (Fig. 6D) compared to sham-operated PGF mice that received FMT with faecal suspensions from saline-injected mice. Interestingly, FMT with faecal suspensions from secondary LPS-exposed mice treated with esketamine attenuated the increased spleen weight (Fig. 6B and C) and spleen weight/body weight ratio (Fig. 6D) in PGF mice without SDV but not in PGF mice with SDV.

**The roles of hippocampal BDNF in secondary LPS exposure-induced cognitive dysfunction**

The beneficial effects of esketamine on the secondary LPS exposure-induced decrease in the number of entries (Fig. 7B) and duration (Fig. 7C) in the novel arm of the Y maze test and the increase in the latency of mice to eat the food (Fig. 7D) in the buried food test were significantly weakened by blocking BDNF signalling with ANA-12. During the training trial in the NORT, mice spent a comparable amount of time exploring the same objects in all groups (Fig. 7E). During the retention trial performed 1 hour following training, esketamine-treated mice spent significantly more time exploring the novel object than the familiar object 1 day after the secondary LPS exposure. However, the esketamine treatment-induced significant increase in the time spent exploring the novel object compared with the familiar object (Fig. 7F) was abolished in mice treated with ANA-12.

**Discussion**

The major findings of this study are presented as follows: First, after recovery from primary LPS injection-mediated endotoxaemia, both the augmented spleen and intestinal flora/subdiaphragmatic vagus nerve played important roles in secondary LPS exposure-mimicked secondary infection-mediated systemic inflammation and cognitive dysfunction; Second, the intestinal flora/subdiaphragmatic vagus nerve exerted proinflammatory effects mediated by the spleen in secondary infection; Third, the downregulation of hippocampal BDNF was involved in intestinal flora/subdiaphragmatic vagus nerve/spleen axis-mediated cognitive dysfunction in secondary infection; Fourth, esketamine treatment immediately after primary LPS exposure could prevent secondary infection-induced systemic inflammation and cognitive dysfunction via intestinal flora/subdiaphragmatic vagus nerve/spleen axis-mediated hippocampal BDNF signalling.

The spleen has a noteworthy influence on systemic inflammation triggered by an upset in intestinal flora, following a systemic injection of LPS (5 mg/kg), and splenectomy abrogated the increased plasma proinflammatory cytokines (IL-6 and TNF-α) and decreased plasma anti-inflammatory cytokines (IL-10) in PGF mice transplanted with faecal bacteria from LPS-induced septic mice subjected to sleep deprivation (32). The spleen also plays important roles in lethal sepsis-induced systemic inflammation and cognitive dysfunction (31, 32). In addition, LPS (10 mg/kg)-induced AKI could be attenuated by splenectomy (38). The same procedure may also reduce systemic inflammation and improve survival rates in mice with caecal ligation and puncture (CLP)-induced polymicrobial sepsis (30). Furthermore, learning impairments and anxiety-like behaviors that arise from the exposure to red light subsequent to LPS (20 mg/kg)-
induced fatal sepsis may likewise be alleviated through the procedure of splenectomy (31). Splenic reservoir Ly-6C$^{\text{high}}$ monocytes could emigrate from the subcapsular red pulp of the spleen to the circulation and ischaemic myocardium in response to surgically induced acute myocardium ischaemia (33). Beyond its key roles in primary infection-induced inflammatory injury, the spleen significantly impacts secondary infection-induced systemic inflammation and cognitive dysfunction, possibly due to trafficking of the initial sensitizing event-induced accumulated monocytes from the enlarged spleen to the circulation and brain (34–37). In our present study, we found that an enlarged spleen 10 days after induction by primary LPS exposure played important roles in the second infection-induced systemic inflammation. However, the underlying mechanisms are unclear and need to be further investigated. The migration of accumulated inflammatory monocytes from the systemic LPS-induced enlarged spleen to the circulation may contribute to the second infection-induced systemic inflammation (32, 33, 36). A study found that splenectomy could cause significant alterations in the diversity of gut microbes (39). In our present study, after 7 days of splenectomy, the mice were given broad-spectrum antibiotics for 14 days; thus, the effects of splenectomy on gut microbiota diversity could not be investigated.

Evidence has also shown that the subdiaphragmatic vagus nerve is essential for communication between the intestinal flora and brain (40, 41). The subdiaphragmatic vagus nerve plays important roles in low-dose LPS (0.5 mg/kg)-induced splenomegaly, systemic inflammation and depression-like behavior in mice (42). Systemic LPS injection combined with sleep deprivation-induced intestinal flora disturbance mediated splenomegaly via the subdiaphragmatic vagus nerve (32), indicating that the subdiaphragmatic vagus nerve could also mediate communication between the intestinal flora and spleen. In our present study, intestinal flora exerted deleterious impacts on cognitive function by means of regulation of the spleen, facilitated by the subdiaphragmatic vagus nerve. These findings align with our previous research, which demonstrated that intestinal flora disturbance causes postoperative splenomegaly via the subdiaphragmatic vagus nerve (28).

BDNF expressed in the hippocampus and cerebral cortex is integral to synaptic plasticity, neuronal survival and neurogenesis and is associated with core features of neuropsychiatric disorders (43). The hippocampal-specific deletion of the BDNF gene by a lentivirus expressing Cre recombinase impairs spatial learning and extinction of aversive memories (44). Downregulation of BDNF in the hippocampus has been proposed to play important roles in repairing behavioral and cognitive changes by LPS-induced systemic and cerebral inflammation (43). LPS-induced proinflammatory cytokines mediate the impairment of BDNF (45). Alleviation of the downregulation of BDNF in the hippocampus through reducing inflammation in LPS-treated mice could enhance memory deficits and rectify behavioral dysfunctions (46). Based on our results, LPS administration was associated with decreased BDNF levels in the hippocampus, and blocking TrkB/BDNF signalling compromised the protective effects of the inhibition of the subdiaphragmatic vagus nerve-spleen axis on cognitive dysfunction, underscoring the relevance of hippocampal BDNF in the recovery of cognitive function (46–48). In our present study, secondary infection-induced cognitive dysfunction was due to intestinal flora/subdiaphragmatic vagus nerve/spleen axis-mediated downregulation of hippocampal BDNF, which further suggests the
importance of BDNF signalling in cognitive function. However, other neurotrophic factors (vascular growth factor (VGF), nerve growth factor (NGF), neurotrophin 3 (NT3), NT4, etc.) also play important roles in cognitive function (49, 50), and whether they could also be involved in intestinal flora/subdiaphragmatic vagus nerve/spleen axis-induced cognitive function caused by secondary infection remains unclear and needs further investigation. Another important limitation of the study is that the alteration of the gut microbiota composition was not examined, given that mouse faeces were not readily available 24 hours after LPS-induced bacteremia. In addition, the prefrontal cortex (PFC) has also been shown to play important roles in cognitive and behavioral functions in humans and rodents (21, 42, 51). Whether BDNF in the PFC could be involved in LPS-induced secondary infection-mediated cognitive dysfunction is also unclear.

It has been well established that \((R,S)\)-ketamine possesses potent anti-inflammatory properties (12–16). \((R,S)\)-Ketamine is an arylcyclohexylamine and has two enantiomers, \((R)\)-ketamine and esketamine. \((R)\)-Ketamine has been shown to be capable of exerting protective effects in a variety of inflammatory diseases, including LPS-induced depression-like behaviors (21), ischaemic stroke (22), Parkinson’s disease (23), and ulcerative colitis (24). Esketamine, as the pure dextrorotatory enantiomer of ketamine, has been available for clinical use in China. Because of its broad spectrum of pharmacologic effects, including analgesia and Anesthesia, anti-depression and anti-inflammatory effects, esketamine has great application prospects in emergency and intensive care medicine (25, 26). Anesthesia with continuous infusion of esketamine after a single intravenous injection during elective coronary artery bypass graft (CABG) surgery decreases plasma proinflammatory cytokines (IL-6 and IL-8) and increases plasma IL-10 during and after cardiopulmonary bypass (26). Combined use of esketamine for induction and maintenance could also exert a systemic anti-inflammatory effects, and increase the absolute counts of CD3+ and CD4+ cells in patients at the end of and 24 h after modified radical mastectomy (27). Our present study further revealed potential anti-inflammatory effects of esketamine on secondary infection-induced systemic inflammation and elucidated the mechanism of its anti-inflammatory effects by attenuating the downregulation of hippocampal BDNF through inhibiting the intestinal flora/subdiaphragmatic vagus nerve/spleen axis. However, an obvious limitation of our study is that there were only eight mice in each group.

**Conclusion**

In the secondary LPS exposure-mimicked secondary infection after recovery from primary LPS injection-mediated endotoxaemia, the intestinal flora/subdiaphragmatic vagus nerve/spleen axis played important roles in systemic inflammation. The downregulation of hippocampal BDNF mediated by the axis contributed to cognitive dysfunction, which could be prevented by esketamine treatment immediately after primary LPS exposure.

**Materials and Methods**
Animals

Male C57BL/6 mice (8 weeks old) were purchased from Vital River Laboratory Animal Technology Co Ltd., Beijing, China. Mice were housed in a 12 h dark/light cycle with *ad libitum* access to food and water. Mice were allowed to acclimatize for 1 week prior to commencement of the study. All experiments were conducted in accordance with the National Institute of Health (NIH) Guide for the Care and Use of Laboratory Animals (publications no. 80 – 23) revised 1996 and approved by the Animal Care and Use Committee of Henan Provincial People's Hospital. Efforts were taken to reduce animal suffering from pain and discomfort.

Grouping and treatment

The mice were randomly assigned to the following groups (8 mice/group): (1) 0.9% saline (10 ml/kg) or lipopolysaccharides (LPS; 5 mg/kg; Sigma–Aldrich, St. Louis, MO, USA) was administered intraperitoneally (i.p.) to mice. Secondary LPS was administered 10 days after the primary administration of LPS or saline; (2) esketamine (10, 15 or 30 mg/kg; Hengrui Pharmaceutical Co., Jiangsu, China) was i.p. administered to mice immediately after the primary administration of LPS or saline; (3) splenectomy, subdiaphragmatic vagotomy (SDV) or their respective sham operation was performed 3 days after the primary LPS exposure; (4) ANA-12 (N-[2-[(hexahydro-2-oxo-1H-azepin-3-yl)amino]carbonyl]phenyl]-benzo[b]thiophene-2-carboxamide; 0.5 mg/kg; Maybridge, Cornwall, UK) was dissolved in phosphate-buffered saline (PBS) containing 17% dimethylsulfoxide (DMSO) (52) and administered i.p. to mice 30 minutes prior to the second administration of LPS; (5) PGF mice with splenectomy, SDV or their respective sham operation received faecal microbiota transplantation (FMT) from secondary LPS-treated mice with or without esketamine treatment. PGF mice underwent splenectomy, SDV or their respective sham operation 7 days prior to antibiotic administration.

Subdiaphragmatic bilateral truncal vagotomy

Bilateral SDV was performed under Anesthesia with 1% sodium pentobarbital (50 mg/kg). After a 1 cm transverse incision was made on the left upper abdominal wall to expose the gastroesophageal junction, the oesophagus was exposed by carefully keeping the costal arc, liver, and stomach out of sight. Under a stereodissection microscope, the ventral and dorsal truncal branches were exposed along the subdiaphragmatic oesophagus and transected. The incisions were closed by suture. For sham-operated mice, both vagus nerves were exposed but not transected. Soft gel feed was provided to mice that received SDV until they could perform normal activities. The completeness of vagotomy was verified at sacrifice by inspection of vagal nerve endings.

Splenectomy

Splenectomy was performed under Anesthesia with 1% sodium pentobarbital (50 mg/kg). After a 1-cm subcostal incision on the left dorsolateral side of the abdomen was made, the spleen was brought out carefully into the field of view through the incision. The afferent and efferent vessels near the spleen underwent ligatures and sectioning, and then the spleen was removed. The abdominal wall was closed in
two layers with sutures. Sham surgery was performed by exteriorizing the spleen without removal. Mice were allowed to recover for 7 days prior to the second administration of LPS or administration of antibiotics.

**PGF mouse model and FMT**

According to previous studies (32, 53), broad-spectrum antibiotics (ampicillin 1 g/L, neomycin sulfate 1 g/L, and metronidazole 1 g/L; Sigma–Aldrich Co. Ltd, USA) dissolved in drinking water were given *ad libitum* to mice for 14 consecutive days. The drinking solution was renewed every 2 days.

The faecal donor mice received secondary administration of LPS or saline with or without esketamine treatment. Twenty-four hours after the second administration of LPS or saline, the mice were placed in a clean cage with sterilized filter paper on the bottom. Stool samples were collected immediately after defecation in a sterile microtube and immediately stored at −80 °C. One gram of faecal sample from donor mice was diluted in 10 ml of PBS and then suspended. Two hundred microlitres of the suspension was given to each mouse recipient by gavage.

**Behavioral assessment**

The Y maze test, buried food test and novel object recognition task (NORT) were performed at 22, 24 and 26 hours after the second administration of LPS or the final FMT, respectively. The Y maze test, buried food test and NORT were performed at 26, 24 and 22 hours prior to the second administration of LPS or the first FMT as the baseline values, respectively.

The Y maze test was performed as previously described (54). Each maze consisted of three equal arms (30 × 8 × 15 cm, length × width × height) with a 120° angle from each other. In the first training trial, the novel arm was blocked, and each mouse was allowed to explore the other two open arms for 10 minutes. The second retention trial (retention) was conducted 1 hour after the first training trial. The mouse was placed back in the start arm with free exploration of all 3 arms for 5 minutes. The number of entries and the time spent in the novel arm were recorded by a video camera.

The buried food test was conducted as described previously (54). On the day of the test, 1 sweetened cereal pellet was hidden 1 cm beneath the surface of the clean bedding (3 cm deep) in a random corner of the clean cage (46 × 23.5 × 20 cm, length × width × height). The mouse was placed in the diagonal corner of the test cage to explore freely for 5 minutes, and the latency to find the buried food was recorded. Latency was defined as the time between when the mouse was placed in the test cage and when the mouse started to eat. If the mouse failed to find the buried food after 5 minutes had elapsed, the test was stopped, and the latency score was recorded as 300 sec.

The NORT was carried out as described previously (55). The mouse was placed in a grey chamber (35 × 35 × 25 cm, length × width × height) for 30 minutes 1 day prior to the NORT. During the training trial, the mouse was allowed to explore two identical objects placed symmetrically in the centre of the chamber for 10 minutes. The time spent exploring each object was recorded. During the retention trial, the mouse was
placed in the chamber and allowed to explore a novel object and a familiar object from the training trial for 5 minutes. The time spent exploring familiar and novel objects was recorded. The mouse was considered to be exploring an object when they faced the object (within 0.5 cm of the object) and actively explored it (by sniffing or physical manipulation). The discrimination index: [time spent exploring any one object (training trial) or the novel object (retention trial)/(total time spent exploring both objects) × 100%].

**Enzyme-linked immunosorbent assay (ELISA)**

The plasma expression of IL-6 (#88-7064), IL-17A (#88-7371), tumor necrosis factor alpha (TNF-α; #88-7324), and interferon-gamma (IFN-γ; #88-8314) was detected using ELISA. The kits were purchased from Invitrogen (Camarillo, CA, USA), and the process was conducted according to the manufacturer's instructions.

**Western blotting**

Tissue samples from the hippocampus containing equal amounts of proteins were separated by 12% sodium dodecyl sulfate–polyacrylamide gel electrophoresis and then transferred onto polyvinylidene difluoride (PVDF) membranes using a Trans Blot Mini Cell (Bio-Rad), followed by blocking with 5% skim milk in TBS with 0.1% Tween 20 (TBST) and incubation with the rabbit polyclonal anti-BDNF antibody and mouse monoclonal anti-β-actin antibody (1:10,000, #A5441, Sigma–Aldrich Co., Ltd., St Louis, MO, USA) overnight at 4°C. Following primary antibody incubation, membranes were washed with TBST and incubated with horseradish peroxidase (HRP)-conjugated anti-rabbit antibody (1:5,000) for 1 hour at room temperature and visualized by enhanced chemiluminescence (ECL) plus the Western blot Detection system (GE Healthcare Bioscience).

**Statistical Analysis**

Data are expressed as the mean ± standard error of the mean (SEM). Data were analysed using either Student's t test (for simple two-sample comparison) or one-way analysis of variance (ANOVA) with Tukey's post hoc test (for multiple comparison). \( P < 0.05 \) was considered to be statistically significant. Statistical analysis was performed using GraphPad Prism 9.0 software (GraphPad Software, Inc., La Jolla, CA, USA).

**Declarations**

**Author contributions**

GZW and SYY had full access to all data in the study and take responsibility for the integrity of the data and the accuracy of the data analysis and contributed to the concept and design. YMW, YJZ, BX and XYZ performed the experiments, analyzed and interpreted the data. YMW contributed to the drafting of the manuscript and the statistical analysis. SYY contributed to the critical revision of the manuscript for important intellectual content and supervised the study. All authors contributed to the article and approved the submitted version.
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Data availability statement

The data sets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Declaration of conflicting interests

The authors declare that there is no conflict of interest.

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Figures
Esketamine treatment alleviated splenomegaly induced by the primary systemic LPS injection. (A)

Treatment schedule. Mice were intraperitoneally (i.p.) administered LPS (5 mg/kg) or 0.9% saline. Mice were i.p. administered esketamine (10, 15 or 30 mg/kg) immediately after the administration of LPS or saline. Spleens were collected 3 and 10 days after LPS injection. Representative picture of spleen (B), spleen weight (C) and spleen weight/body weight ratio (D) 3 days after LPS injection. Representative picture of spleen (E), spleen weight (F) and spleen weight/body weight ratio (G) 10 days after LPS injection. Data are shown as the mean ± SEM (n = 8/group). *P < 0.05, **P < 0.01, ***P < 0.0001; NS, not significant. LPS, lipopolysaccharide; S-KT, esketamine.
Figure 2

Esketamine prevented the secondary LPS exposure-induced increase in plasma proinflammatory cytokines and cognitive dysfunction. (A) Treatment schedule. Mice were intraperitoneally (i.p.) administered LPS (5 mg/kg) or 0.9% saline. Secondary LPS was administered 10 days after the primary administration of LPS or saline. Mice were i.p. administered esketamine (10, 15 or 30 mg/kg) immediately after the primary administration of LPS or saline. The Y maze test, buried food test and novel object recognition task (NORT) were performed at 22, 24 and 26 hours after the secondary LPS exposure. The Y maze test, buried food test and NORT were performed at 26, 24 and 22 hours prior to the secondary LPS exposure as the baseline values. Blood and the hippocampus were collected 24 hours after the secondary LPS exposure. Enzyme-linked immunosorbent assay (ELISA) detection of plasma interleukin (IL)-6 (B), IL-17A (C), TNF-α (D), and IFN-γ (E). (F) Western blotting analysis of the expression
of BDNF in the hippocampus. Entries in the novel arm (G) and duration in the novel arm (H) in the Y maze test. (I) The latency to eat food in the buried food test. (J) Time spent exploring the familiar objects in all groups during the training trial. (K) Time spent exploring the familiar and novel objects in all groups during the test trial. Data are shown as the mean ± SEM (n = 8/group). *P < 0.05, **P < 0.01, ***P < 0.0001; NS, not significant. LPS, lipopolysaccharide; S-KT, esketamine; TNF-α, tumor necrosis factor alpha; IFN-γ, interferon-gamma.
Figure 3

The effects of the spleen on the secondary LPS exposure-induced increase in plasma proinflammatory cytokines and cognitive function. (A) Treatment schedule. Mice were intraperitoneally (i.p.) administered LPS (5 mg/kg) or 0.9% saline. Secondary LPS or saline was administered 10 days after the primary administration of LPS or saline. Mice were i.p. administered esketamine (30 mg/kg) immediately after the primary administration of LPS or saline. Splenectomy or sham operation was performed 7 days before the secondary LPS exposure. The Y maze test, buried food test and novel object recognition task (NORT) were performed at 22, 24 and 26 hours after the secondary LPS exposure. The Y maze test, buried food test and NORT were performed at 26, 24 and 22 hours prior to the secondary LPS exposure as the baseline values. Blood and the hippocampus were collected after behavioral tests. Enzyme-linked immunosorbent assay (ELISA) detection of plasma interleukin (IL)-6 (B), IL-17A (C), TNF-α (D), and IFN-γ (E). (F) Western blotting analysis of the expression of BDNF in the hippocampus. Entries in the novel arm (G) and duration in the novel arm (H) in the Y maze test. (I) The latency to eat food in the buried food test. (J) Time spent exploring the familiar objects in all groups during the training trial. (K) Time spent exploring the familiar and novel objects in all groups during the test trial. Data are shown as the mean ± SEM (n = 8/group). *P < 0.05, **P < 0.01; NS, not significant. LPS, lipopolysaccharides; S-KT, esketamine; SPX, splenectomy; TNF-α, tumor necrosis factor alpha; IFN-γ, interferon-gamma.
Figure 4

The roles of the subdiaphragmatic vagus nerve in the secondary LPS exposure-induced increase in plasma proinflammatory cytokines and cognitive function. (A) Treatment schedule. Mice were intraperitoneally (i.p.) administered LPS (5 mg/kg) or 0.9% saline. Secondary LPS or saline was administered 10 days after the primary administration of LPS or saline. Mice were i.p. administered esketamine (30 mg/kg) immediately after the primary administration of LPS or saline. SDV or sham...
operation was performed 7 days before the secondary LPS exposure. The Y maze test, buried food test and novel object recognition task (NORT) were performed at 22, 24 and 26 hours after the secondary LPS exposure. The Y maze test, buried food test and NORT were performed at 26, 24 and 22 hours prior to the secondary LPS exposure as the baseline values. Blood and the hippocampus were collected after behavioral tests. Enzyme-linked immunosorbent assay (ELISA) detection of plasma interleukin (IL)-6 (B), IL-17A (C), TNF-α (D), and IFN-γ (E). (F) Western blotting analysis of the expression of BDNF in the hippocampus. Entries in the novel arm (G) and duration in the novel arm (H) in the Y maze test. (I) The latency to eat food in the buried food test. (J) Time spent exploring the familiar objects in all groups during the training trial. (K) Time spent exploring the familiar and novel objects in all groups during the test trial. Data are shown as the mean ± SEM (n = 8/group). *P < 0.05, **P < 0.01; NS, not significant. LPS, lipopolysaccharides; S-KT, esketamine; SDV, subdiaphragmatic vagotomy; BDNF, brain-derived neurotrophic factor; TNF-α, tumor necrosis factor alpha; IFN-γ, interferon-gamma.
The effects of the spleen on intestinal flora-mediated cognitive function after secondary LPS exposure.

(A) Treatment schedule. Splenectomy or sham operation was performed 21 days before FMT. After 7 days, the mice were given broad-spectrum antibiotics for 14 days. Then, the mice were orally administered faecal microbiota from secondary LPS (5 mg/kg)-exposed mice that had been treated with esketamine (30 mg/kg) or 0.9% saline. The Y maze test, buried food test and novel object recognition...
task (NORT) were performed at 22, 24 and 26 hours after the final FMT. The Y maze test, buried food test and NORT were performed at 26, 24 and 22 hours prior to the first FMT as the baseline values. After the behavioral tests, hippocampi were collected. (B) Western blotting analysis of the expression of BDNF in the hippocampus. Entries in the novel arm (C) and duration in the novel arm (D) in the Y maze test. (E) The latency to eat food in the buried food test. (F) Time spent exploring the familiar objects in all groups during the training trial. (G) Time spent exploring the familiar and novel objects in all groups during the test trial. Data are shown as the mean ± SEM (n = 8/group). *P < 0.05, **P < 0.01; NS, not significant. LPS, lipopolysaccharides; S-KT, esketamine; SPX, splenectomy; FMT, faecal microbiota transplantation; BDNF, brain-derived neurotrophic factor.

Figure 6

The effects of the subdiaphragmatic vagus nerve on the modulation of intestinal flora in the spleen after secondary LPS exposure. (A) Treatment schedule. SDV or sham operation was performed 21 days before FMT. After 7 days, the mice were given broad-spectrum antibiotics for 14 days. Then, the mice were orally administered faecal microbiota from secondary LPS (5 mg/kg)-exposed mice that had been treated with esketamine (30 mg/kg) or 0.9% saline. Twenty-four hours after the final FMT, spleens were collected and weighed. Representative picture of spleen (B), spleen weight (C) and spleen weight/body weight ratio (D). Data are shown as the mean ± SEM (n = 8/group). *P < 0.05, **P < 0.01; NS, not significant. LPS, lipopolysaccharides; S-KT, esketamine; SDV, subdiaphragmatic vagotomy; FMT, faecal microbiota transplantation.
The roles of hippocampal BDNF in secondary LPS exposure-induced cognitive dysfunction. (A) Treatment schedule. Mice were intraperitoneally (i.p.) administered LPS (5 mg/kg) or 0.9% saline. Secondary LPS or saline was administered 10 days after the primary administration of LPS or saline. Mice were i.p. administered esketamine (30 mg/kg) immediately after the primary administration of LPS or saline. ANA-12 or 17% DMSO was given i.p. 30 minutes prior to the secondary LPS exposure. The Y maze test, buried food test and novel object recognition task (NORT) were performed at 22, 24 and 26 hours after the secondary LPS exposure. The Y maze test, buried food test and NORT were performed at 26, 24 and 22 hours prior to the secondary LPS exposure as the baseline values. Entries in the novel arm (B) and duration in the novel arm (C) in the Y maze test. (D) The latency to eat food in the buried food test. (E)
Time spent exploring the familiar objects in all groups during the training trial. (F) Time spent exploring the familiar and novel objects in all groups during the test trial. Data are shown as the mean ± SEM (n = 8/group). *P < 0.05, **P < 0.01; NS, not significant. LPS, lipopolysaccharides; S-KT, esketamine; DMSO, dimethylsulfoxide; ANA-12, N-[2-[[hexahydro-2-oxo-1H-azepin-3-yl]amino]carbonyl]phenyl]-benzo[b]thiophene-2-carboxamide.