学位論文

The Impact of Obesity on Postoperative Cognitive Dysfunction and the Neuroprotective Effects of Anesthetics Against Sepsis-Associated Encephalopathy

(術後認知機能障害における肥満の影響および敗血症性関連脳症に対する麻酔薬の神経保護作用)

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Catalogue

Published papers directory

This research has been published by <Biomedical Research (Tokyo)>. The paper is as follows:

1. Wei Wang, Yosuke Uchida, and Yuji Morimoto

Remimazolam and dexmedetomidine prevent cognitive decline by affecting lipopolysaccharide-induced brain injury in mice: Possible involvement of the vagus nerve pathway

Biomed Res 2025; 46(4):177-187. Doi:10.2220biomedres.46.177.

Abstract

【Background】

With the aging population and increasing demand for surgical procedures, postoperative cognitive dysfunction (POCD) and inflammation-induced cognitive impairment have become significant medical concerns.

This thesis aims to investigate:

(1) POCD represents a significant challenge in modern medical care, especially affecting the elderly. With the increase in the number of surgeries, the incidence of POCD is increasing, affecting the quality of life of patients and placing a heavy burden on the global healthcare system. Although the occurrence of POCD is related to a variety of factors, increasing evidence suggests that neuroinflammation plays a crucial role in its occurrence and development. Advanced age, metabolic syndrome, obesity and other risk factors are associated with increased susceptibility to POCD. However, the exact mechanisms linking these factors to cognitive decline remain unclear. The relationship between obesity and POCD is of particular interest given the global obesity epidemic and its potential impact on surgical outcomes.

(2) Administration of lipopolysaccharide (LPS) is a common experimental model used to induce systemic inflammation, mimicking conditions like sepsis. This LPS-induced systemic inflammation can subsequently lead to sepsis-associated encephalopathy (SAE) in the brain. SAE, in this context, frequently causes acute cognitive decline, which is associated with neuroinflammation, microglial activation, and disruption of the blood-brain barrier (BBB). Anesthetic agents like dexmedetomidine (DEX) possess known neuroprotective properties potentially linked to the vagus nerve-mediated cholinergic anti-inflammatory pathway (CAP) via α7 nicotinic acetylcholine receptors (α7nAChRs). However, the neuroprotective potential and mechanisms of the newer ultra-short-acting benzodiazepine, remimazolam (REMI), particularly concerning the CAP, remain largely unexplored. There is a lack of studies simultaneously evaluating the neuroprotective effects of REMI and DEX and their dependence on vagal pathways in relevant models.

【Methods】

1. To investigate the effect of obesity on POCD, diet-induced obesity (DIO) mouse model was established. C57BL/6 mice were fed with normal diet (NOR) and high fat diet (HFD) for 8 weeks, respectively. POCD was induced by tibial fracture surgery or

intraperitoneal injection of high mobility group box-1 (HMGB-1). Cognitive function was assessed using the trace-fear conditioning (TFC) test, a well-established method for assessing hippocampal-dependent memory in rodents. A test was performed 72 h after the intervention to evaluate the short-term cognitive effect. Blood and hippocampus tissues were collected for determination of inflammatory markers (IL-1β, TNF-α, IL-6) and leptin levels by enzyme-linked immunosorbent assay (ELISA).

2. C57BL/6 mice received intraperitoneal injection of LPS (1 mg/kg). Treatment groups included Control (saline), LPS, REMI (16 mg/kg IP + 8 mg/kg SC), DEX (30 μg/kg IP), REMI+LPS, and DEX+LPS. Additional groups received the selective α7nAChR antagonist methyllycaconitine (MLA; 4 mg/kg IP) 30 min prior to REMI/DEX/LPS. Cognitive function (TFC) was assessed 24 h post-LPS. Hippocampal CA3 neuropathology was assessed via NeuN immunohistochemistry. Serum/hippocampal cytokines (TNF-α, IL-1β, IL-6) were measured by ELISA. BBB integrity was assessed via hippocampal albumin western blot. Vagal pathway involvement was probed by measuring lung Netrin-1 expression via western Blot.

【Results】

(1) HFD mice exhibited significant weight gain and impaired baseline cognitive function (reduced TFC freezing time) compared to NOR mice. However, neither tibial fracture surgery nor HMGB-1 injection resulted in a significantly greater cognitive deficit in HFD mice compared to NOR mice under these experimental conditions. HMGB-1 injection significantly increased hippocampal IL-1β and IL-6 levels, with HFD+HMGB-1 mice showing significantly higher levels than NOR+HMGB-1 mice. Serum leptin was elevated in HFD mice, but hippocampal leptin levels were similar to NOR mice.

(2) LPS administration significantly impaired TFC freezing time, induced significant CA3 neuronal damage, increased serum/hippocampal TNF-α, IL-1β, and IL-6, increased hippocampal albumin (indicating BBB disruption), and decreased lung Netrin-1 expression. Pretreatment with either REMI or DEX significantly attenuated LPS-induced cognitive impairment, reduced CA3 neuronal damage, decreased cytokine levels, limited hippocampal albumin extravasation, and reversed the downregulation of lung Netrin-1. Crucially, the neuroprotective effects of both REMI and DEX were significantly blocked by pretreatment with the α7nAChR antagonist MLA.

【Discussion】

1. These findings suggest the interaction between obesity and acute postsurgical/inflammatory cognitive decline may be complex and model-dependent, potentially influenced by factors like obesity duration. The lack of exacerbated decline post-insult in HFD mice, despite heightened hippocampal inflammation after HMGB-1, warrants further investigation into underlying mechanisms and model limitations.
2. Both REMI and DEX demonstrate significant and comparable neuroprotective effects against LPS-induced acute cognitive impairment, neuroinflammation, hippocampal neuronal injury, and BBB disruption. The profound blockade of cognitive protection by MLA strongly indicates that α7nAChR activation is a critical component mediating the beneficial effects of both anesthetic agents. The restoration of lung Netrin-1, a marker modulated by vagal activity, further supports the involvement of the CAP. This suggests REMI, like DEX, engages α7nAChR-dependent cholinergic anti-inflammatory signaling.

【Conclusion】

1. Diet-induced obesity impairs baseline cognition in mice, but its role in exacerbating acute post-insult cognitive decline appears complex under the specific conditions tested, emphasizing the need for further research.
2. REMI and DEX exert robust neuroprotective effects against LPS-induced cognitive dysfunction and neuropathology. These effects involve mitigating neuroinflammation and BBB damage and are critically dependent, at least partially, on the activation of α7nAChRs, strongly implicating the vagus nerve-mediated cholinergic anti-inflammatory pathway. These findings establish REMI as a potent neuroprotective agent comparable to DEX in this model and reveal a shared reliance on α7nAChR signaling, suggesting potential therapeutic applications for both drugs in mitigating acute neurological complications associated with systemic inflammation, such as SAE.

Abbreviation

The abbreviations used in this article and in the figure are shown below.

BBB	Blood-Brain Barrier
CAP	Cholinergic Anti-inflammatory Pathway
CNS	Central Nervous System
DEX	Dexmedetomidine
DIO	Diet-induced obesity
GABA	Gamma-Aminobutyric Acid
HFD	High-Fat Diet
HMGB-1	High Mobility Group Box 1
IL-1β	Interleukin 1 Beta
IL-6	Interleukin 6
LPS	Lipopolysaccharide
MLA	Methyllycaconitine
NDs	Neurodegenerative Disease
NeuN	Neuronal Nuclei
NOR	Normal Diet
POCD	Postoperative Cognitive Dysfunction
REMI	Remimazolam
SAE	Sepsis-Associated Encephalopathy
TFC	Trace Fear Conditioning
TLR	Toll-like Receptor
TNF-α	Tumor Necrosis Factor Alpha

α7nAChR Alpha-7 Nicotinic Acetylcholine Receptor

Introduction

Cognitive dysfunction is a growing concern in modern healthcare and has a significant impact on the quality of life of patients and the global healthcare system. This thesis explores cognitive impairment within two critical contexts: postoperative cognitive dysfunction (POCD), particularly in relation to obesity, and inflammation-induced cognitive decline, modeled by lipopolysaccharide (LPS) administration often associated with conditions like sepsis-associated encephalopathy (SAE). Both conditions share neuroinflammation as a key pathological feature.

POCD is becoming more and more common with the increase of surgical procedures, especially in the elderly population (Terrando et al, 2011a; Bedford, 1955; Monk et al, 2008). POCD is a complex, multifactorial disorder characterized by cognitive decline after surgery, affecting memory, attention, and executive function. Although multiple factors lead to POCD, increasing evidence suggests that neuroinflammation plays a key role in its development (Deiner et al, 2017; Shoair et al, 2015; Silbert et al, 2015; Evered et al, 2016; Nemeth et al, 2017). Risk factors such as advanced age and metabolic syndrome are associated with increased susceptibility to POCD, while obesity is also being actively investigated as a potential contributing factor (Hovens et al, 2016; Culley and Crosby, 2015).

In addition to the aging population, obesity has also become a pressing contemporary issue. The prevalence of obesity has increased dramatically in recent decades, with 4 million deaths globally attributed to high body mass index (BMI) between 1990 and 2015 (Hansen, 2014). With the increase in obesity, there is growing evidence that obesity has adverse effects on brain and cognitive function (Dodds and Allison, 1998; Bryson and Wyand, 2006; Jungwirth et al, 2009; Mandal et al, 2009; Culley et al, 2007; Wei and Xie, 2009). Obesity also aggravates brain damage and accelerates brain aging, thereby increasing the risk of neurodegenerative diseases (Dodds and Allison, 1998; Bryson and Wyand, 2006). In the past decade, research in this field has made significant progress, and many new concepts have emerged, revealing the cellular and molecular mechanisms by which obesity causes cognitive impairment in the elderly. These lines of evidence suggest that the physical changes that accompany overweight and obesity eventually lead to central nervous system dysfunction. However, the pathophysiological and molecular mechanisms by which obesity exacerbates POCD are not yet clearly defined.

Beyond the postoperative setting, systemic inflammation, as seen in sepsis,

frequently leads to SAE, characterized by acute cognitive dysfunction (Iwashyna et al, 2010; Gofton and Young, 2012; Tauber et al, 2021). LPS, a component of Gram-negative bacteria, serves as a potent inducer of systemic inflammation and is widely used to model neuroinflammation and related cognitive decline relevant to SAE (Sun et al., 2019, Jimenez et al., 2008; Park and Lee, 2013; Semmler et al, 2007; Zhou et al, 2024). LPS activates Toll-like receptor 4 (TLR4), primarily on microglia, triggering the release of pro-inflammatory cytokines (e.g. Tumor necrosis factor- alpha, Interleukin-1 beta, Interleukin-6), blood-brain barrier (BBB) disruption, and neuronal dysfunction (Jimenez et al., 2008; Wee Yong, 2010; Barichello et al, 2021; Widmann and Heneka, 2014).

In recent years, there has been increasing interest in the potential neuroprotective effects of various anesthetic and sedative agents. Dexmedetomidine (DEX), a highly selective α2-adrenergic receptor agonist, is commonly used in intensive care units and has good anti-inflammatory properties (Xiang et al, 2014; Wu and Li, 2015; Sezer et al, 2010). Several studies have demonstrated its ability to reduce neuroinflammation and may improve cognitive outcomes in various clinical situations (Baierle et al, 2015; Pandharipande et al, 2010; Taniguchi et al, 2004; Venn et al, 1999).

Remimazolam (REMI), a new ultra-short-acting benzodiazepine developed in Japan, has attracted much attention in the field of anesthesiology in recent years. It is mainly used for intravenous sedation and general anesthesia and has a rapid reversal of sedation and recovery compared with other drugs in the same class (Wang et al, 2020; Dardalas et al, 2019; Kilpatrick et al, 2007). Although the clinical use of REMI is expanding, its potential anti-inflammatory effects and effects on cognitive function remain largely unexplored. This gap in knowledge presents an important area of research, especially with the increasing use of REMI in clinical practice.

Crucially, the nervous system actively regulates inflammation via pathways like the cholinergic anti-inflammatory pathway (CAP). Mediated by the vagus nerve, the CAP involves the release of acetylcholine, which acts on α7 nicotinic acetylcholine receptors (α7nAChRs) on immune cells to suppress pro-inflammatory cytokine production (Tracey, 2007; Gavilán et al., 2007; Tracey, 2002; Borovikova et al., 2000; Rosas-Ballina et al., 2011; van Maanen et al., 2009; Chavan and Tracey, 2017). DEX's anti-inflammatory effects have been linked to this pathway (Xiang et al, 2014; Wu and Li, 2015), but whether REMI engages similar mechanisms is unknown. Furthermore, a simultaneous evaluation of REMI and DEX regarding their neuroprotective effects and vagal mechanisms in relevant models is lacking.

1. The first study investigated the effect of obesity on POCD using a diet-induced obesity (DIO) mouse model. It investigated the role of high mobility group box 1 (HMGB-1), a key inflammatory mediator, in the pathogenesis of POCD. The effect of obesity on POCD was mainly evaluated.

2.The second study focused on the effects of REMI and DEX on LPS-induced cognitive dysfunction. It elaborates on the neuroprotective properties of both drugs, given the limited research on its anti-inflammatory effects. The study explored the positive effects of these drugs on neuroinflammation, BBB integrity, and vagal-mediated inflammatory responses.

Chapter 1

Study on postoperative cognitive dysfunction in diet-induced obese mice (食餌誘発肥満マウスにおける術後認知機能障害の検討)

Introduction

Together with an increase in demand for surgical procedures, POCD has become a growing concern (Terrando et al, 2011a; Bedford, 1955; Monk et al, 2008). POCD is a recognized multifactorial and complex disease, and its occurrence can be affected by a variety of preoperative, intraoperative, and postoperative risk factors. At present, there are several hypotheses regarding the pathogenesis of POCD, which are related to central neuroinflammation, cholinergic nervous system dysfunction, nerve cell apoptosis, and oxidative stress damage. Above all, an increasing number of studies have shown that neuroinflammation is the main pathological mechanism of POCD (Hovens et al, 2016; Culley and Crosby, 2015). Neuroinflammation, particularly the activation of microglia, is central to the pathogenesis of POCD. As immune cells of the central nervous system (CNS), microglia are activated by surgical trauma, anesthesia, or perioperative stress, releasing pro-inflammatory cytokines such as IL-6 and IL-1β. These cytokines trigger inflammatory responses, disrupt the BBB, and lead to neuronal dysfunction and cognitive impairment. Elevated levels of IL-6 and IL-1β have been consistently observed in both animal models and clinical studies of POCD, highlighting their critical roles (Terrando et al., 2011b; Zhang et al., 2020).

As the background of patients prone to POCD, increasing age and metabolic syndrome are listed. And studies have shown that obesity is a very important related factor (Burns et al, 2023). However, the pathophysiological mechanisms underlying the association between obesity and POCD have not yet been well clarified.

Diet-induced obesity model (DIO mouse model) can be induced by feeding mice with high-fat food (HFD) or other obesity-inducing foods, and the daily excess caloric intake of mice can be artificially controlled. According to the previous studies, most DIO models use inbred mouse strains, and although many mouse strains are susceptible to DIO, C57BL/6 is the most common one (Pettersson et al, 2012; Surwit et al, 1995). Thus, in this thesis, I used C57BL/6 mice, which are commonly employed in obesity-related research. Moreover, studies have shown that dietary induction with a fat content of 60% is more effective than a diet with a low-fat content (e.g., 45%) (Donovan et al, 2009; Johnston et al, 2007). Therefore, C57BL/6 mice fed with HFD were used as a model for related research and pathological mechanism elucidation.

Leptin is a hormone secreted primarily by adipose tissues and is known to increase obesity due to increased fat mass (Murakami, 1996). Leptin has been reported to play a dual role in neuroinflammation and cognitive function. First, leptin can exacerbate

neuroinflammation by activating microglia, leading to the secretion of pro-inflammatory cytokines such as IL-6 and IL-1β (Konturek et al, 2002). On the other hand, some studies suggest that leptin may have a neuroprotective effect and may improve cognitive function (Morrison, 2009). Therefore, I measured leptin along with IL-6 and IL-1β to assess its role in the cognitive changes observed in DIO mice.

HMGB-1 is an abundant inflammatory protein in the brain, which may be involved in the pathogenesis of cognitive decline related diseases, such as Alzheimer's disease (AD) (Paudel et al, 2020). It has been reported that during cytoreductive surgery and hyperthermic intraperitoneal chemotherapy in adults, the levels of HMGB-1 *in vivo* are significantly increased in conjunction with perioperative short-term cognitive dysfunction (Yu et al, 2017). In mammals, HMGB-1 is present in the nucleus (Lotze and Tracey, 2005). In contrast, HMGB-1 acts as an injury-causing factor, which can activate inflammatory responses and promote the generation of immune cells (Faraco et al, 2007; Scaffidi et al, 2002). According to existing studies, the binding and activation of TLR2 and TLR4 by HMGB-1 is a possible mechanism (Aucott et al, 2018; Yu et al, 2006). It also activates the pattern recognition receptor for the receptor for advanced glycation end products (RAGE) (Nan et al, 2019; Matsuura et al, 2018). Damaged or necrotic cells passively release nuclear HMGB-1, triggering an immediate inflammatory response via proinflammatory cytokines such as TNF-α (Ma et al, 2012). Since systemically administered HMGB-1 has previously been documented to reproduce the surgical phenotype with high fidelity, I am ethically able to study a surrogate of the “surgery only” cohort without use of anesthesia/hypnotics (Vacas et al, 2014; Hu et al, 2018). Therefore, it is possible to create a state of POCD without anesthesia/hypnotics.

In this thesis, DIO mice were treated with tibial fracture surgery or single intraperitoneal injection of HMGB-1 to induce POCD, and the effect of obesity on POCD was evaluated. My hypothesis is that obesity exacerbates neuroinflammation, thereby worsening POCD. I also evaluated the contribution of leptin to explore its potential role in obesity-related cognitive dysfunction.

Methods

1. Animals and Drug preparation

Male C57BL/6 (WT) mice (4 weeks of age) were purchased from SLC (SLC Japan Inc., Shizuoka, Japan). The mice were housed in a temperature-controlled environment with a 12-h: 12-h light-dark cycle and allowed access to food and water. All experiments were performed in accordance with the guidelines for the Care and Use of Laboratory Animals of Hokkaido University and were approved by the Animal Care and Use Committee of Hokkaido University (20-0097).

After a week of acclimatization, some mice were fed a normal diet (NOR), and some were fed a high-fat diet (HFD) with 60% of calories from fat (DIETS D12492 from Research Diets, EPtrading, Japan) for eight weeks. HMGB-1(R&D Systems, Minneapolis, MN, USA) was dissolved in normal saline.

2. Surgery

Mice were subjected to general anesthesia under 2.1% isoflurane and then to intramedullary fixation of open tibial fractures of the left hind paw under aseptic surgical conditions (Steinmetz et al, 2009; Nishikawa et al, 2007). Under sterile conditions, a 20 g needle was inserted into the left tibial intramedullary canal, and the osteotomy was performed after stripping the periosteum. Temperature was monitored and maintained at 37°C with the aid of a heating pad during the procedure, which lasted approximately 10 min. Buprenorphine (0.1 mg/kg) was administered subcutaneously after induction of anesthesia and before skin incision to provide analgesia. Mice in the nonsurgical group were given anesthesia and analgesia as described above.

3. Cognitive test

Trace fear conditioning (TFC) was used to assess learning and memory in rodents (Feng et al, 2017; Terrando et al, 2011b).

The training consisted of two parts: noise stimulation and foot shocks. Mice were placed in the TFC chamber and allowed to explore freely. Mice were then subjected to 20 seconds of noise stimulation (75-80 Db, 5 kHz, conditioned stimulation) followed by 2 seconds of plantar electrical stimulation (0.8 mAmp; Unconditioned stimulus). The stimulation cycle was repeated nine times.

Situational testing was performed 24 h after the end of training. This time, mice were returned to the same TFC chamber in which they had been trained previously, but

without any noise or shocks, for 4 min. The proportion of freezing time to total time was calculated using the remaining memory at each time point after drug intervention as a means.

4. Design of Experiment

4.1 Experiment 1 Tibia Fracture Surgery

The mice were randomly divided into four groups (n=5 each): NOR, HFD, NOR+SURGERY and HFD+SURGERY.

Th NOR group was fed a normal diet. The HFD group was fed with high-fat diet. The NOR+ SURGERY group underwent tibial fracture surgery within 30 min of the end of behavioral training, and then continued to be fed with normal mouse diet. HFD + SURGERY group at the end of the behavioral training tibial fracture surgery within 30 min, and then continue with high fat feed. Behavioral tests were performed 72 h after TFC training. At the end of the test, mice were sacrificed under isoflurane anesthesia. (Figure 1)

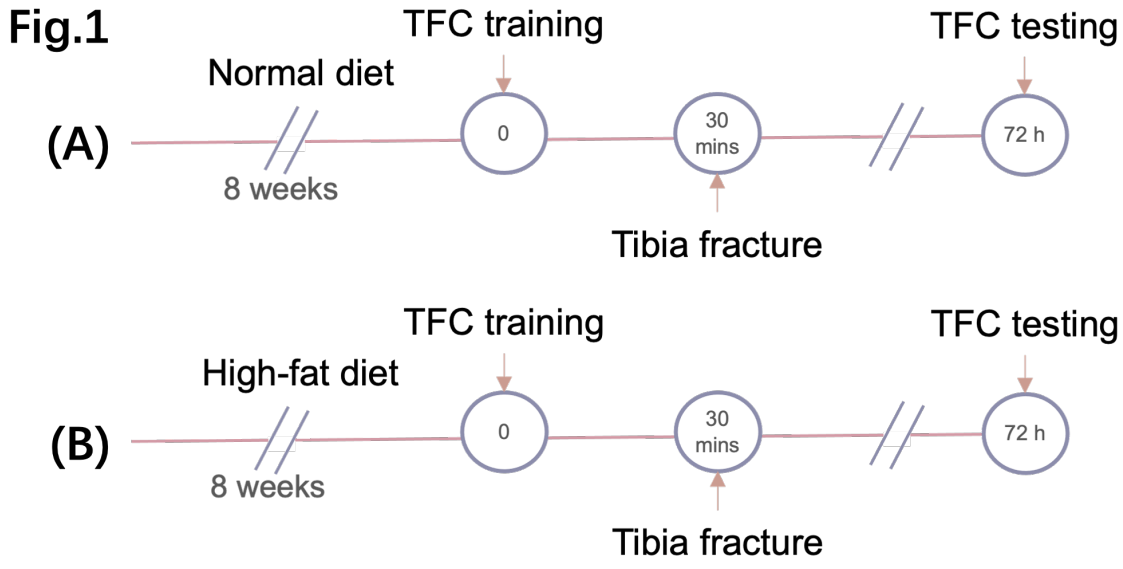

Figure 1. Schematic diagram depicting the process of trace fear conditioning (TFC) training, dosing time, and TFC testing. (A) is the schematic flow diagram of mice undergoing tibial fracture surgery and TFC training after 8 weeks of normal diet feeding. (B) is a schematic flow diagram of mice undergoing tibial fracture surgery and TFC training after 8 weeks of high-fat diet feeding.

4.2 Experiment 2 HMGB-1 injection

The mice were randomly divided into four groups (n=5 each): NOR, HFD,

NOR+HMGB-1 and HFD+HMGB-1.

NOR group was fed with normal mouse diet. HFD group was fed high fat diet. NOR + HMGB-1 group was given a dosage of 10 mg/kg HMGB-1 within 30 min after behavioral training, and then continued to feed normal mice. HFD + HMGB-1 group was a dosage of 10 mg/kg HMGB-1 within 30 min after behavioral training, and then continued to be fed high-fat diet. Behavioral tests were performed 72 h after TFC training. At the end of the test, mice were sacrificed under isoflurane anesthesia. Blood samples were collected through the abdominal aorta and centrifuged at 4,000 rpm for 15 min at 4 °C. The supernatant was collected and used to examine inflammatory markers. The hippocampus tissues were taken and quickly frozen on dry ice and stored at -80 °C for subsequent analysis, including enzyme-linked immunosorbent assay (ELISA). (Figure 2)

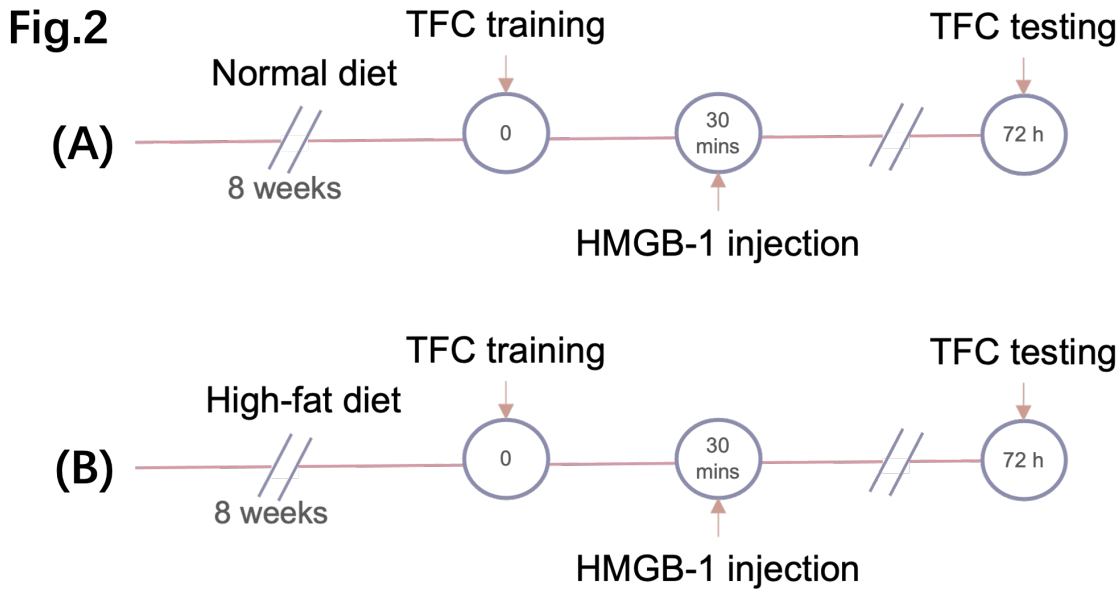

Figure 2. Schematic diagram depicting the process of trace fear conditioning (TFC) training, dosing time, and TFC testing. (A) is a schematic diagram of the process of HMGB-1 injection and TFC training of mice after 8 weeks of normal diet feeding. (B) is a schematic diagram of the flow of HMGB-1 injection and TFC training of mice after 8 weeks of high-fat diet feeding.

5. Measurement of Cytokines (Leptin, IL-6, and IL-1β)

The levels of leptin, IL-6 and IL-1β in serum and hippocampal tissue homogenate were measured by ELISA. In Experiment 2, hippocampal tissues and blood samples were obtained under anesthesia after TFC testing. Hippocampal tissue homogenates were prepared immediately by the ultrasonic grinding ice bath method. After centrifugation

of the tissue homogenates, the supernatant was collected and stored at -80 °C. Meanwhile, the blood samples were centrifuged at 4,000 rpm for 15 min at 4 °C, and the supernatants were collected and stored at -80 °C. Then, the levels of leptin (MOB00B), IL-6 (M6000B-1), and IL-1β (MLB00C-1) in serum and supernatants were measured by ELISA kits (R&D Systems) according to the manufacturer's instructions.

6. Data Analysis

Data were reported as mean ± standard deviation (SD). T-test was performed to compare the body weight between NOR and HFD groups. In other statistical analyses, two-way ANOVA was conducted followed by Tukey's post hoc test. GraphPad Prism software (version 9, GraphPad software, San Diego, CA, USA) was used for analysis. Values with $P < 0.05$ were considered statistically significant, and $P<0.001$ were considered extremely significant.

Results

1. Weight changes in mice fed with obesity

According to the t-test results (Figure 3) of the independent samples showed that there was a significant difference in the mean value of the weight of the mice between NOR group (mean value = 30.03 g) and HFD group (mean value = 46.54 g) ($t = -12.592$, $p < 0.001$), and the mean value of HFD group was significantly higher than that of NOR group.

Fig.3

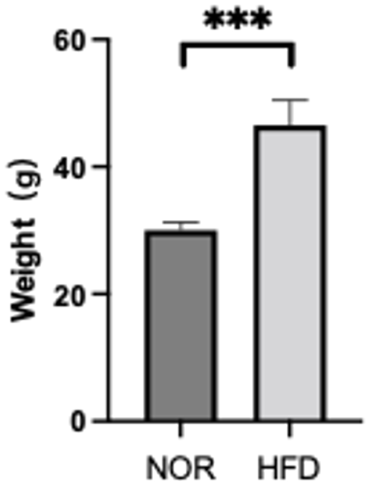

Figure 3. The weight of mice after eight weeks of feeding normal diet and high-fat diet respectively. Data were presented as mean ± SD (n=5). ***P < 0.001, for comparisons shown.

2. Effect of obesity on surgically induced and HMGB-1 induced cognitive impairment

To investigate the effect of obesity on postoperative cognitive dysfunction, I first characterized the mice that received tibial fracture surgery or sham treatment, as illustrated in Figure 4A. Two-way ANOVA showed that obesity ($F (1, 16) = 7.64$, $p = 0.014$, $\eta^2 = 0.32$) and surgery ($F (1, 16) = 30.25$, $p < 0.001$, $\eta^2 = 0.65$) had significant main effects on the freezing time. There was significant interaction between them ($F (1, 16) = 10.92$, $p = 0.004$, $\eta^2 = 0.41$). Under the HFD condition without surgery, the percentage of freezing time was significantly lower than that in the normal diet non-surgery group ($p < 0.05$). In the NOR condition, surgery did not significantly reduce the freezing time ($p = 0.12$). Importantly, in the surgery groups, the difference in freezing time between HFD and NOR was not significant.

Subsequently, HMGB-1 was administered to mimic POCD, as illustrated in Figure 4B. Two-way ANOVA showed that obesity ($F(1, 16) = 1.78$, $p = 0.003$, $\eta^2 = 0.42$) and injection of HMGB-1 ($F(1, 16) = 2.35$, $p = 0.003$, $\eta^2 = 0.44$) had significant main effect on freezing time. There was significant interaction between them ($F(1, 16) = 0.55$, $p = 0.005$, $\eta^2 = 0.40$). In the non-HMGB-1 injection group, the HFD group showed significantly reduced freezing time compared to the NOR group ($p < 0.05$). Under NOR conditions, HMGB-1 injection significantly reduced the freezing time ($p < 0.001$). Notably, in the HMGB-1 injection groups, there was no significant difference in freezing time between HFD and NOR conditions ($p > 0.05$).

These results indicate that obesity itself has a significant effect on cognitive function in mice without surgery or HMGB-1 intervention. However, obesity did not affect cognitive function in the surgical and HMGB-1 injection groups.

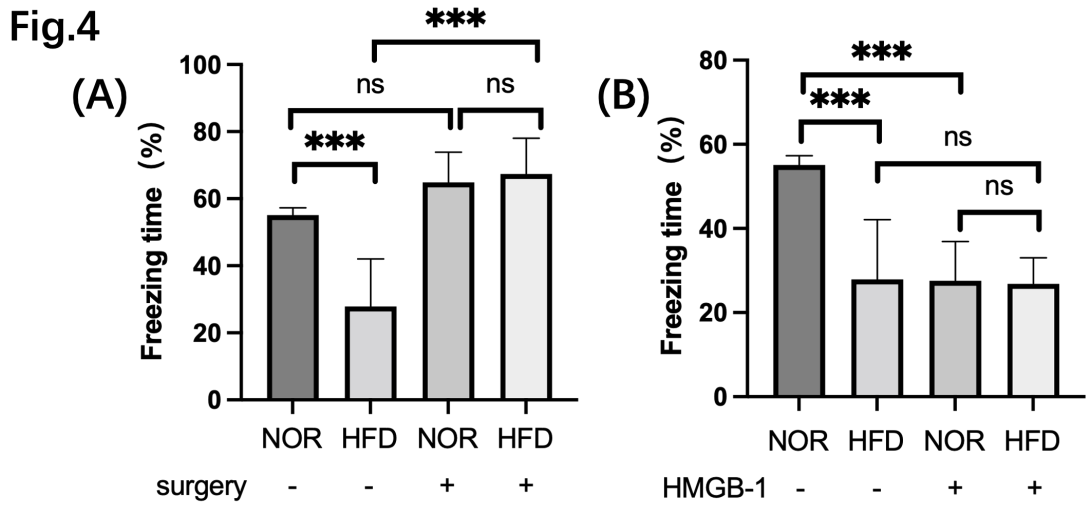

Figure 4. (A) Testing for freezing behavior in the trace fear conditioning context was undertaken 72h after tibial fracture surgery. Data were presented as mean ± standard deviation (n=5). Comparison showed that ***P < 0.001, *P<0.05.(B) Testing for freezing behavior in the trace fear conditioning context was undertaken 72h after HMGB-1 injection. Data were presented as mean ± SD (n=5). Comparison showed that ***P < 0.001, *P<0.05.

3. Effects of obesity on IL-1β, IL-6 and leptin levels in Mice

As mentioned earlier, there is a significant correlation between cognitive impairment and neuroinflammation. First, the levels of IL-6 and IL-1β in plasma and hippocampus were measured by ELISA to evaluate the inflammatory response of the body and brain. For IL-1β (Figure 5A, 5B), two-way ANOVA showed that obesity had a significant effect on the hippocampus ($F(1, 16) = 19.517$, $p < 0.001$, $\eta^2 = 0.550$), and HMGB-1

injection had a significant effect on both the hippocampus and plasma (hippocampus: $F(1, 16) = 239.249$, $p < 0.001$, $\eta^2 = 0.937$; plasma: $F(1, 16) = 79.373$, $p < 0.001$, $\eta^2 = 0.832$). There was significant interaction between obesity and HMGB-1 injection on hippocampus ($F(1, 16) = 14.628$, $p = 0.001$, $\eta^2 = 0.478$), but no significant interaction on plasma ($F(1, 16) = 0.063$, $p = 0.805$). In the non-HMGB-1 injection group, IL-1β levels did not significantly increase with obesity in either the hippocampus or plasma. In the NOR group, HMGB-1 injection resulted in significant increases in IL-1β levels in both the hippocampus and plasma. Most importantly, under HMGB-1 injection conditions, the HFD group showed significantly higher IL-1β levels in the hippocampus compared to the NOR group, while no similar increase was observed in plasma

For IL-6 (Figure 5C, 5D), two-way ANOVA showed that HMGB-1 had significant main effects on the hippocampus and plasma (hippocampus: $F(1, 16) = 221.121$, $p < 0.001$, $\eta^2 = 0.933$; plasma: $F(1, 16) = 13.181$, $p < 0.001$, $\eta^2 = 0.891$), while the main effect of obesity was not significant (hippocampus: $F(1, 16) = 2.570$, $p = 0.128$; plasma: $F(1, 16) = 0.407$, $p = 0.532$). The interaction between obesity factors and HMGB-1 injection was not significant (hippocampus: $F(1, 16) = 2.011$, $p = 0.175$; plasma: $F(1, 16) = 0.555$, $p = 0.467$). In the non-HMGB-1-injected group, IL-6 levels in both the hippocampus and plasma did not significantly increase with obesity. In the NOR group, HMGB-1 injection significantly elevated IL-6 levels in both the hippocampus and plasma. Most importantly, under HMGB-1 injection conditions, the HFD group exhibited significantly higher IL-6 levels in the hippocampus compared to the NOR group, whereas no significant increase in plasma IL-6 levels was observed.

I also measured leptin levels in the samples (Figure 6). Two-way ANOVA showed that obesity had a significant main effect on plasma ($F(1, 16) = 270.667$, $p < 0.001$, $\eta^2 = 0.944$), and had a significant main effect on hippocampus ($F(1, 16) = 4.941$, $p = 0.041$, $\eta^2 = 0.236$). The interaction between obesity and HMGB-1 injection was not significant. In the serum, leptin levels were significantly higher in HFD groups compared to NOR groups, regardless of HMGB-1 injection. However, in the hippocampus, the differences in leptin levels between HFD and NOR groups were not as pronounced.

Fig.5

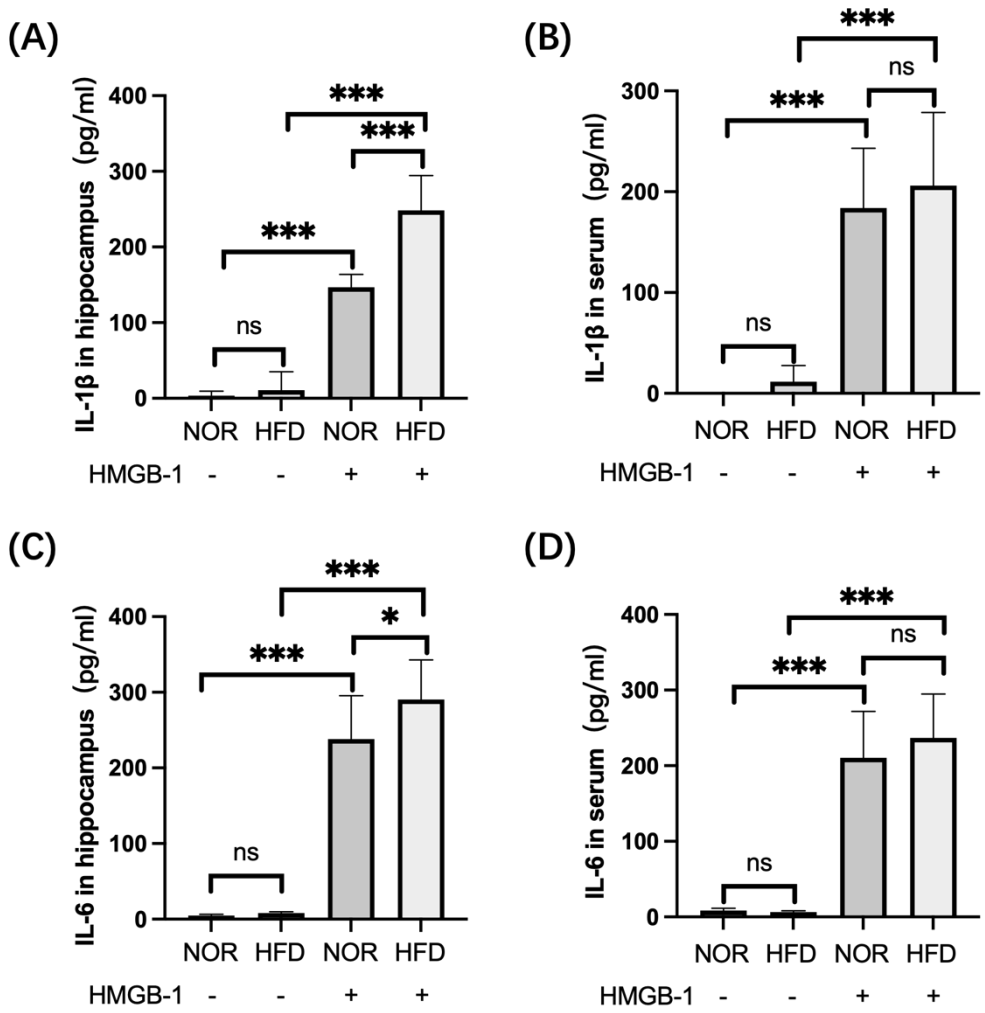

Figure 5. (A), (B) At 72h after HMGB-1 injection, the proinflammatory cytokine IL-1β levels in hippocampus and serum were measured. (C), (D) At 72h after HMGB-1 injection, the proinflammatory cytokine IL-6 levels in hippocampus and serum were measured. Data were presented as mean ± SD (n=5). ***P < 0.001 for comparisons shown.

Fig.6

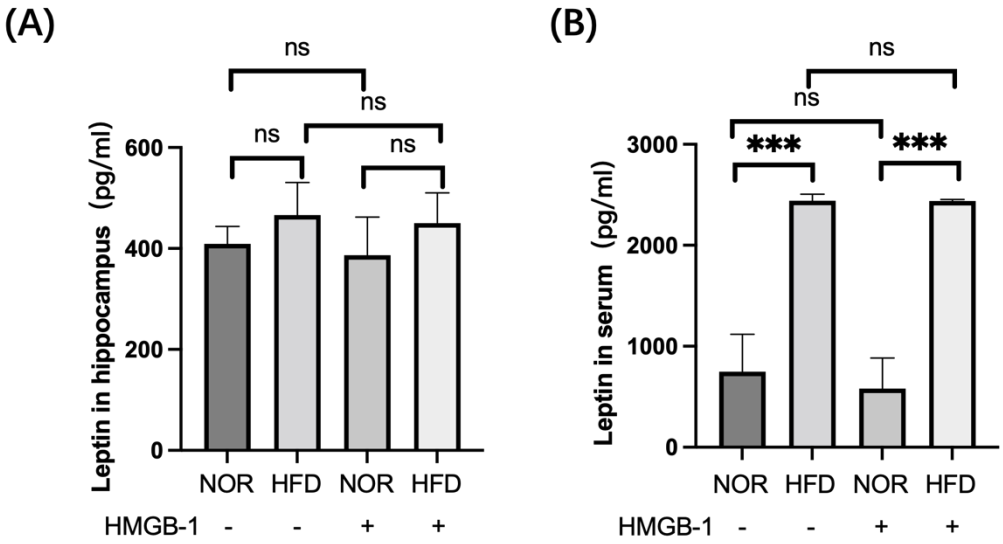

Figure 6. (A), (B) Leptin levels in hippocampus and serum were measured 72h after HMGB-1 injection. Data were presented as mean ± SD (n=5). ***P < 0.001 for comparisons shown.

Discussion

My thesis investigated the effects of obesity on POCD in mice. Results showed that HFD mice significantly gained weight. Obesity notably impacted cognitive function in mice without surgery or HMGB-1 intervention. However, cognitive differences between HFD and NOR groups were less apparent after surgery or HMGB-1 intervention, although HMGB-1 injection increased IL-6 and IL-1β levels in hippocampal tissue.

In my thesis, the percentage of freezing time in HFD group was significantly lower than that in NOR group in mice without surgery or HMGB-1 treatment. However, IL-6 and IL-1β levels did not increase, which suggests that cognitive impairment by obesity is not related to neuroinflammation. Previous reports (Toda et al, 2014) have shown that obesity causes cognitive impairment, potentially through mechanisms involving endothelial dysfunction. Endothelial dysfunction could potentially disrupts blood flow, reduces vascular tone, and impairs the BBB (Wardlaw et al, 2013). This might be, in part, by the disruption of specific receptors on endothelial cells that transact mechanical and chemical stimuli that promote the release of signaling molecules such as nitric oxide, endothelin, and prostaglandins. In addition, it has been suggested that other mechanisms such as oxidative stress, mitochondrial dysfunction and neurotrophic factors are also closely related to cognitive impairment in obese people (de Mello et al, 2018; Sripetchwandee et al, 2018). Obesity may thus create a feedforward loop between these different mechanisms, potential leading to endothelial dysfunction and further cognitive dysfunction.

In my thesis, I observed that after tibial fracture surgery, mice in the surgery group showed an increased tendency to freeze time, contrary to my expectations. It is possible that the pain caused by the operation led to the reduced activity of the mice, or it may be due to the limited movement caused by the operation of the legs of the mice that causes the reduced activity of the mice. Therefore, I used injection of HMGB-1 instead of tibial fracture surgery. The injection of HMGB-1 produced an inflammatory response that affected the cognitive function of the mice. Unfortunately, no significant difference in cognitive function was observed after injection of HMGB-1 between obese and non-obese mice.

My results showed that serum leptin levels were significantly higher in the HFD group compared to the NOR group, which is consistent with the expected increase in leptin due to obesity. However, in the hippocampus, there was no significant difference

in leptin levels between the HFD and NOR groups, regardless of whether HGMB-1 was injected.

In summary, my thesis provides evidence that diet-induced obesity negatively impacts cognitive function. However, obesity did not exacerbate cognitive impairment, despite intensifying neuroinflammation in response to HMGB-1 injection, which serves as a model of surgical stimuli. Therefore, my hypothesis was not supported.

The involvement of leptin was initially considered as a potential factor; however, this was not supported, as leptin levels in the hippocampus did not increase, Intracellular signaling pathways induced by leptin should be investigated in future studies. Other factors may explain the observed results, and the following possibilities could be considered:

1. Duration of obesity: The 8-week feeding period may not fully reflect the long-term effects of chronic obesity.
2. Cognitive test: I adopted TFC as a representative test. The unexpected results obtained in the tibial fracture surgery model may have been influenced by pain and reduced activity. Although this issue was addressed with HMGB-1 injection, additional behavioral tests might have been necessary.

Regarding point 1 above, a longer feeding period implies a prolonged experimental duration. As for point 2,due to current equipment limitations, I was unable to perform additional behavioral tests; however, I aim to include them in future research. Additionally, factors such as the selection of optimal animal strains, types of feed, and the age and sex of the animals could potentially lead to unexpected experimental outcomes.

CHAPTER 2

Remimazolam and dexmedetomidine prevent cognitive decline by affecting lipopolysaccharide-induced brain injury in mice: Possible involvement of the vagus nerve pathway

(レミマゾラムおよびデクスメデトミジンは、リポポリサッカライド誘発性脳障害に影響を与えることで認知機能低下を予防する：迷走神経経路の関与の可能性)

Introduction

Sepsis, defined as a life-threatening organ dysfunction resulting from a dysregulated host response to infection, remains a paramount challenge in modern critical care medicine and a leading cause of mortality worldwide (Singer et al, 2016). Beyond its immediate systemic effects, sepsis frequently precipitates profound neurological complications, the most common of which is sepsis-associated encephalopathy (SAE). SAE presents as an acute or subacute cerebral dysfunction, with clinical manifestations ranging from mild delirium and confusion to deep coma (Gofton and Young, 2012; Tauber et al, 2021). The onset of SAE is a grim prognostic indicator, significantly associated with increased mortality, prolonged hospital stays, and, for many survivors, debilitating long-term cognitive impairments that severely diminish their quality of life (Iwashyna et al, 2010; Widmann and Heneka, 2014).

The pathophysiology of SAE is remarkably complex and multifactorial, with a growing consensus that neuroinflammation serves as a central pathogenic hub (Barichello et al, 2021; Zhang et al, 2022). The cascade is often initiated by systemic inflammatory mediators or pathogens that breach a compromised BBB. This breach permits an influx of cytokines and immune cells into the CNS, triggering the activation of resident immune cells, primarily microglia (Hoogland et al, 2015). Once activated, microglia adopt a pro-inflammatory phenotype, releasing a barrage of cytotoxic mediators, including pro-inflammatory cytokines such as TNF-α, IL-1β, and IL-6, which further propagate neuronal injury and synaptic dysfunction (Sweeney et al, 2019).

To effectively elucidate the intricate mechanisms of SAE and evaluate potential therapeutic interventions, robust animal models are indispensable. The systemic administration of LPS, a major endotoxin component of the outer membrane of Gram-negative bacteria, is a widely accepted and utilized experimental model. LPS is a potent agonist forTLR4, and its administration effectively recapitulates many of the hallmark features of the acute phase of sepsis and SAE, including a robust systemic inflammatory response, microglial activation, BBB disruption, and subsequent cognitive deficits (Park and Lee, 2013; Semmler et al, 2007).

In the midst of this inflammatory cascade, the nervous system is not a passive victim but an active regulator. A key endogenous anti-inflammatory mechanism is the CAP, a neuro-immune reflex mediated primarily by the vagus nerve (Tracey, 2002). Afferent signals from the periphery inform the CNS of inflammation, which in turn activates efferent vagal fibers. The release of acetylcholine from these fibers acts upon

α7 nicotinic acetylcholine receptors (α7nAChRs) expressed on macrophages and other immune cells, potently inhibiting the production and release of pro-inflammatory cytokines (Borovikova et al, 2000; Chavan and Tracey, 2017). Pharmacological modulation of the CAP therefore represents a promising therapeutic strategy for a host of inflammatory conditions, including SAE.

In this context, certain anesthetic and sedative agents used in the perioperative and intensive care settings have garnered interest for their potential immunomodulatory and neuroprotective properties.

Dexmedetomidine (DEX), a highly selective α2-adrenergic receptor agonist, is one such agent. Beyond its sedative and analgesic effects, DEX has demonstrated significant anti-inflammatory and neuroprotective capabilities in various preclinical and clinical settings (Su et al, 2016). Notably, its anti-inflammatory effects have been linked, at least in part, to the activation of the CAP (Xiang et al, 2014; Wu and Li, 2015).

Remimazolam (REMI) is a more recently developed anesthetic from the benzodiazepine class, characterized by its ultra-short-acting profile due to rapid metabolism by tissue esterases (Keam, 2020). While its primary mechanism of action is the enhancement of $GABA_A$ receptor activity, its potential roles in neuroprotection and immunomodulation are far less established than those of DEX. Although some evidence suggests potential organ-protective effects (Shi et al, 2022), and benzodiazepines can interact with peripheral receptors like the translocator protein (TSPO) on immune cells (Gavish et al, 1999), a comprehensive investigation into its neuroprotective capacity in the context of SAE has been lacking.

To date, no studies have simultaneously evaluated the neuroprotective effects and underlying vagal mechanisms of both DEX and REMI under identical experimental conditions relevant to SAE. This knowledge gap is significant, given the increasing clinical use of REMI. Based on the known anti-inflammatory potential of the CAP and the reported effects of DEX, I hypothesized that REMI, like DEX, ameliorates LPS-induced cognitive dysfunction by modulating neuroinflammation, potentially through mechanisms involving the vagus nerve and α7nAChRs. Therefore, the primary aims of this thesis were: To evaluate the effects of REMI and DEX on cognitive function and hippocampal neuropathology in an LPS-induced mouse model of acute neuroinflammation relevant to SAE. To investigate the underlying mechanisms by assessing neuroinflammation, BBB integrity, and markers related to the vagus nerve pathway such as Netrin-1 expression in a vagally-innervated organ like the lung. To explore the potential involvement of the vagus nerve pathway, the impact of

methyllycaconitine (MLA), anα7nAChR antagonist, on cognitive function was assessed, along with the expression of Netrin-1 in the lung, a representative vagally-innervated organ.

Methods

1.Animals and Drug Preparation

All experimental procedures were performed using male C57BL/6 (WT) mice, aged 7 weeks, obtained from SLC Japan Inc. (Shizuoka, Japan). Upon arrival, mice were allowed a one-week acclimatization period. They were housed in a temperature-controlled environment with a standard 12-hour light/dark cycle and had *ad libitum* access to standard chow and water. All procedures were conducted in strict accordance with the Hokkaido University's Guidelines for the Care and Use of Laboratory Animals and received formal approval from the Hokkaido University Animal Care and Use Committee (No. 20-0097). For the experiments, 8-week-old mice with an average body weight of approximately 25 g were selected.

LPS (E. coli O111:B4; Sigma-Aldrich, St. Louis, MO, USA) was dissolved in 0.9% sterile saline to a final concentration for administration at 1 mg/kg via intraperitoneal injection. DEX (Sigma-Aldrich) was also dissolved in 0.9% sterile saline and administered intraperitoneally at a dose of 30 μg/kg. REMI (Mundipharma K.K., Tokyo, Japan) was prepared in 0.9% sterile saline. For experiments involving LPS challenge, it was administered in a two-part regimen: an initial intraperitoneal injection of 16 mg/kg followed by a subcutaneous injection of 8 mg/kg 1 h later, a protocol designed to maintain a stable level of sedation. MLA (Sigma-Aldrich), a selective α7nAChR antagonist, was dissolved in 0.9% sterile saline and intraperitoneally administered at 4 mg/kg, a dose previously demonstrated to effectively block α7nAChR-mediated responses in similar models (Terrando et al, 2011c).

2.Experimental Design

2.1 Cognitive Testing

As cognitive testing, I adopted TFC testing. There were two components to the training: foot shock and noise stimulation. After being placed in the TFC chamber, the mice were given free rein to explore. The TFC chamber (30 × 24 × 21 cm; MED Associates Inc., St. Albans, VT, USA) was maintained under controlled conditions with ambient lighting and background white noise. Freezing behavior was defined as a complete absence of movement other than breathing and was scored using manual methods. After that, for 20 sec, they were conditioned to respond to noise (75-80 Db, 5 kHz), and then, for 2 sec, they were given plantar electric stimulation (0.8 mAmp, unconditional stimulus). This conditioning cycle was repeated nine times.

The test, termed the contextual test, was conducted 24 h after training. Mice were reintroduced to the same TFC chamber where they had been trained, but without any noise or electric shocks, for 4 mins. The proportion of freezing time to total time was measured to assess residual memory at time points post-drug intervention.

Fifty-five mice were randomly divided into 11 groups (n=5 each): CON, LPS, REMI, REMI+LPS, DEX, DEX+LPS, LPS+MLA, REMI+MLA, REMI+LPS+MLA, DEX+MLA and DEX+LPS+MLA. The LPS group was injected with LPS at a dose of 1 mg/kg intraperitoneally (Figure 7(A)). Group CON received an equivalent volume of normal saline (Figure 7(A)). The REMI group was administered an initial injection of 16 mg/kg REMI intraperitoneally and, 1 h later, another injection of 8 mg/kg REMI subcutaneously (Figure 7(B)). The REMI+LPS group similarly received this treatment: The prototype 16 mg/kg REMI intra-peritoneal dose 30 min before the 1 mg/kg LPS and a subcutaneous 8 mg/kg REMI 30 min post LPS administration (Figure 7(B)). The DEX group was given a dosage of 30 μg/kg DEX (Figure 7(C)). Mice in the DEX+LPS group were pre-treated with DEX 30 min earlier in a dose of 30 μg/kg and then received a challenge in LPS at 1 mg/kg (Figure 7(C)). In the groups treated with MLA (4 mg/kg), the compound was administered 30 min prior to the start of the behavioral test.

Behavioral training was conducted. Following this training, the experimental drug was administered. LPS treatment was then initiated 30 min after this administration. Motor performance was assessed 24 h after the initial behavioral training session to determine if the mice had learned and remembered the task.

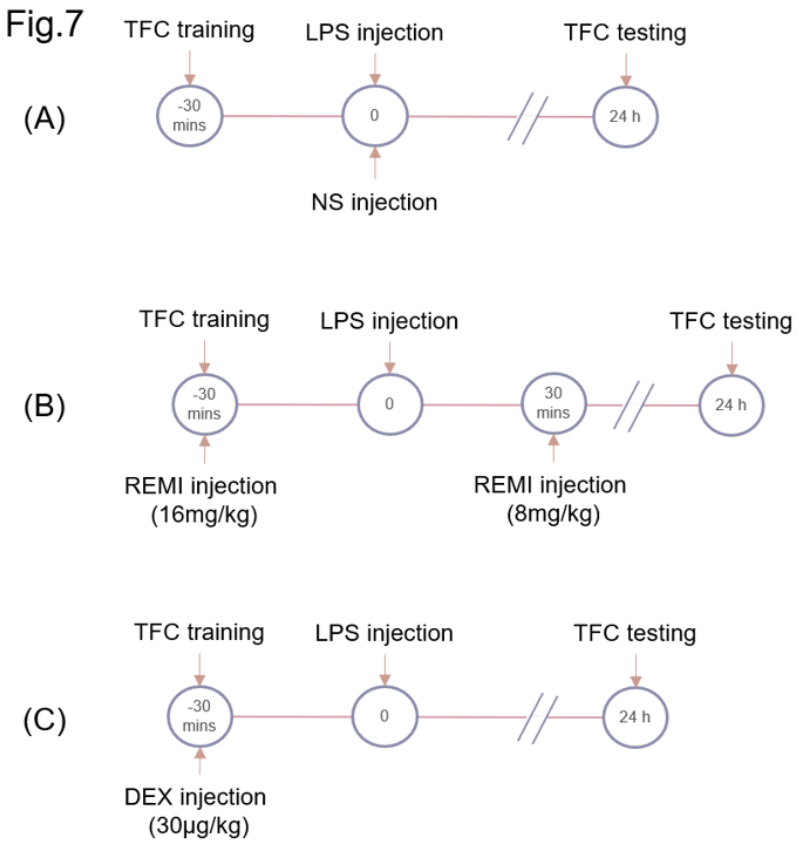

Figure 7. Schematic diagram depicting the process of TFC training, dosing time, and TFC testing. (A) is the schematic diagram of the process of LPS injection and TFC training of mice in the saline injection group. (B) is the schematic diagram of the process of LPS injection and TFC training in the REMI injection group. (C) is the schematic diagram of the process of LPS injection and TFC training in the DEX injection group.

2.2 Blood and Tissue Sample Harvesting

Sixty mice were randomly allocated into 6 groups (n=10 each): CON, LPS, DEX, DEX+LPS, REMI, and REMI+LPS. The LPS group was injected with LPS at a dose of 1 mg/kg intraperitoneally (Figure 8(A)). Group CON received an equivalent volume of normal saline (Figure 8(A)). The REMI group was administered an initial injection of 16 mg/kg REMI intraperitoneally and, 1 h later, another injection of 8 mg/kg REMI subcutaneously (Figure 8(B)). The REMI+LPS group similarly received this treatment: The prototype 16 mg/kg REMI intra-peritoneal dose 30 min before the 1 mg/kg LPS and a subcutaneous 8 mg/kg REMI 30 min post LPS administration (Figure 8(B)). The DEX group was given a dosage of 30 μg/kg DEX (Figure 8(C)). Mice in the DEX+LPS group were pre-treated with DEX 30 min earlier in a dose of 30 μg/kg and then received a challenge in LPS at 1 mg/kg (Figure 8(C)).

Isoflurane was used for anesthesia 24 h after LPS administration, and blood was

drawn from the abdominal aorta, then centrifuged at 4,000 rpm at 4°C for 15 min. The supernatant was harvested for inflammation marker analysis. The brains of five mice from each group were immediately preserved in cold 4% paraformaldehyde (PFA) and stored at 4°C for immunohistochemical analysis, while hippocampus and lung tissues from the remaining five mice were quickly frozen with dry ice and maintained at -80°C for subsequent ELISA and western blot analyses.

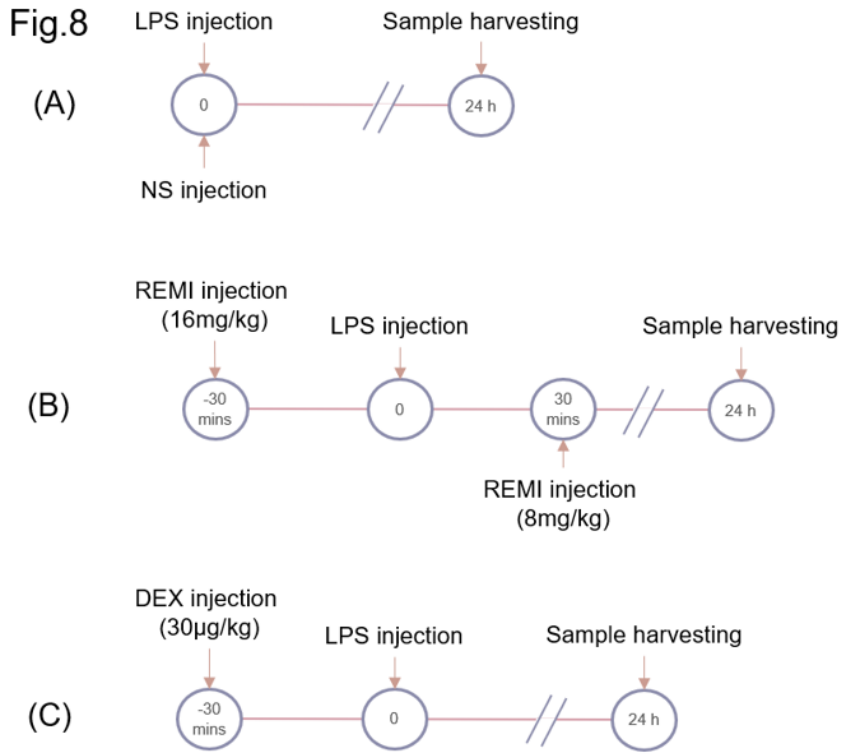

Figure 8. A diagram describing the time of administration. (A) is the schematic diagram of the process of LPS injection and tissue sample extraction in the saline injection group. (B) is the schematic diagram of the process of LPS injection and tissue sample extraction in the REMI injection group. (C) is the schematic diagram of the process of LPS injection and tissue sample extraction in the DEX injection group.

3. Immunohistochemistry

PFA-fixed brains were cryoprotected by sequential immersion in 20% and 30% sucrose solutions at 4 °C until they sank. Brains were then embedded in Optimal Cutting Temperature (O.C.T.) compound (Tissue-Tek; Sakura Finetek, Japan), frozen, and sectioned into 30 μm-thick coronal sections using a cryostat (CM1850; Leica

Biosystems, Wetzlar, Germany). Sections containing the hippocampus were mounted on glass slides. For neuronal staining, sections were incubated with a primary antibody against Neuronal Nuclei (NeuN) (1:1,000, rabbit polyclonal, Cell Signaling Technology, cat. # 24307). Immunoreactivity was visualized using a Rabbit-specific HRP/DAB (ABC) kit (ab64261; Abcam, Cambridge, UK) according to the manufacturer's protocol. Images were captured using an Olympus IX-80 microscope. Within each section, the CA3 region was first identified under low magnification (10x). Then, three non-overlapping fields within the CA3 pyramidal layer were randomly selected and imaged at 20x magnification for cell counting. The number of NeuN-positive cells, identified by their distinct brown nuclear staining, was manually counted using the 'Cell Counter' plugin in ImageJ software (NIH, Bethesda, MD, USA). To ensure counting accuracy, only cells with a clearly defined nucleus located entirely within the field boundaries were included. The average number of NeuN-positive cells per field was then determined for each animal. Additionally, neuronal morphology and tissue organization were qualitatively assessed at a higher magnification (40x).

4. Measurement of Cytokines

Hippocampal tissue was homogenized on an ice bath in cell lysis buffer using an ultrasonic grinder. The tissue homogenate was then centrifuged, and the resulting supernatant was collected and immediately stored at -80 °C until analysis. Concentrations of TNF-α, IL-6, and IL-1β in both plasma and hippocampal supernatants were determined using commercially available ELISA kits (R&D Systems,; Cat# MTA00B-1 for TNF-α, M6000B-1 for IL-6, MLB00C-1 for IL-1β), following the manufacturer's instructions. Samples were diluted 1:10 to ensure concentrations fell within the assay's dynamic range. Standard curves were generated using a four-parameter logistic (4PL) regression.

5. Western Blotting

Hippocampus tissue was homogenized in RIPA lysis (Thermo Fisher 89900) and extraction buffer containing a protease inhibitor mixture (Thermo Fisher 78442). Total protein concentration was determined using the BCA protein assay kit (Thermo Fisher) and samples were normalized to ensure equal protein loading (30 μg per lane). Protein samples were mixed with 4× Laemmli buffer, denatured at 95 °C for 5 min, and separated by 10% SDS-PAGE. Proteins were electrophoretically transferred to PVDF membranes (0.45 μm, Millipore) using a wet transfer system at 300 mA for 90 min at

4 °C. Transfer efficiency was verified using Ponceau S staining. The membrane was blocked with 5% skim milk (Bio-Rad) in TBS-T for 1 h at room temperature, then incubated overnight at 4 °C with primary antibodies against GAPDH (1:1,000, Rabbit, Cell Signaling Technology, cat. # 5174S) as loading control, Albumin (1:500, Rabbit, GeneTex, cat. # GTX102419), and Netrin-1 (1:500, Rabbit, GeneTex, cat. # GTX133213). After washing with TBS-T (3×10 min), membranes were incubated with HRP-conjugated secondary rabbit antibodies (1:10,000, Cell Signaling Technologies, cat. # 7074P2) for 1 h at room temperature. Protein bands were visualized using Pierce ECL Western Blotting Substrate (Thermo Fisher 32132) and detected using an Image Quant LAS 4000 scanner. Band intensities were quantified using ImageJ software, with protein levels normalized to GAPDH expression.

6. Statistical Analysis

All data are presented as mean ± standard deviation (SD). Statistical analyses were performed using GraphPad Prism software (version 9; GraphPad Software, San Diego, CA, USA). Two-way ANOVA was used to analyze the effects of LPS treatment and drug treatment, followed by Tukey's post hoc test for multiple comparisons. A P-value < 0.05 was considered statistically significant, and $P<0.001$ were considered extremely significant.

Results

1. Remimazolam and Dexmedetomidine Improve LPS-Induced Cognitive Impairment

A two-way ANOVA on the TFC data revealed a significant main effect of LPS treatment ($F(1, 29) = 70.825$, $p < .001$), confirming that LPS profoundly impaired contextual fear memory. A significant interaction was observed between LPS treatment and drug treatment ($F(1, 29) = 12.085$, $p = .002$). Post-hoc analysis (Figure 9) showed that mice subjected to LPS administration exhibited a dramatic reduction in freezing time compared to control mice. Pre-treatment with either REMI or DEX significantly attenuated this cognitive decline, with freezing times in these groups being markedly higher than in the LPS-only group. This finding indicates that both anesthetic agents confer a protective effect against LPS-induced memory impairment. Crucially, the neuroprotective cognitive effects of both REMI and DEX were completely abrogated by pre-treatment with the selective α7nAChR antagonist, MLA. The REMI+LPS+MLA and DEX+LPS+MLA groups showed freezing times that were statistically indistinguishable from the LPS-only group, suggesting that the beneficial effects of both anesthetics on cognitive function are mediated through the α7nAChR pathway.

Fig.9

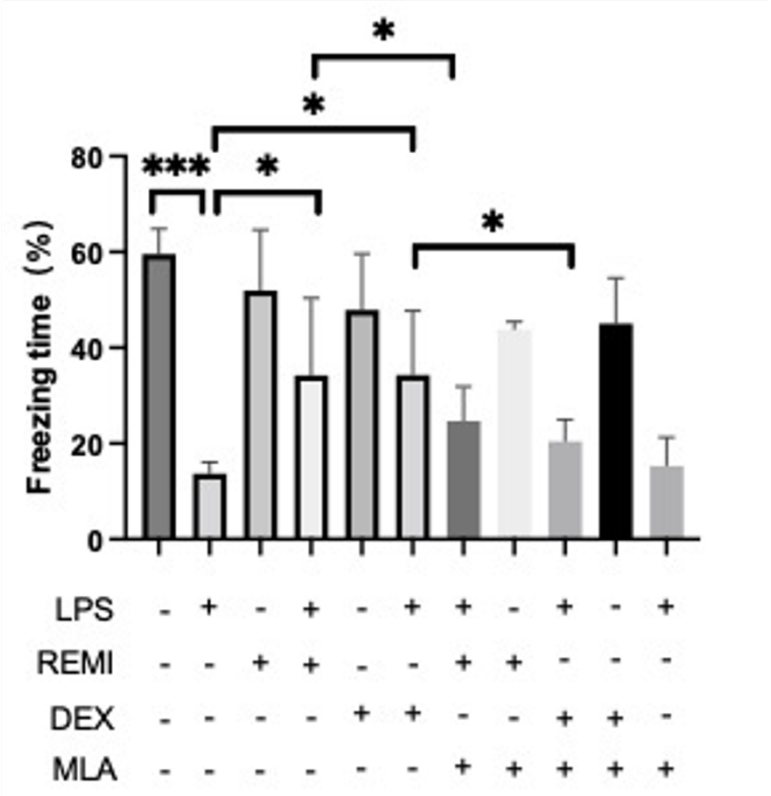

Figure 9. Testing for freezing behavior in the trace fear conditioning context was undertaken

24h after LPS injection. Data were presented as mean ± SD (n=3). ***P < 0.001, *P<0.05 for comparisons shown.

2. Remimazolam and Dexmedetomidine Improved LPS-Induced Hippocampal Structural Lesions

Histopathological examination of the hippocampus via NeuN immunostaining revealed distinct structural changes. In the CON, REMI, and DEX groups, neurons in the CA3 region were well-organized, densely packed, and displayed clear nuclear morphology. In stark contrast, sections from the LPS-treated group showed a disorganized and sparse neuronal arrangement, with significant apparent cell loss, indicative of severe neuronal injury (Figure 10). Two-way ANOVA of the cell counts (Figure 11) revealed a significant effect of LPS treatment ($F(1, 14) = 41.195$, $p < .001$) and a significant interaction between LPS and drug treatment ($F(1, 14) = 23.172$, $p < .001$). Post-hoc analysis confirmed that LPS alone caused a significant decrease in NeuN-positive cell numbers. This neuronal loss was substantially mitigated in mice pre-treated with either REMI or DEX. The REMI+LPS and DEX+LPS groups exhibited a much more preserved and orderly neuronal arrangement compared to the LPS group, demonstrating a structural correlate for the observed functional cognitive protection.

Fig.10

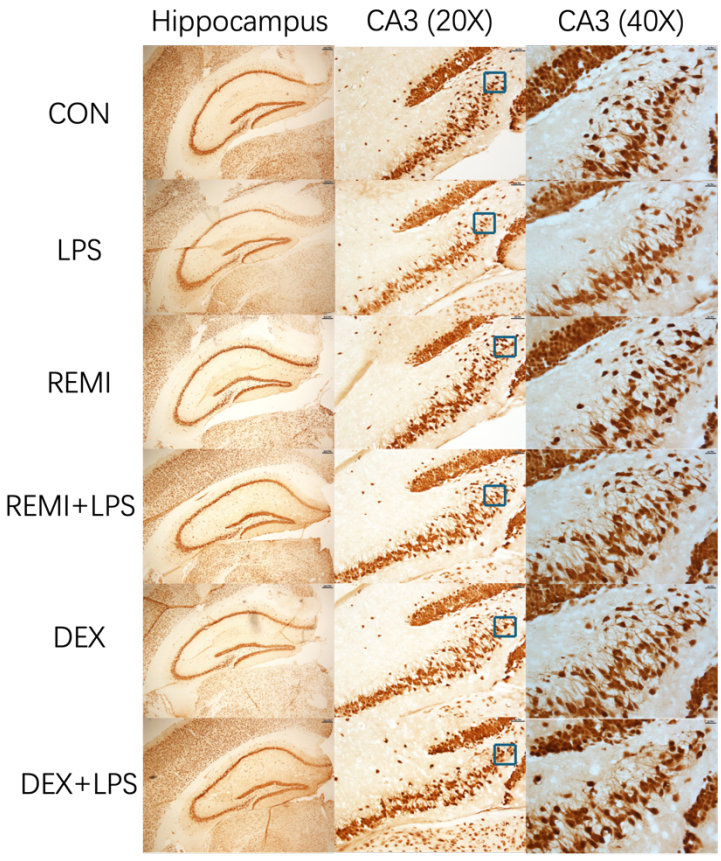

Figure 10. Remimazolam and dexmedetomidine improved LPS-induced hippocampal structural damage. Histopathological observation of hippocampal CA3 region of mice in CON group, LPS group, REMI group, REMI+LPS group, DEX group and DEX+ LPS group.

Images show: an overview of the hippocampus (left column); the CA3 region at 20x magnification (middle column, scale bar = 50 μm); and the CA3 region at 40x magnification (right column, scale bar = 20 μm), which is a further enlargement of the area within the blue box in the middle column (20x magnification).

Fig.11

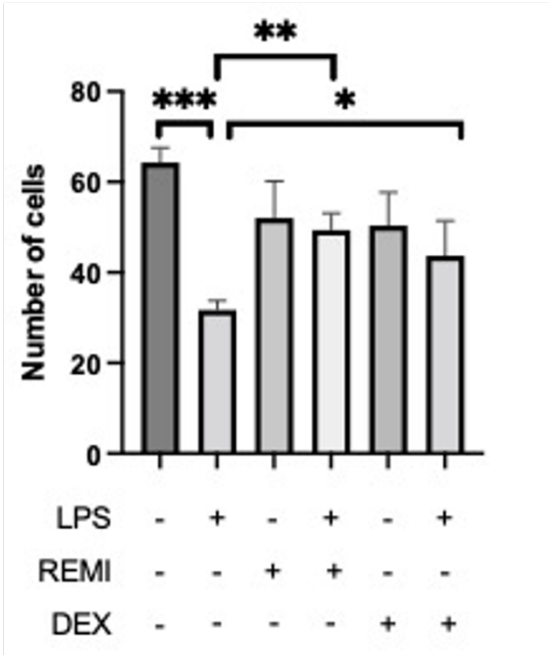

Figure11. Remimazolam and dexmedetomidine improved LPS-induced hippocampal structural damage. The number of cells in CA3 region of hippocampus of mice in CON group, LPS group, REMI group, REMI+LPS group, DEX group and DEX+ LPS group. Data were presented as mean±SD (n=3). ***P < 0.001, **P < 0.01, *P<0.05 for comparisons shown.

3. Effect of Remimazolam and Dexmedetomidine on IL-1β, TNF-α, and IL-6 Expression in Mice

To elucidate the anti-inflammatory effects, I measured key pro-inflammatory cytokine levels. Two-way ANOVA revealed that LPS caused a massive increase in the levels of IL-6, IL-1β, and TNF-α in both the hippocampus and the serum ($p < 0.001$ for all main effects of LPS). Pre-treatment with either REMI or DEX significantly counteracted this

inflammatory surge (Figure 12). Post-hoc tests confirmed that both drugs markedly reduced the LPS-induced elevation of IL-6 (Hippocampus: $F(1, 26)=29.358$, $p< 0.001$; Serum: $F(1, 26)=75.889$, $p< 0.001$), IL-1β (Hippocampus: $F(1, 26)=38.654$, $p< 0.001$; Serum: $F(1, 26)=82.560$, $p< 0.001$), and TNF-α (Hippocampus: $F(1, 26)=590.130$, $p< 0.001$; Serum: $F(1, 26)=590.130$, $p< 0.001$) in both compartments. These results demonstrate that both REMI and DEX exert potent anti-inflammatory effects both systemically and within the CNS.

Fig.12

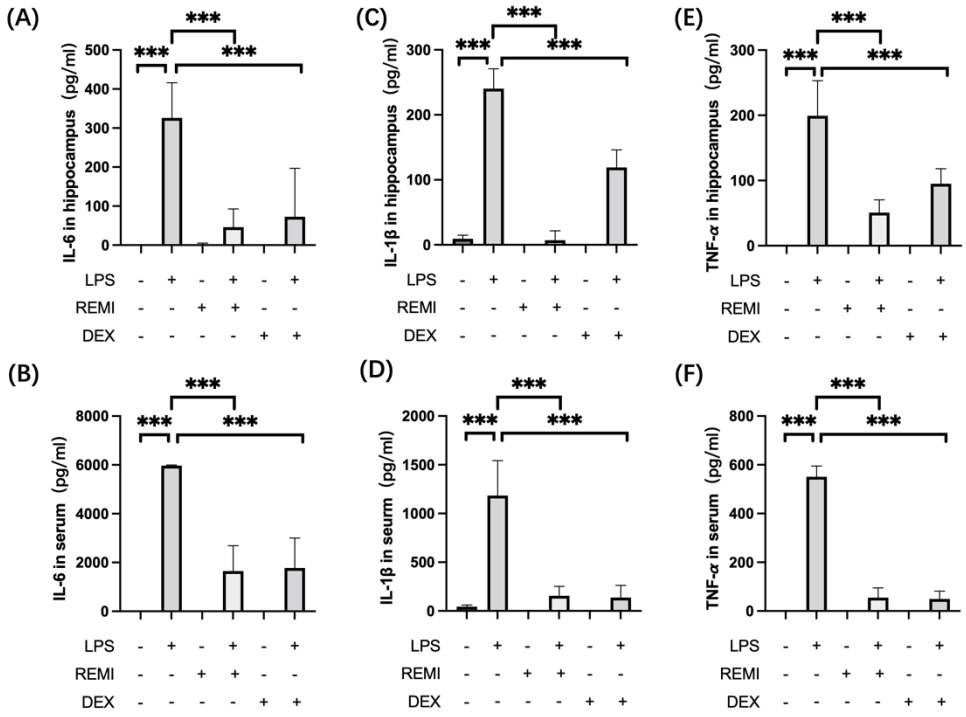

Figure12. (A), (B)IL-6 levels in hippocampus and serum. (C), (D) IL-1β levels in hippocampus and serum. (E), (F) TNF-α levels in hippocampus and serum. Data were presented as mean±SD (n=5). ***P < 0.001 for all indicated comparisons.

4. Effect of Remimazolam and Dexmedetomidine on the Amount of Albumin

Western blot analysis for albumin in hippocampal lysates was performed to estimate the degree of BBB diruption. A two-way ANOVA showed a significant main effect of LPS treatment ($F(1,14) = 50.316$, $p < 0.001$) and a significant interaction ($F(1,14) = 16.357$, $p = 0.001$). As shown in Figure 13, LPS exposure led to a significant increase in hippocampal albumin levels, indicating a compromised BBB. Pre-treatment with both REMI and DEX significantly reduced this albumin extravasation, with levels in the REMI+LPS and DEX+LPS groups being significantly lower than in the LPS-only group. This indicates that both agents help to maintain or restore BBB integrity during

systemic inflammation.

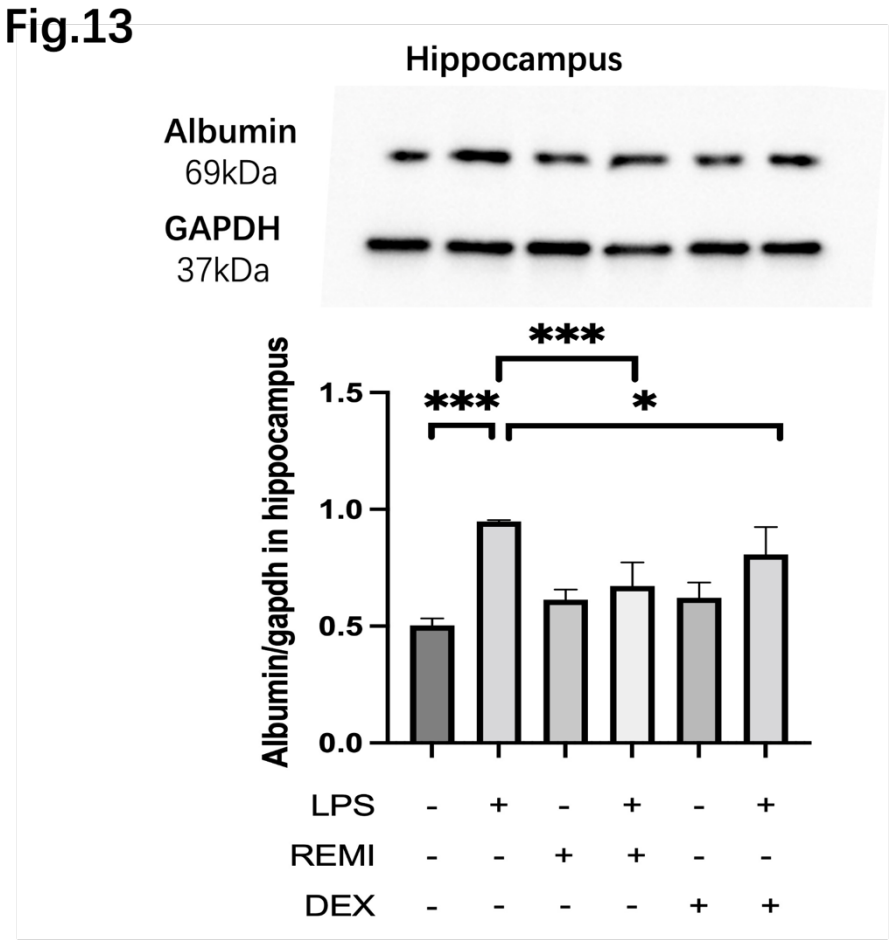

Figure13. Pretreatment with remimazolam and dexmedetomidine reversed LPS-induced increased albumin amount in mice; brains were subsequently collected for albumin expression immunoblotting. Data were presented as mean ± SD (n=3). ***P < 0.001, *P<0.05 for comparisons shown.

5. Effect of Remimazolam and Dexmedetomidine on the Expression of Netrin-1

To probe the involvement of the vagus nerve pathway, I measured the expression of Netrin-1 in the lung. Two-way ANOVA of Netrin-1 expression revealed significant main effects for both LPS treatment ($F(1,14) = 11.993$, $p = 0.004$) and drug treatment ($F(1,14) = 46.687$, $p < 0.001$), with a significant interaction ($F(1, 14) = 12.723$, $p = 0.001$). LPS administration significantly decreased lung Netrin-1 expression (Figure 14). This effect was significantly ameliorated by pre-treatment with both REMI and DEX. This finding suggests that both drugs can modulate vagal nerve-related pathways, potentially enhancing CAP signaling, which is reflected in peripheral Netrin-1 levels.

Fig.14

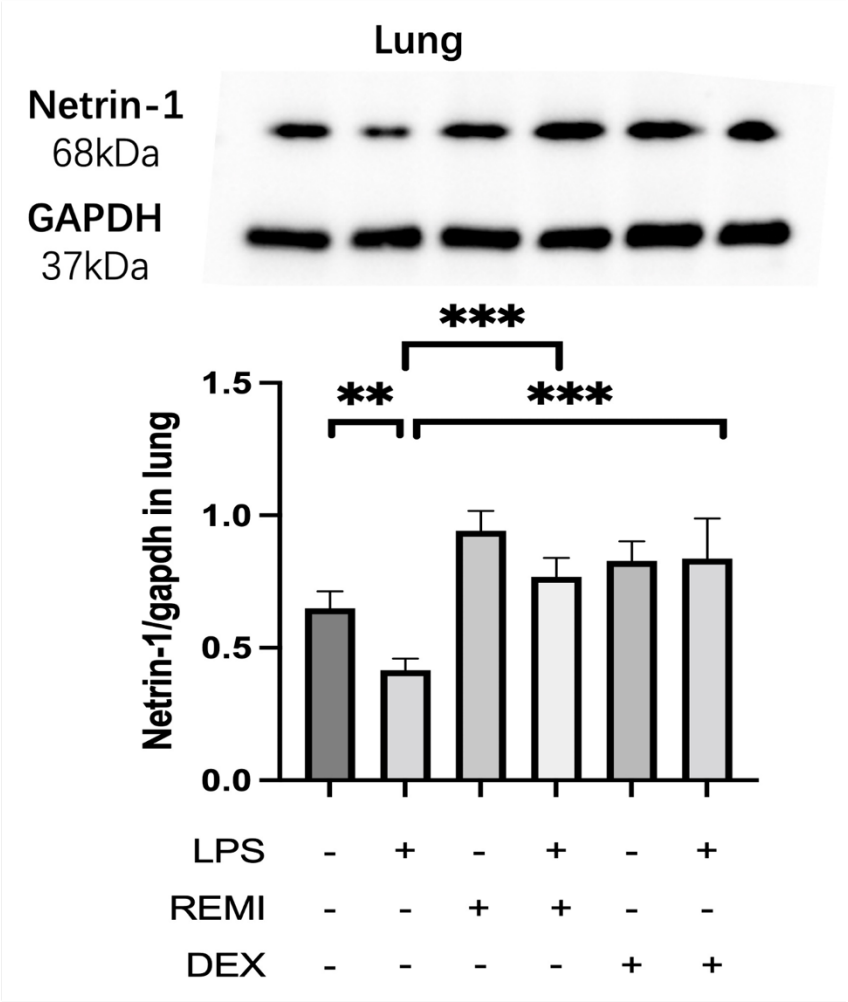

Figure14. Remimazolam and dexmedetomidine pretreated mice reversed LPS-induced lung netrin-1 expression. Data were presented as mean ± SD (n=3). ***P < 0.001, **P < 0.01 for comparisons shown.

Discussion

In this thesis, I demonstrated that systemic administration of LPS induced cognitive deficits (impaired contextual fear memory) and hippocampal pathology (neuronal damage in CA3) in mice, accompanied by elevated systemic and central inflammation (increased TNF-α, IL-6, IL-1β), BBB disruption (increased hippocampal albumin), and altered Netrin-1 expression in the lung. Both REMI and DEX pre-treatment significantly ameliorated these LPS-induced changes. Furthermore, the protective effects of both drugs on cognitive function were blocked by the α7nAChR antagonist, MLA, suggesting involvement of this receptor pathway.

The administration of LPS induced a robust and reliable cognitive deficit, specifically in contextual fear memory, a process highly dependent on the integrity of the hippocampus (Kesner, 2007). Both REMI and DEX treatments led to a significant improvement in memory performance, indicating a preservation or restoration of hippocampal function. The most compelling evidence regarding the mechanism of action comes from the experiments involving the α7nAChR antagonist, MLA. The complete blockade of the cognitive benefits of both REMI and DEX by MLA strongly supports my hypothesis that these agents exert their neuroprotective effects, at least in part, through the activation of the CAP. This finding aligns with previous reports on DEX (Hu et al, 2018) and provides novel evidence for a similar mechanism for REMI. The functional recovery observed in the behavioral tasks was strongly correlated with the preservation of cellular integrity within the hippocampus. My histological analysis focused on the CA3 subfield, a region known to be particularly vulnerable to ischemic and excitotoxic insults due to its dense recurrent collateral network, making it a sensitive area for assessing neuroprotection in models of neuroinflammation(Schmidt-Kastner and Freund, 1991; Liang et al, 2020). The marked neuronal loss and disorganization seen in the LPS-treated group were significantly attenuated by both REMI and DEX, suggesting that the structural preservation of this critical memory-related circuit is a key contributor to the observed cognitive benefits.

Consistent with the established pathophysiology of SAE (Barichello et al, 2021), my thesis confirmed that LPS administration triggered a potent systemic and central inflammatory response. The marked increases in TNF-α, IL-6, and IL-1β underscore the penetration of the inflammatory cascade into the CNS. The ability of both REMI and DEX to effectively counteract this cytokine storm in both the serum and the hippocampus highlights their potent anti-inflammatory properties. This targeted anti-

inflammatory action represents a cornerstone of their neuroprotective efficacy, mitigating the harmful environment that drives neuronal dysfunction in SAE.

Furthermore, I indicated that the integrity of the BBB might be a critical target of both LPS and the protective anesthetics. The BBB is essential for maintaining CNS homeostasis, and its disruption during sepsis allows the influx of peripheral inflammatory mediators, exacerbating neuroinflammation and contributing directly to cognitive impairment (Sweeney et al, 2019). The finding that both REMI and DEX effectively counteracted the LPS-induced increase in hippocampal albumin strongly suggests that they help maintain or restore BBB integrity. This action likely represents another crucial mechanism contributing to their overall neuroprotective profile.

To further explore the link between the observed neuroprotection and the vagus nerve pathway, I investigated Netrin-1 expression. I chose the lung as a peripheral readout organ due to its extensive vagal innervation and its role as a major site where the CAP exerts its effects (Mazzone and Undem, 2016). The finding that LPS decreased, and both REMI and DEX treatment restored, lung Netrin-1 expression provides a peripheral molecular correlate for the modulation of the vagus nerve pathway. This suggests that both anesthetics may positively modulate vagal activity, thereby enhancing α7nAChR-mediated CAP signaling, which is then reflected in peripheral Netrin-1 levels (Mirakaj et al, 2014).

This thesis possesses several limitations that warrant careful consideration when interpreting the findings:

(1) Mechanism of Action - Vagus Nerve & Alternatives: While my data, particularly the effects of the α7nAChR antagonist MLA and the changes in lung Netrin-1, suggest the involvement of the vagus nerve and the cholinergic anti-inflammatory pathway, this remains correlational and not definitively proven. To confirm the necessity and sufficiency of the vagus nerve pathway for the observed protective effects of REMI and DEX, further specific experiments are required. These would include performing subdiaphragmatic vagotomy prior to LPS and drug administration to assess if the benefits are abolished, or potentially directly measuring vagal nerve electrical activity in response to treatment. Furthermore, alternative mechanisms cannot be excluded and may contribute significantly. These include:

Direct actions within the CNS after crossing the BBB, independent of vagal signaling. For instance:

DEX: As an α2-adrenergic agonist, DEX can directly act on central α2 receptors (e.g., in the locus coeruleus and spinal cord) to reduce sympathetic outflow, induce sedation,

provide analgesia, and potentially exert neuroprotective effects by limiting excitotoxicity or modulating central neuroinflammatory responses.

REMI: As a positive allosteric modulator of $GABA_A$ receptor, beyond potent analgesia, this could directly influence neuronal function, potentially modulate glial cell activity (e.g., microglia activation), or trigger mechanisms like preconditioning that protect neurons from subsequent insults.

Peripheral anti-inflammatory effects mediated through non-vagal pathways. Examples include the direct α2-receptor agonism on peripheral immune cells by DEX, or potential interactions with the TSPO – expressed on immune and glial cells – for REMI, influencing inflammatory signaling.

Involvement of other intracellular signaling pathways beyond the CAP might also play a role.

(2) Behavioral Assessment: The evaluation of cognitive function was limited solely to the TFC task, which assesses contextual fear memory. This provides only a narrow view of the potential cognitive effects. A more comprehensive assessment using a broader battery of behavioral tests is needed to evaluate other cognitive domains potentially affected by SAE and protected by these drugs, such as spatial learning and memory (e.g., using the Morris water maze) or recognition memory (e.g., using the novel object recognition test).

(3) Histological Assessment - Regions & Apoptosis: My histological analysis was predominantly focused on the hippocampal CA3 region. While informative, this neglects other crucial brain areas involved in learning, memory, and the response to inflammation, such as the hippocampal CA1 subfield, the dentate gyrus (DG), and the amygdala (particularly relevant given the use of fear conditioning). A more complete neuropathological assessment should include these regions. Additionally, while morphological changes like nuclear pyknosis observed in the LPS group were suggestive of cell death, this thesis did not employ specific assays to confirm the mechanism of cell death. Techniques such as TUNEL staining or immunohistochemistry for cleaved caspase-3 were not performed. Therefore, I cannot definitively conclude whether the observed neuroprotection by REMI and DEX involves the inhibition of apoptosis specifically, as opposed to necrosis or other forms of neuronal injury.

Despite these limitations, this thesis provides novel insights. This is the first thesis to simultaneously evaluate the neuroprotective effects of REMI and DEX in an LPS-induced model relevant to SAE. This thesis demonstrates that REMI, similar to DEX,

can ameliorate LPS-induced cognitive dysfunction and hippocampal pathology. The findings suggest the involvement of the α7nAChR pathway in the protective mechanisms of both drugs in this context.

In conclusion, this thesis is the first to simultaneously evaluate the neuroprotective effects of REMI and DEX in an LPS-induced model relevant to SAE. I have demonstrated that remimazolam, similar to dexmedetomidine, can effectively ameliorate LPS-induced cognitive dysfunction and hippocampal pathology. The findings strongly suggest that these effects are mediated by the suppression of neuroinflammation and the preservation of BBB integrity, and that these actions are critically dependent on the α7nAChR-related cholinergic anti-inflammatory pathway. These results highlight the potential therapeutic relevance of these commonly used anesthetic agents in mitigating acute neurological complications like SAE, encouraging further research into their precise mechanisms and clinical applicability.

Conclusion

1. New findings obtained from these studies

Chapter 1

Obesity significantly affected cognitive function in mice without surgery or HGMB-1 intervention. By contrast, no deterioration of cognitive function was observed in a POCD model, despite increased hippocampal cytokine levels.

Chapter 2

(1) REMI and DEX can effectively improve LPS-induced cognitive dysfunction in mice. It can alleviate LPS-induced hippocampal structural damage in CA3 region.

(2) REMI and DEX inhibited LPS-induced increase of serum and hippocampal inflammatory cytokines (IL-1β, TNF-α, IL-6).

(3) REMI and DEX might counteract LPS-induced BBB disruption, as indicated by reduced hippocampal albumin extravasation. Both drugs reversed the LPS-induced downregulation of Netrin-1 expression in the lung, a vagally-innervated organ.

(4) The protective effects of both REMI and DEX on cognitive function were significantly blocked by pretreatment with MLA, a selective α7nAChR antagonist.

2. Significance of new findings

Chapter 1

Demonstrating that obesity is a risk factor for cognitive dysfunction highlights the need for more careful perioperative management in obese patients.

Chapter 2

(1) REMI demonstrated neuroprotective effects comparable to DEX in an LPS-induced neuroinflammation model.

(2) The effects of both agents appear to involve α7nAChRs and the vagus nerve-mediated cholinergic anti-inflammatory pathway.

(3) These findings suggest that REMI and DEX have therapeutic potential as neuroprotective agents.

3. Future studies

Chapter 1

(1) Longer durations of high-fat diet exposure may help to more accurately model the effects of chronic obesity.

(2) Including additional cognitive tests could provide a more comprehensive view of

functional impairments across different cognitive domains.

Chapter 2

(1) Cognitive function was assessed only with the TFC task, limiting the scope of evaluation. Broader behavioral testing may better capture cognitive domains affected by SAE and responsive to treatment.

(2) Histological analysis focused on the CA3 region, overlooking other relevant areas like CA1, DG, and the amygdala, which are important for learning, memory, and inflammation.

(3) Although neuroprotection was observed, the thesis did not directly assess the mechanism of cell death. Future studies may benefit from apoptosis-specific assays such as TUNEL staining or cleaved Caspase-3 immunohistochemistry.

4. Future challenges

Chapter 1

Optimization of obesity models (considering duration and conditions of diet-induced obesity)

Chapter 2

(1) To better understand the neuroprotective effects of REMI and DEX, future studies should investigate their underlying molecular mechanisms and confirm the involvement of the vagal pathway through approaches such as subdiaphragmatic vagotomy and direct measurement of vagal nerve activity.

(2) It is also important to explore the optimal dosing and timing strategies that maximize their neuroprotective efficacy.

(3) Long-term studies are needed to evaluate the sustained effects of REMI and DEX on cognition and neuroinflammation, as well as to explore the potential synergistic benefits of their combined use.

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Conflict of Interest

The author declares no conflict of interest.

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