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Effects of MFHAS1 on cognitive impairment and dendritic pathology in the hippocampus of septic rats

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ABSTRACT

Aims: To investigate the effects of malignant fibrous histiocytoma amplified sequence 1 (MFHAS1) on cognitive dysfunction, the expression of tumor necrosis factor-α (TNF-α), interleukin (IL)-1β and amyloid β peptide (Aβ) in the hippocampus, as well as dendritic pathology in the hippocampal CA1 region in sepsis-associated encephalopathy (SAE) rats.

Main methods: The rats were randomly divided into four groups: 1) control group (subjected to sham surgery), 2) control plus *Mfhas1* siRNA group (rats received intracerebroventricular injection of *Mfhas1* siRNA after sham surgery), 3) CLP plus control siRNA group (rats received intracerebroventricular injection of control siRNA after cecal ligation and puncture (CLP)), 4) CLP plus *Mfhas1* siRNA group (rats received intracerebroventricular injection of *Mfhas1* siRNA after CLP). The learning and memory capabilities of the rats were examined by means of fear conditioning and Barnes maze test. The concentration of TNF-α and IL-1β was determined by enzyme-linked immunosorbent assay. The efficiency of siRNA transfection, MFHAS1 and Aβ expression were detected by Western blotting. Total branch lengths of pyramidal dendrites of the CA1 basilar trees and spine density were determined by Golgi staining.

Key findings: We observed that MFHAS1 knock-down by *Mfhas1* siRNA intracerebroventricular injection could improve cognitive impairment, reduce the expression of TNF-α, IL-1β and Aβ in the hippocampus induced by CLP, and alleviate the dendritic spinal loss of the pyramidal neurons, as well as increase the dendritic branching of the CA1 basilar trees of septic rats.

Significance: MFHAS1 knock-down can alleviate cognitive impairment, neuroinflammation and dendritic spinal loss in SAE rats.

1. Introduction

Sepsis is a leading cause of morbidity and mortality with the dysfunction and failure of multiple organs, including acute brain dysfunction (The third international consensus definitions for sepsis and septic shock (sepsis-3)). Sepsis-associated encephalopathy (SAE) is a common complication of sepsis [1]. The prevalence of SAE can be as high as 30% among the admitted of sepsis patients [2,3]. Approximately 30–70% of all in-hospital sepsis and systemic inflammatory response syndrome patients develop SAE [4,5]. The pathogenesis of encephalopathy in sepsis is still not clearly understood. It is reported that microcirculatory dysfunction, under-perfusion, mitochondria-dependent apoptosis and necrosis of peripheral organs may yield a systemic inflammatory state involving leukocyte, particularly microglial-activation, lysosomal-exocytosis, cytokine release, and free-radical generation and cause the SAE happening [6–9]. The dysfunction of the central nervous system (CNS), which is a secondary complication of sepsis, is associated with the local generation of pro- and anti-inflammatory cytokines resulting in cognitive impairment. Microglia activation and subsequent release of proinflammatory cytokines, including tumor necrosis factor-α (TNF-α) and interleukin (IL)-1β, are hypothesized to play a key role in the development of SAE [10,11]. An early but transitory oxidative stress response has been documented in various areas of the brain of septic rats, especially in the hippocampus

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and the cortex [12]. In addition, exposure to lipopolysaccharide depresses neuronal excitability, notably of the pyramidal neurons of the hippocampus [13]. In this context, sepsis is associated with the increase in the levels of proinflammatory cytokines and neuronal pathology in the hippocampus.

As the smallest member of ROCO protein family, malignant fibrous histiocytoma amplified sequence 1 (MFHAS1) has a role in innate immunity [14]. Numerous studies have linked this protein family to human pathologies, but it is the link to PD (Parkinson's disease) through one of its family members [LRRK (leucine-rich repeat kinase) 2] that has put these proteins in the spotlight of CNS dysfunction. LRRK points to the MFHAS1 role in signal transduction and control of cell growth in humans. Also, MFHAS1 is reported to cause inflammation through the Raf/MEK/ERK pathway and JNK/NF-κB/AP-1 signaling pathway [15,16]. Our previous studies demonstrated that MFHAS1 is ubiquitinated by E3 ubiquitin ligase praja2, which activates the TLR2 (Toll-like receptor 2) signaling pathway, promotes the polarization of M1 macrophages as well as the inflammatory response [17–20]. We have also found that the plasma concentration of MFHAS1 in septic patients is significantly higher than that in non-septic patients. According to its relationship with PD, the CNS disorder, we speculated that MFHAS1 should play an important role in CNS injury caused by sepsis [14]. Therefore, in continuation with our previous research, the purpose in this study is to understand the role of MFHAS1 in septic rats. To this end, we evaluated the effects of MFHAS1 knock-down on dendritic pathology in the hippocampus by Golgi staining.

Since 2011, Golgi staining (also called Golgi silver staining) technique has been used in neuropathological examination of the visual cortex in patients with Alzheimer's disease [21]. Thereafter, Golgi staining has also been used for the morphological assessment of the capillaries in patients with early Alzheimer's disease [22]. Golgi staining can effectively stain one or more neuron cells present among many cells, without affecting the other non-neuron cells. In a study conducted by Ji et al. in 2018, Golgi staining of neurons was used for the first time to study SAE in mice [23].

In the present study, we detected the expression of MFHAS1 in the hippocampus of septic rats, performed intracerebroventricular injection of MFHAS1 siRNA into rats to investigate the effect of MFHAS1 knock-down on cognitive dysfunction induced by CLP (the gold standard for experimental induction and the investigation of the pathogenesis of sepsis [24]), the expression of TNF-α, IL-1β and amyloid β peptide (Aβ) in the hippocampus, as well as dendritic branching and spine density of the CA1 basilar tree. Our findings provide valuable insights into the possible influence of MFHAS1 on the pathophysiological characteristics and molecular mechanism of SAE, as well as its potential to act as a novel target for the treatment of SAE.

2. Materials and methods

2.1. Animal groups

Male Wistar rats (3–4 months old, 220–300 g) were housed in individual cages in a temperature-controlled environment with 12 h light:dark cycle. The animal protocol was approved by the Department of Laboratory Animal Science, Fudan University (Shanghai, China) (No. 2019 Cancer Hospital JS-262).

The rats were randomly divided into four groups of six to eight rats each using a random number table. The four groups were 1) control group (subjected to sham surgery), 2) control plus *Mfhas1* siRNA group (rats received intracerebroventricular injection of *Mfhas1* siRNA after sham surgery), 3) CLP plus control siRNA group (rats received intracerebroventricular injection of control siRNA after CLP), 4) CLP plus *Mfhas1* siRNA group (rats received intracerebroventricular injection of *Mfhas1* siRNA after CLP). The rats received intracerebroventricular injection of *Mfhas1* or control siRNA immediately after the CLP procedure or sham surgery. The rats were monitored for 10 days after the surgery and intracerebroventricular injection in the animal survival rate experiment (eight rats per group). Seven days after the intracerebroventricular injection, the rats were subjected to fear conditioning test or Barnes maze test, and following that, they were euthanized to harvest the brain tissues. Six rats from each group were euthanized 0 h, 24 h, 3 d, 5 d and 7 d after intracerebroventricular injection to detect TNF-α and IL-1β levels, as well as Aβ levels only on 7 d after intracerebroventricular injection).

2.2. Anesthesia and surgery

To perform CLP on the rats, they were anesthetized with a mixture of ketamine (80 mg/kg) and xylazine (10 mg/kg) administered intraperitoneally (i.p). Then, a 3-cm midline laparotomy was performed to allow exposure of the cecum. The cecum was tightly ligated with a 3.0-silk suture at its base, below the ileocecal valve, and perforated once with a 14-gauge needle. Then the cecum was gently squeezed to extrude a small amount of feces from the perforation site and then returned into the peritoneal cavity. The laparotomy was closed with 4.0-silk sutures. All animals were returned to their cages with free access to food and water. In the sham-operated group, the rats were subjected to all surgical procedures, but the cecum was neither perforated nor ligated. After the surgery, all the groups received 'basic support': 50 mL/kg saline immediately and then, 12 h after CLP.

2.3. Intracerebroventricular injection of siRNA

The rats received intracerebroventricular injections of 10 μL control siRNA (250 μmol/L, D-001810-02, Thermo Scientific.) or 10 μL *Mfhas1* siRNA (250 μmol/L), each, along with the transfection reagent Entranster™-in vivo (Engreen Biosystem Co, Ltd., Beijing, China) immediately after CLP under anesthesia with a mixture of ketamine (80 mg/kg) and xylazine (10 mg/kg) administered i.p. The intracerebroventricular injection was performed with the aid of a stereotactic apparatus (SAS-5100, ASI Instruments, Inc., Warren, MI) using the following coordinates: 0.4 mm posterior to bregma, 1.5 mm lateral from the midline and 4.5 mm ventral from the surface of the skull. After the injection, the needle was kept in place for 1 min to prevent backflow of the injected solution.

2.4. Western blotting analysis

The tissue was lysed in lysis buffer (Beyotime, Shanghai, China). The proteins obtained were separated by SDS-PAGE on a 10% gel, transferred onto Hybond TM-P membrane (GE Healthcare, Little Chalfont, UK) and blocked with 8% skimmed milk in TBST (20 mmol/L Tris HCl (pH 8.0), 150 mmol/L NaCl and 0.05% Tween 20) for 1 h at room temperature. Next, the membranes were incubated overnight at 4 °C with an anti-MFHAS1 antibody (1:500 dilution, sc-390,556, Santa Cruz, CA, USA) or an anti-Aβ antibody (1:1000 dilution, no. 2454, Cell Signaling Technology, USA). β-actin was used as the loading control. After washing thrice with TBST, the membranes were incubated with peroxidase-conjugated secondary antibodies for 1 h. After washing, the blots were treated with a chemiluminescent reagent (Merck Millipore, Billerica, MA, USA) and were exposed to X-ray films (MidSci, St. Louis, MO, USA). The protein bands obtained were quantified with Image J software (NIH, Bethesda, MD, USA).

2.5. Barnes maze

The animals were subjected to Barnes maze to test their spatial learning and memory [25]. Animals were first placed in the middle of a circular platform with 20 equally spaced holes (SD Instruments, San Diego, CA). One of these holes was connected to a dark chamber called target box. Aversive noise (85 dB) and bright light (200 W) shed on the platform was used to encourage rats to find the target box. They had a

spatial acquisition phase that lasted for 4 days with 3 min per trial, 4 trials per day and 15 min between each trial. Animals then went through the reference memory phase to test the short-term retention on day 5 and long-term retention on day 12. No test or handling was performed from day 5 to day 12. The latency to find the target box during each trial was recorded with the assistance of ANY-Maze video tracking system (SD Instruments).

2.6. Fear conditioning

Behavioral procedures were conducted between 8 a.m. and noon. The cognitive function of rats was tested by fear conditioning [25,26]. Each rat was placed into a test chamber wiped with 70% alcohol and exposed to 3 tone-foot shock pairings (tone: 2000 Hz, 85 dB, 30 s; foot shock: 1 mA, 2 s) with an intertrial interval of 1 min in a relatively dark room. The rat was removed from this test chamber 30 s after the conditioning stimuli. The animal was then placed back into the same chamber without the tone and shock 24 h later for 8 min. Then, 2 h later, animal was placed into another test chamber that had different context and smell from the first test chamber and was kept in a relatively lighted room. This second chamber was wiped with 1% acetic acid. Freezing was recorded for 3 min without the tone stimulus. The tone was then turned on for 3 cycles, each cycle for 30 s followed by 1-min inter-cycle interval (4.5 min in total). The animal behavior in these two chambers was video recorded. The freezing behavior during the 8 min treatment in the first chamber (context-related) and the 4.5 min treatment in the second chamber (tone-related) was scored at an interval of 8 s by an observer who was blind to the group assignment.

2.7. Harvesting of the brain tissue

The rats were deeply anesthetized with 5% isoflurane for 2 min and perfused transcardially with physiological saline. Their right hippocampus was dissected out immediately and used for enzyme-linked immunosorbent assay (ELISA), while the left hippocampus was used for Golgi staining.

2.8. ELISA analysis

The hippocampus sample obtained were homogenized in a buffer containing 10 mmol/L HEPES, 1.5 mmol/L $MgCl_2$, 10 mmol/L KCl, 0.5 mmol/L dithiothreitol and 0.05% NP40 (pH 7.9) as well as protease inhibitor cocktail (10 mg/mL aprotinin, 5 mg/mL pepstatin, 5 mg/mL leupeptin, and 1 mmol/L phenylmethanesulfonylfluoride) and incubated on ice for 10 min. The samples were then centrifuged at 13000 rpm for 20 min at 4 °C. The supernatant was stored as the cytoplasmic fraction. The concentration of the cytokines (TNF-α and IL-1β) was determined by ELISA (R&D Systems, Minneapolis, MN). The absorbance was measured at 492 nm using a plate reader (Emax, Molecular Devices, Minneapolis, MN, USA). All the results were normalized to the protein concentration measured by the BCA assay.

2.9. Golgi staining

This experiment was performed using the FD Rapid Golgi Stain™ Kit (FD Neurotechnologies, Inc., USA) according to the manufacturer's instructions. In principle, after euthanizing the rats, the brains were removed (pre-processing) and transferred into Golgi–Cox impregnation solution (impregnation). Following sectioning and mounting onto slides (slide preparation), the staining procedure (staining) was initiated.

2.9.1. Pre-processing

The rats were deeply anesthetized with isoflurane and euthanized without perfusion. The brains were removed and rinsed rapidly with physiological saline.

2.9.2. Impregnation

The brain tissues were incubated in the Golgi impregnation solutions separately for a specific duration: 14 d (recommended time for neuronal staining according to the kit's manual) at 26 °C ± 1 (RT) in the dark. The incubation solution was prepared by mix of equal volumes of solution A and solution B (containing mercuric chloride, potassium dichromate and potassium chromate) at least 24 h prior to the incubation and was kept at RT in an aluminium foil-covered bottle in the dark to avoid any exposure to light. The volume of the impregnation solution used for incubation was five times that of the volume of the brain tissue. The impregnation solution was changed after the first 24 h of incubation.

2.9.3. Slide preparation

Following the impregnation, the brain tissues were transferred into solution C and incubated at 4 °C for 7 d in the dark. Solution C was changed every 24 h.

2.9.4. Staining

After drying, the slides were rinsed with Milli-Q water twice (4 min each time) and then placed in a mixture consisting of 1 part solution D, 1 part solution E and 2 parts Milli-Q water for 10 min at RT. The slides were then rinsed twice again in double Milli-Q water (4 min each time). Next, the slides were dehydrated in solutions with increasing ethanol concentration (50%, 70% and 95%) for 4 min each and in absolute ethanol for 16 min. Finally, the slides were cleared in xylene for 12 min before being coverslipped in Permount. The slides were allowed to dry at RT and then analyzed under a microscope.

2.9.5. Microscopy

One hundred and 50 μm brain sections were cut with a vibratome (Microslicer® 10110, Ted Pella, Inc. California, USA) around −2.7 mm from bregma and +1.0 mm from the interaural relative to the rat's atlas. More than ten well individualized neurons from the CA1 region of hippocampus were randomly selected from each rat, and sequential optical image stacks of 1388 × 1040 pixels were taken at 1.0 μm intervals along the z-axis (ZEISS, Axio Imager Z2, Germany) with 5×, 10×, 20×, and 60× oil objective. The MBF software (MBF Bioscience, Williston, USA) was used for the dimensional reconstruction. The total branch numbers and dendritic lengths were measured with Fiji software (Fiji-win64, NIH, USA). For spine density measurement, 20 neurons were selected from each group and the matching regions of distal branch dendrites were photographed with a 63× objective [27]. The spine numbers in the 40 μm segments were counted by an observer who was blind to the group assignment. The results were expressed as the number of spines per μm.

2.10. Statistical analysis

The parametric results in the normal distribution were presented as mean ± S.D. ($n \geq 6$). The data were analyzed by one-way or two-way analysis of variance followed by the Tukey test if the data were normally distributed or by one-way or two-way analysis of variance on ranks followed by the Tukey test if the data were not normally distributed. Differences were considered significant at $P < 0.05$ based on two-tailed hypothesis testing. All statistical analyses were performed with SigmaPlot14.0 (Systat Software, Point Richmond, CA).

3. Results

3.1. Intracerebroventricular injection of Mfhas1 siRNA could improve cognitive impairment in septic rats

In this study, MFHAS1 upregulation was observed in the rat's hippocampus upon sepsis induction by CLP (Fig. 1A, B). Then, the efficiency of the intracerebroventricular injection of *Mfhas1* or control

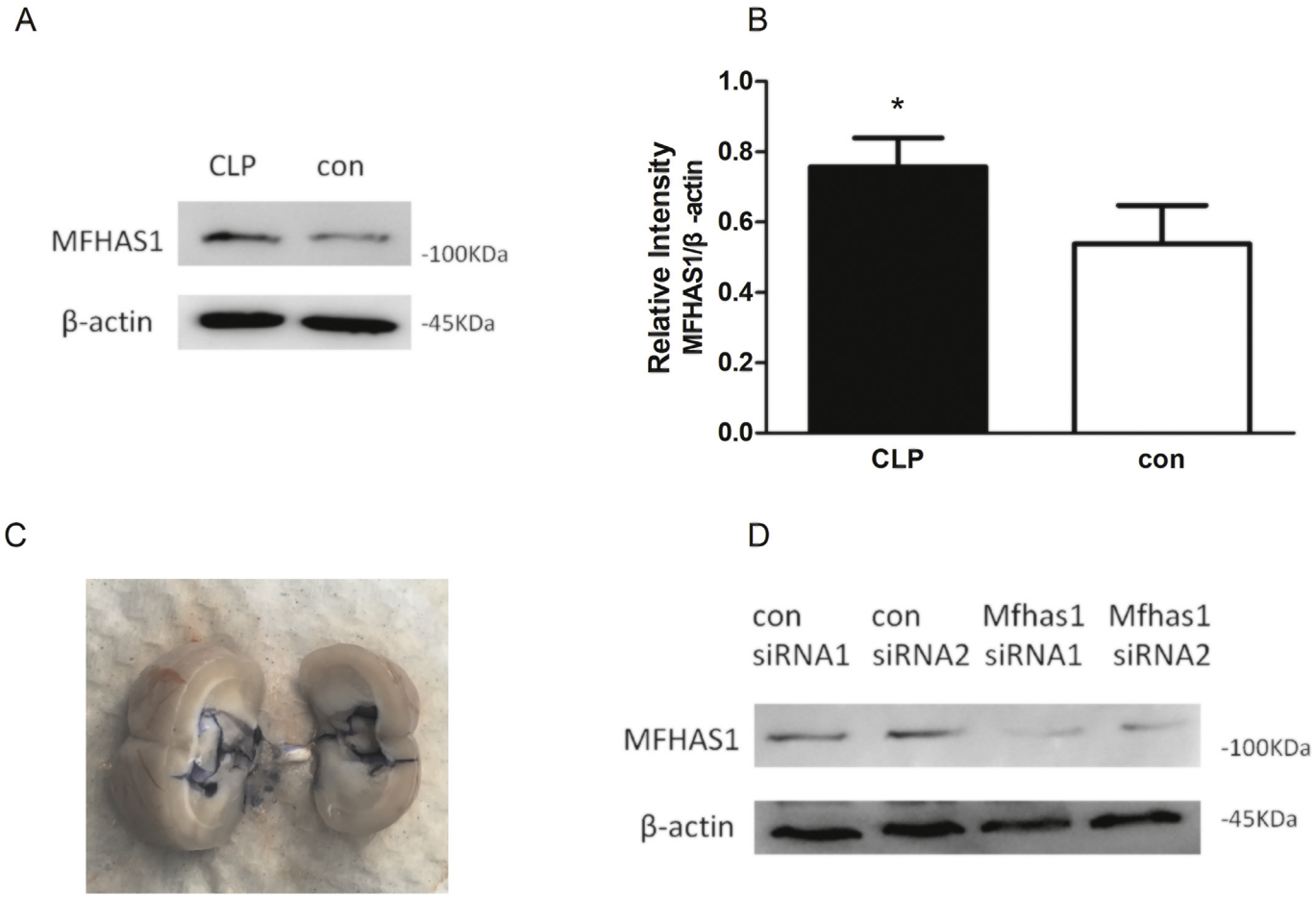

Fig. 1. The expression of MFHAS1 in the hippocampus of rats after CLP or intracerebroventricular injection of *Mfhas1* siRNA. (A) The expression of MFHAS1 in the hippocampus of the rats was detected by Western blotting 24 h after sham surgery (control group) or CLP. (B) Band intensity on Western blotting was semi-quantified using Image J software. The data were shown as mean ± SD ($n = 4$). (* $P < 0.05$). (C) Intracerebroventricular injection of dye into septic rats was done to determine the success of the intracerebroventricular injection. (D) The rats received intracerebroventricular injection of either *Mfhas1* siRNA (250 μmol/L, 10 μL) or control siRNA (250 μmol/L, 10 μL) plus 20 μL of transfection reagent Entranster™-in vivo immediately after the CLP. The effects of *Mfhas1* siRNA or control siRNA on the expression of MFHAS1 in the hippocampus were detected by Western blotting, 3 d after intracerebroventricular injection of *Mfhas1* siRNA or control siRNA in rats. con: control group. CLP, cecal ligation and puncture.

Table 1
Sequences of *Mfhas1* and control siRNA for intracerebroventricular injection.

Gene	siRNA sequences
Mfhas1	Sequence 1: 5′-CCAAAUAUAUGGACCGCAU-3′ Sequence 2: 5′-GCUCUAUCUUAGUCGCAAU-3′ Sequence 3: 5′-GAACAACGGCCUCGAGGAU-3′ Sequence 4: 5′-CUGGAUUGUUCGCACGUUA-3′
Control	5′-UGGUUUACAUGUUGUGUGA-3′

siRNA in septic and control rats was validated by intracerebroventricular injection of dye (Fig. 1C) and by Western blotting (Fig. 1D). The intracerebroventricular injection of *Mfhas1* siRNA significantly reduced the expression of MFHAS1 in the hippocampus (Fig. 1D). The sequences of *Mfhas1* and control siRNA were showed in Table 1. The rats in the CLP plus control siRNA group displayed less freezing behavior than those in the control group and the control plus *Mfhas1* siRNA group in context-related fear conditioning test ($P < 0.05$) (Fig. 2A). However, there was no significant difference between CLP plus *Mfhas1* siRNA group and control group ($P > 0.05$) (Fig. 2A, B). Of note, septic rats took longer than control rats to identify the target box on day 1 and day 8 after the training sessions of the Barnes maze test. This increase was not affected by intracerebroventricular injection of *Mfhas1* siRNA (Fig. 2C, D). Septic rats also exhibited high mortality, and the intracerebroventricular injection of *Mfhas1* siRNA or control siRNA did not significantly affect the mortality in septic rats (Fig. 2E). These results showed that the intracerebroventricular injection of *Mfhas1* siRNA could improve cognitive dysfunction induced by CLP but not the mortality in septic rats.

3.2. Intraventricular injection of Mfhas1 siRNA reduced the expression of TNF-α, IL-1β and Aβ in hippocampus induced by CLP

Compared to the control group and control plus *Mfhas1* siRNA group, the expression of TNF-α and IL-1β in the CLP plus control siRNA group and CLP plus *Mfhas1* siRNA group increased significantly 24 h and 3 d after CLP ($P < 0.05$) (Fig. 3A, B). However, the expression of TNF-α 3 d after intracerebroventricular siRNA injection and IL-1β 24 h and 3 d after intracerebroventricular siRNA injection in the CLP plus *Mfhas1* siRNA group were significantly lower than those in the CLP plus control siRNA group ($P < 0.05$) (Fig. 3A, B). These results suggest that intracerebroventricular injection of *Mfhas1* siRNA suppressed the CLP-induced expression of TNF-α and IL-1β in the hippocampus. CLP also significantly induced the expression of Aβ in the hippocampus ($P < 0.05$) (Fig. 3C, D). However, there was no significant difference in the expression of Aβ between the CLP plus *Mfhas1* siRNA group and the control group ($P > 0.05$).

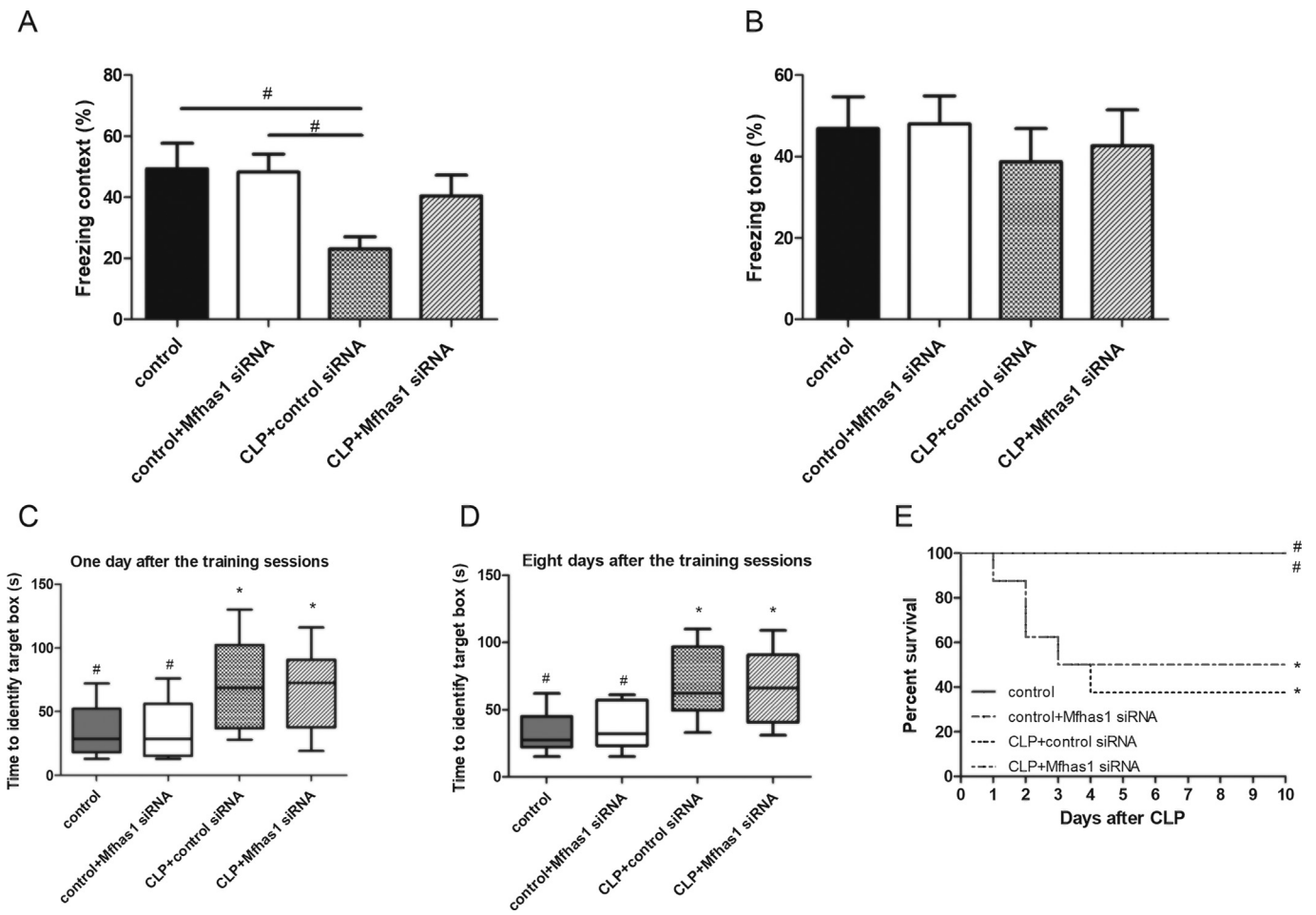

Fig. 2. The learning and memory abilities of septic rats after intracerebroventricular injection of *Mfhas1* siRNA. Freezing context (A) and tone (B) were detected on the 7th and 8th day after intracerebroventricular injection of *Mfhas1* siRNA, respectively. The data were shown as mean ± SD. (C, D) Barnes maze memory phases were detected one day and eight days after the training sessions of the Barnes maze test. The test started on the 7th day after the surgery and intracerebroventricular injection. Results in C and D are in box plot format. (E) The survival rate following the surgery and intracerebroventricular injection of siRNA in rats. The rats were injected intracerebroventricularly with *Mfhas1* siRNA or control siRNA immediately after the CLP or sham surgery. Animals were monitored for 10 days after the CLP or sham surgery. * $P < 0.05$, significantly from control group. # $P < 0.05$, significantly from CLP plus control siRNA group. ($n = 8$ for each group). CLP, cecal ligation and puncture.

3.3. Golgi staining demonstrated more dendritic branching of the CA1 basilar tree in septic rats after intracerebroventricular injection of Mfhas1 siRNA

The dendrites of the hippocampal CA1 pyramidal neurons can be divided into basal and apical systems. The basal tree arising from the cell body and ramifying into the stratum oriens can be shown by Golgi staining (Fig. 4A). The average total branch length of pyramidal dendrites of the basilar trees of the isolated neurons in the CA1 region of the rats in the CLP plus control siRNA group was significantly shorter than that in the control group, the control plus control siRNA group and the CLP plus *Mfhas1* siRNA group ($P < 0.05$) (Fig. 4B). The rats in the CLP plus control siRNA group had less branching at branch points compared with the control group ($P < 0.05$) (Fig. 4C). However, there was no significant difference in both the average total branch length of pyramidal dendrites and the branching points in CA1 region between the CLP plus *Mfhas1* siRNA group and the control group ($P > 0.05$) (Fig. 4B, C).

3.4. Effects of intracerebroventricular injection of Mfhas1 siRNA on hippocampal dendritic spines in septic rats

The effects of intracerebroventricular injection of *Mfhas1* siRNA on the density of the secondary dendritic spines of the isolated pyramidal neurons in the hippocampal CA1 region of septic rats were also analyzed. The results showed that intracerebroventricular injection of *Mfhas1* siRNA prevented the decline of dendritic spine density of the isolated pyramidal neurons in the stratum radiatum area of the hippocampal CA1 region in septic rats (Fig. 5).

4. Discussion

MFHAS1 was reported to have close relationship with the CNS disorder, thus, we speculated that MFHAS1 should play an important role in CNS injury caused by sepsis. In this study, we found that the expression of MFHAS1 increased in the hippocampus upon sepsis induction by CLP. Down-regulation of *Mfhas1* could improve cognitive impairment in septic rats. And this verified our hypothesis.

Ng et al.'s study showed that MFHAS1 regulates Toll-like receptor-dependent signaling and IL-6 production [28]. Our previous study indicated that the E3 ubiquitin ligase, praja2 ubiquitylates MFHAS1 and promotes M1 macrophage polarization as well as transformation from M2 to M1 by activating the JNK and p38 pathways [18]. This study further demonstrated that the expression of MFHAS1 increased in the hippocampus upon sepsis induction by CLP. It is known that the p38 MAPK activation in neurons and astrocytes may be induced by the

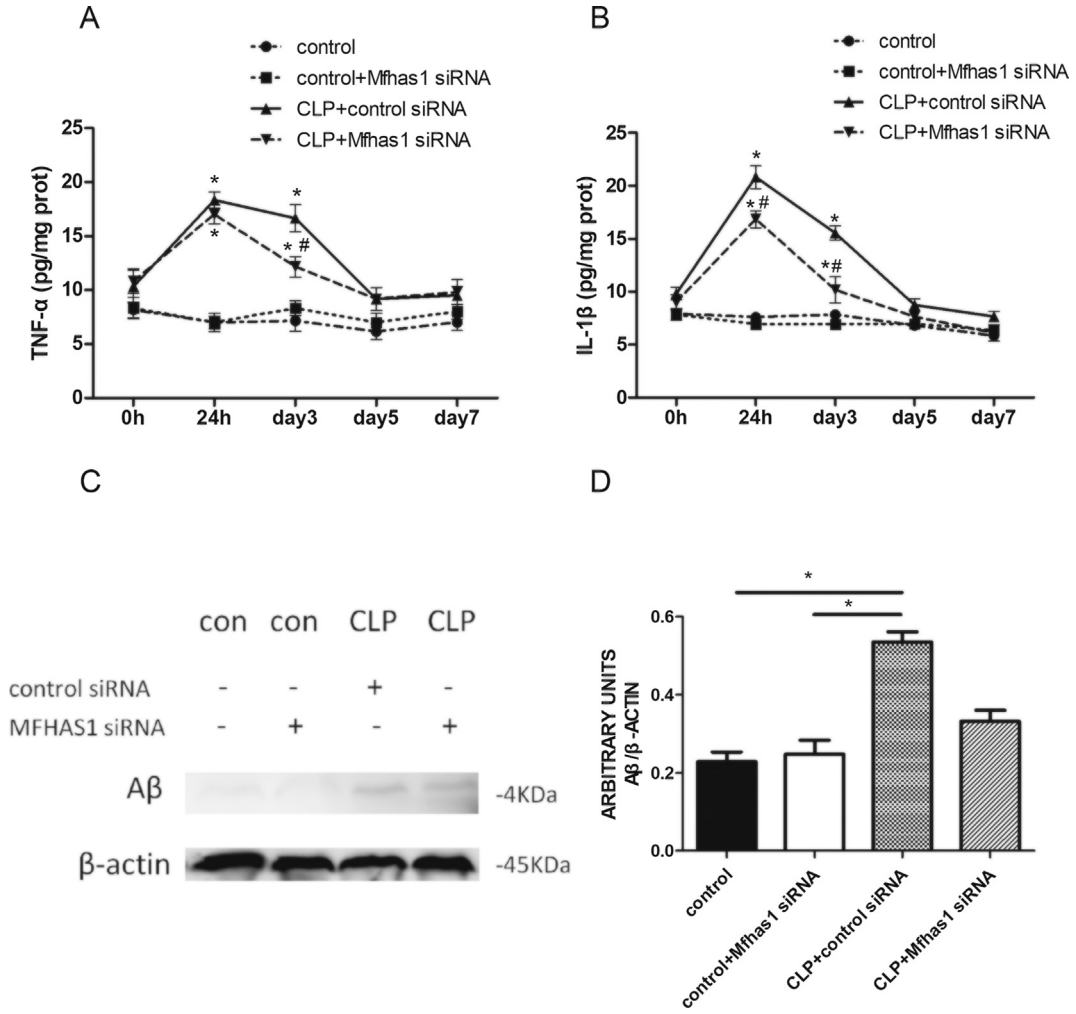

Fig. 3. The expression of TNF-α, IL-1β and Aβ in the hippocampus of septic rats after intracerebroventricular injection of *Mfhas1* siRNA. (A–D) The control siRNA or *Mfhas1* siRNA (250 μmol/L, 10 μL) along with transfection reagent Entranster™-in vivo (20 μL) were injected intracerebroventricularly into both the control and CLP groups. The levels of TNF-α (A) IL-1β (B) protein in the hippocampus were detected 0 h, 24 h, 3 d, 5 d and 7 d after intracerebroventricular injection by ELISA. The Aβ levels (C) in the hippocampus were assessed 7 d after intracerebroventricular injection by Western blotting. (D) Band intensity on Western blotting was semi-quantified using Image J software. *$P < 0.05$ was compared with control group, # $P < 0.05$ was compared with CLP plus control siRNA group. The data were shown as mean ± SD ($n = 6$ for each group). CLP, cecal ligation and puncture. Aβ, amyloid β peptide. TNF-α, tumor necrosis factor-α. IL-1β, interleukin-1β.

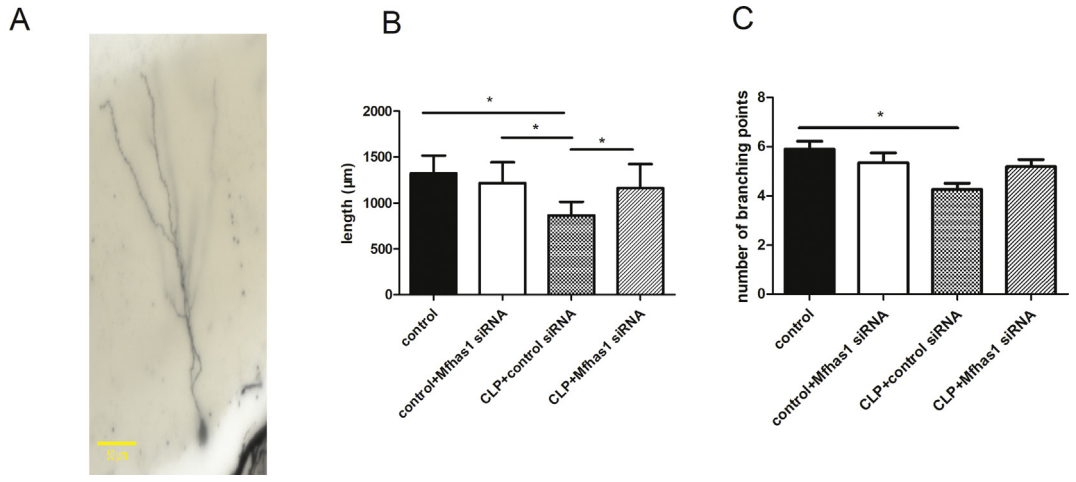

Fig. 4. Effects of *Mfhas1* siRNA treatment on the dendritic length and branching pattern of CA1 pyramidal neurons in septic rats. (A) A picture of one whole neuron with dendrites and their branching from a 20X-STACK image for dendrites length and branching points counting. (B) The average total lengths of the basal dendrites of the isolated pyramidal neurons ($n = 20$, for each group). (C) Branching points. The graph represents the number of dendrites from the cell body of basal dendrites in the CA1 isolated pyramidal neurons (n = 20, for each group). *$P < 0.05$. CLP, cecal ligation and puncture.

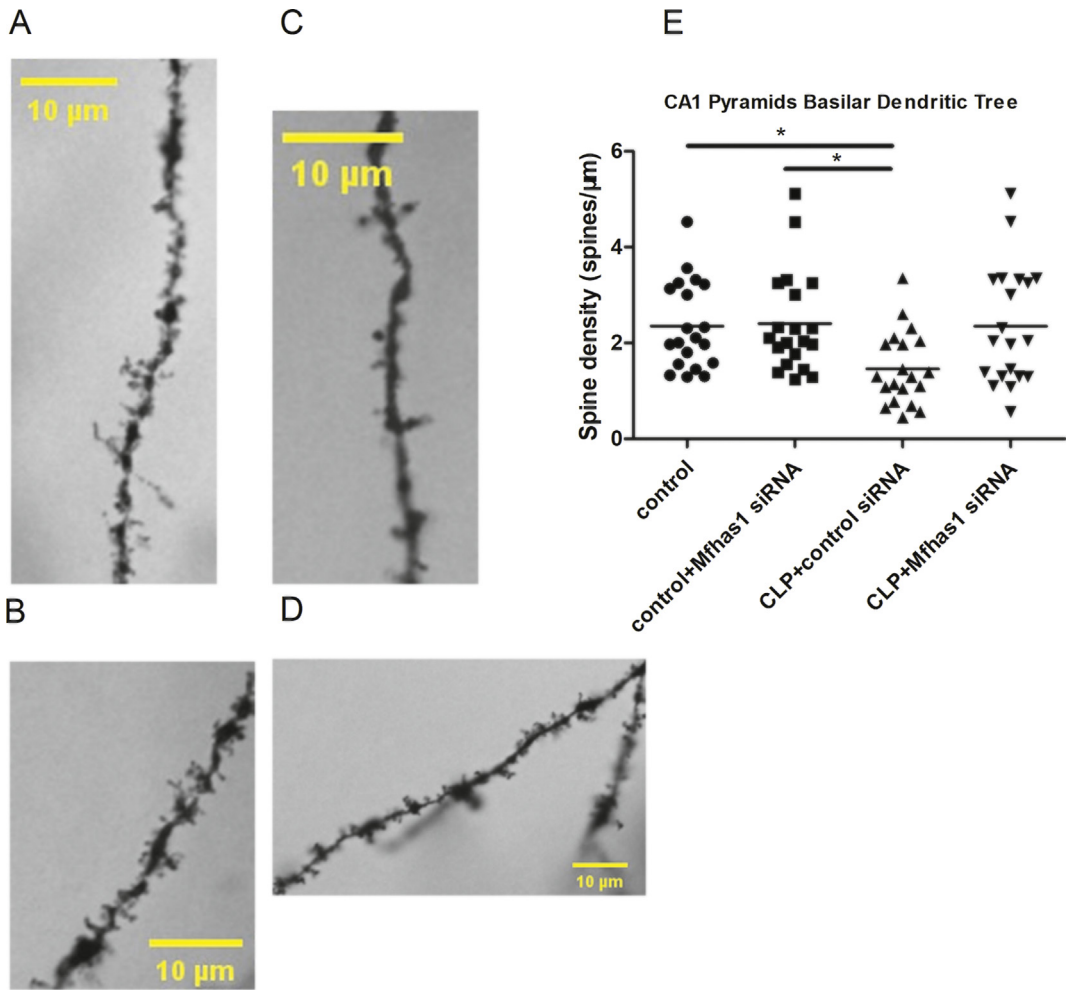

Fig. 5. Effects of *Mfhas1* siRNA treatment on the dendritic spine density of CA1 hippocampal pyramidal neurons in septic rats. (A–D) The representative enlarged images of the secondary dendritic branches of the isolated pyramidal neurons in the hippocampal CA1 region of septic rats were displayed by neuron Golgi staining and used to calculate the number of spine density. (A) control group ; (B) control plus *Mfhas1* siRNA group ; (C) CLP plus control siRNA group ; (D) CLP plus *Mfhas1* siRNA group. (E) The scatter plot of the dendritic spine density of the secondary dendrites of the pyramidal neurons in the hippocampal CA1 region of control and septic rats, the number of spines per μm (n = 20 in each group). CLP, cecal ligation and puncture.

release of cytokines, such as TNF-α and IL-1β [29]. It was reported that the increase of TNF-α and IL-1β levels in the cerebrospinal fluid (CSF) and brain tissue such as hippocampus and cortex is associated with sepsis. IL-1β, as a potent pro-inflammatory cytokine is important for host's defense responses to infection and injury, and it serves as soluble and principally extracellular activators of the IL-1 system [30]. The release of IL-1β is verified to depend on a secondary signal, such as the extracellular adenosine triphosphate (ATP) that triggers the assembly of the inflammasome and subsequent caspase activation that in turn processes pro-IL-1β to its mature form. P2X7 receptor is believed to relate with this process. In addition, blocking of IL-1β signaling attenuates cognitive deficits after sepsis [31]. Moreover, IL-1 pathway regulates inflammation, hematopoiesis and cognition [32] and that a sustained systemic inflammation might aggravate or prolong brain dysfunction [33]. Due to its widespread effects on immune signaling, CNS functions, and its prominence in many disease states, IL-1 can be considered the prototypic multifunctional and pleiotropic cytokine [30].

Lignitto et al., reported that intracerebral infusions of praja2 siRNA reduced the levels of praja2 in the areas surrounding the perfused hemispheres (striatum and hippocampus) [34]. The results of this study suggest that intracerebroventricular injection of *Mfhas1* siRNA reduced the expression of TNF-α and IL-1β in the hippocampus induced by CLP in no > 3 d. Therefore, the protective effect of *Mfhas1* siRNA on cognitive dysfunction may be due to its effect on reduced Aβ expression in hippocampus and dendritic pathology. MFHAS1 knock-down in the hippocampus could alleviate the cognitive impairment and suppress Aβ increase induced by CLP, suggesting that the effect of MFHAS1 on cognitive dysfunction in rats might be related to the MAPK signaling pathway. These results are consistent with previous reports [35,36] showing that the accumulation of Aβ may trigger the generation of proinflammatory cytokines. In this study, the cognitive function which was detected by fear conditioning test in rats in CLP plus *Mfhas1* siRNA group was better than that of rats in CLP plus control siRNA group, but there was no significant difference. This may be because there are many mechanisms of CLP-induced cognitive impairment in rats, not only related to MFHAS1. The intracerebroventricular injection of *Mfhas1* siRNA did not affect the locomotor activity and the mortality rate in septic rats, suggesting that sepsis-induced motor incoordination is not responsible for the behavioral effects seen in this study.

For more than a century, the Golgi method has been used for qualitative and quantitative histology. However, this method provides a unique view of the nervous system and hence, is indispensable for its study. Morphometric evaluation of the Golgi impregnation provides an unsurpassed advantage for the early detection of various forms of neuronal damage and neurodegeneration such as dendritic pathology and spine loss. Therefore, the Golgi method represents a keystone in neurobiology and neuropathology, especially in the study of

degenerative diseases of the nervous system [21]. Dendritic morphology from stack images was traced and reconstructed with the MBF software. The whole neuron with dendrites and their branching can be analyzed by this method [37]. With careful selection of optical sectioning distances, it is possible to achieve a more precise interpretation of the morphology and organization of complex structures by the use of a suitable stack of sections, such as those of the nervous system [38]. The present findings suggest that the loss of dendritic spines might be the morphological representation of neuroplasticity as a response to sepsis. On the other hand, the decrease in the dendritic spine density and dendritic length is a mechanism of cognitive dysfunction in SAE. Further research needs to be carried out with techniques such as electron microscopy, on the signaling pathways involved to establish the appearance or preference for specific parts and states of affected neurons.

In summary, the effect of MFHAS1 knock-down by *Mfhas1* siRNA intracerebroventricular injection can improve cognitive impairment in SAE rats, reduce the expression of TNF-α, IL-1β and Aβ as well as alleviate the dendritic spinal loss of the isolated pyramidal neurons in the hippocampal CA1 region. It also increases the dendritic branching of the CA1 basilar tree of septic rats. We expect that these results will help in designing more effective target-oriented therapeutic strategies for treating cognitive dysfunction in SAE patients.

Author contributions

JZ and CG: conceived, designed and did statistical analysis & writing of the manuscript; NS and CM: review and final approval of the manuscript. WH: participated in revising the manuscript.

Declaration of competing interest

The authors declare that they have no conflict of interest. The work described has not been submitted elsewhere for publication, in whole or in part, and all the authors listed have approved the manuscript that is enclosed.

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