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ORIGINAL PAPER

Higher Circulating Trimethylamine *N*-oxide Sensitizes Sevoflurane-Induced Cognitive Dysfunction in Aged Rats Probably by Downregulating Hippocampal Methionine Sulfoxide Reductase A

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Abstract
Gut microbiota-derived metabolite trimethylamine *N*-oxide (TMAO) has recently been shown to promote oxidative stress and inflammation in the peripheral tissues, contributing to the pathogenesis of many diseases. Here we examined whether pre-existing higher circulating TMAO would influence cognitive function in aged rats after anesthetic sevoflurane exposure. Aged rats received vehicle or TMAO treatment for 3 weeks. After 2 weeks of treatment, these animals were exposed to either control or 2.6% sevoflurane for 4 h. One week after exposure, freezing as measured by fear conditioning test, microglia activity, proinflammatory cytokine expression and NADPH oxidase-dependent reactive oxygen species (ROS) production in the hippocampus (a key brain structure involved in learning and memory) were comparable between vehicle-treated rats exposed to control and vehicle-treated rats exposed to sevoflurane. TMAO treatment, which increased plasma TMAO before and 1 week after control or sevoflurane exposure, significantly reduced freezing to contextual fear conditioning, which was associated with increases in microglia activity, proinflammatory cytokine expression and NADPH oxidase-dependent ROS production in the hippocampus in rats exposed to sevoflurane but not in rats exposed to control. Moreover, hippocampal expression of antioxidant enzyme methionine sulfoxide reductase A (MsrA) was reduced by TMAO treatment in both groups, and TMAO-induced reduction in MsrA expression was negatively correlated with increased proinflammatory cytokine expression in rats exposed to SEV. These findings suggest that pre-existing higher circulating TMAO downregulates antioxidant enzyme MsrA in the hippocampus, which may sensitize the hippocampus to oxidative stress, resulting in microglia-mediated neuroinflammation and cognitive impairment in aged rats after sevoflurane exposure.

Keywords Anesthetic · Cognitive impairment · TMAO · Oxidative stress · MsrA

Introduction

Postoperative cognitive dysfunction (POCD), defined as a new impairment in cognitive function arising after a surgical procedure, is an important complication and has significant clinical and social impacts [1, 2]. Although transient POCD can occur in patients of any age, it is more common in elder populations [1]. It is estimated that nearly 40% of all patients over age 60 who undergo surgery exhibit POCD at 1 week after surgery, and nearly 10% of older patients have POCD 3 months later [1]. Oxidative stress and neuroinflammation in the brain, particularly in the hippocampus that is associated mainly with learning and memory, have been suggested to play an essential role in the pathogenesis of POCD [2, 3]. Anesthetics or surgical stress can increase NADPH oxidase activity and ROS generation in the hippocampus, which activates microglia to release proinflammatory cytokines [2, 4]. In addition, pre-existing some risk factors may enhance oxidative stress or neuroinflammation in the hippocampus, promoting the development of POCD [2].

Many experimental studies demonstrated that the onset of POCD can be induced by general anesthetic agents [2, 5]. Notably, anesthesia-induced cognitive impairment may depend on many factors, such as anesthetic agent, dosage,

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duration, number of exposures and age [6]. Sevoflurane (SEV) is a widely used volatile anesthetic in clinical practice. Previous studies showed that a moderate duration of SEV exposure is not sufficient to cause cognitive impairment in both young adult and aged animals under physiological conditions [7, 8]. However, a moderate duration of SEV exposure can induce POCD in the presence of risk factors [9, 10].

Trimethylamine *N*-oxide (TMAO) is a gut microbiota-derived metabolite of specific dietary nutrients and has recently been implicated in the pathogenesis of cardiovascular and neurological disorders [11, 12]. Previous studies demonstrated that higher circulating TMAO may cause oxidative stress and inflammation in many peripheral tissues [13–15]. Moreover, the presence of higher circulating TMAO leads to increased susceptibility to cardiovascular disease [16, 17]. Higher circulating TMAO has been reported in patients or animal models with multiple diseases including heart failure, atherosclerosis, kidney failure, obesity, diabetes, Alzheimer's disease and cancer [11, 18]. TMAO can rapidly cross the blood–brain barrier [19]. Here we examined whether pre-existing higher circulating TMAO would influence hippocampal oxidative stress, neuroinflammation and cognitive function in aged rats after SEV exposure.

Methods

Animals

Animal procedures were approved by the 960th Hospital of the PLA Animal Care and Use Committee. Male Sprague–Dawley rats (20 months of age) were obtained from Beijing Laboratory Animal Research Center (Beijing, China). All animals were housed in a climate-controlled room on a 12-h light–dark cycle with free access to food and water.

TMAO Treatment and SEV Exposure

Rats were randomly divided into four groups (n = 14–16 per group): (1) vehicle (VEH)-treated rats exposed to control (CON) (CON + VEH); (2) TMAO-treated rats exposed to CON (CON + TMAO); (3) VEH-treated rats exposed to SEV (SEV + VEH); (4) TMAO-treated rats exposed to SEV (SEV + TMAO). TMAO (120 mg/kg in drinking water) or VEH (tap water) was given for 3 weeks. After 2 weeks of TMAO or VEH treatment (before SEV exposure), increased circulating TMAO was confirmed in TMAO-treated rats via tail vein blood samples, and all animals were then trained for fear conditioning. Within 30 min after training, the animals were exposed to 2.6% SEV in 30% oxygen or CON (30% oxygen only) in an anesthetic induction chamber for 4 h. The dose of SEV was based on previous work showing that this exposure could not result in cognitive impairment in rats under physiological conditions [9]. Rectal temperature was monitored and regulated to 37 ± 0.5 °C by placing animals on a warmed heating pad. One week after SEV exposure, fear conditioning and open field tests were carried out, and animals were then euthanized to collect blood samples and brains for biochemical and molecular studies. Some animals from each group (n = 6–7 per group) were perfused with fixative for immunofluorescent study. The experimental protocol was illustrated in Fig. 1.

Fear Conditioning Test

Fear conditioning test was performed as described previously [20, 21]. Briefly, the rats were placed into a conditioning chamber (Med Associates Inc., St Albans, VT, USA). The chamber consisted of a speaker in the side wall and a stainless steel grid floor that was connected to a shock stimulator. During the training, rats were allowed to habituate the chamber for 3 min, after which they received 3 tone (2000 Hz, 90 db)-shock (1 mA, 2 s) pairings, separated by 1 min [22]. After the final shock presentation, rats were allowed to stay in the chamber for an additional 30 s. SEV

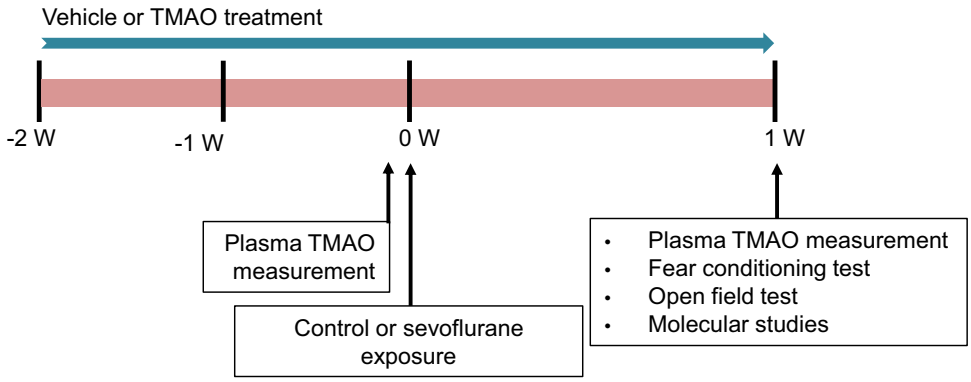

Fig. 1 Experimental protocol of the study

exposure was carried out within 30 min after training. One week after exposure, all rats were tested for the contextual fear conditioning (a hippocampus-dependent task) and the cued fear conditioning (a hippocampus-independent task). For the contextual fear conditioning, each rat was returned to the original training chamber, observed for 6 min without tone or foot shock presentation, and scored for the freezing behavior. The cued fear conditioning was performed 2 h after the contextual fear conditioning in a new chamber without grid floor. Each rat was placed into the new chamber and allowed to habituate for 3 min. The tone was then presented for an additional 3 min and the freezing behavior was scored. Freezing, defined as the absence of movement except for respiration, was expressed as the percentage of the observation period.

Open Field Test

General locomotor activity was evaluated using the open field test as described previously [23]. After fear conditioning was completed, rats were gently placed in a corner of the open-field chamber in which the floor was subdivided into 25 blocks (9″ square) with white stripes. Rats were allowed to move freely for 5 min, and the activity of each animal was automatically recorded by a video camera connected to the Any-Maze animal tracking system (Xinruan Information Technology Co. Ltd., Shanghai, China). The number of line crossings and rearings performed in 5 min observation periods was scored.

Western Blot Analysis

The whole hippocampus was isolated from the brain and snap-frozen in liquid nitrogen. After this, CA1 region was isolated under a dissection microscope at 4 °C from the frozen hippocampus as described previously [24]. The total protein from CA1 region was homogenized in ice-cold RIPA buffer with protease inhibitor cocktails (Sigma Aldrich, Missouri, USA). Forty micrograms of protein extracts were separated by 10% polyacrylamide gel electrophoresis and transferred to PVDF membranes (Millipore Corporation, Bedford, MA, USA). The membrane was blocked for 1 h with 4% non-fat dry milk in TBS-T and then immunoblotted with primary antibodies to interleukin (IL)-1β (17 kDa), tumor necrosis factor (TNF)-α (25 kDa), methionine sulfoxide reductase (Msr) A (26 kDa, Abcam, Cambridge, MA, USA) and β-actin (43 kDa, Santa Cruz Biotechnology, CA, USA) overnight at 4ºC. Following removal of the primary antibodies, HRP-conjugated second antibodies (Santa Cruz Biotechnology, CA, USA) were added for 1 h at room temperature. The immunoreactive bands were detected by using a chemiluminescence detection system according to the manufacturer's instructions (GE Healthcare, WI, USA) and developed on a film. ImageJ software e (NIH, Bethesda, Maryland, USA) was used to quantify the optical density of each protein band. Each lane of protein band density was normalized with corresponding β-actin protein density.

NADPH Oxidase Activity Assay

NADPH oxidase activity in the hippocampus was assessed using a NADPH oxidase activity assay kit (Genmed Scientifics Inc., Wilmington, DE, USA) according to the manufacturer's instructions. Briefly, the CA1 region of the hippocampus was isolated and homogenized in ice-cold PBS buffer. After centrifugation at 2500×*g* for 10 min, the supernatant was collected and incubated with NADPH. NADPH oxidase activity was assessed by monitoring the rate of NADPH consumption and measured with spectrophotometry at 340 nm.

Hydrogen Peroxide Assay

The hippocampal levels of hydrogen peroxide (H_2O_2), a major component of reactive oxygen species (ROS), were assessed using a Hydrogen Peroxide assay kit (Invitrogen, OR, USA) according to the manufacturer's instructions. Briefly, the CA1 region of the hippocampus was isolated and homogenized in ice-cold PBS buffer containing 5 mM sodium azide at 4 °C for 1 min. After centrifugation at 4000×*g* for 10 min, the supernatant was collected and measured with an EnVision multilabel reader (Perkin Elmer, MA, USA) at 560 nm.

Immunofluorescent Study

Immunofluorescent staining for microglia was performed as described before [25]. Briefly, brain sections (20-µm-thick) were cut in a cryostat at −23 °C. Following incubation with an anti-CD11b primary antibody (Chemicon, Temecula, USA), the sections were incubated for 2 h at room temperature with a secondary antibody (Alex Fluor 488; Invitrogen, USA). Sections were examined under a confocal laser scanning microscope (Zeiss LSM700). Activated microglia were defined by strong CD11b immunoreactivity, an enlarged soma, fewer and shorter processes [25]. The numbers of total and activated microglia in the CA1 region were counted and activated microglia were expressed as a percentage of the total number of microglia.

Circulating TMAO and Proinflammatory Cytokine Measurements

Plasma levels of TMAO were determined by liquid chromatography coupled with triple-quadrupole mass spectrometry as described previously [17]. Plasma levels of TNF-α and

IL-1β were measured using ELISA Kits (R&D Systems, Inc., MN, USA).

Statistical Analysis

Statistical analysis was performed with GraphPad Prism 6.0 (GraphPad software for Science, San Diego, CA). All data were expressed as means ± SEM. A two-way ANOVA followed by a Bonferroni post hoc test was applied for statistical analysis. Spearman correlation was applied to determine the associations between hippocampal neuroinflammation and contextual fear conditioning test results or between hippocampal neuroinflammation and MsrA expression. The criterion for statistical significance was $P < 0.05$.

Results

Plasma TMAO Levels are Increased in TMAO-Treated Rats Before and 1 Week After SEV Exposure

Before and 1 week after SEV or VEH exposure, plasma TMAO levels were comparable between two VEH-treated groups (Fig. 2a, b). However, plasma TMAO levels were similarly increased in CON + TMAO rats and SEV + TMAO rats at both time points, compared with the respective VEH-treated groups.

Pre-existing Higher Circulating TMAO Leads to Cognitive Impairment After SEV Exposure

The fear conditioning test was applied to assess fear learning and memory in rats at 1 week after SEV exposure. The freezing behavior is an innate defensive fear response in rodents and a reliable assessment of learned fear [22]. As shown in Fig. 3, the freezing in the contextual fear conditioning test was similar in CON + TMAO rats and CON + VEH rats (Fig. 3a). Compared with the respective CON groups, SEV exposure significantly reduced the freezing in TMAO-treated rats but not in VEH-treated rats. For the cued fear conditioning test, no difference in the freezing to the tone was observed among four experimental groups (Fig. 3b).

Previous studies have suggested that altered locomotor activities should be taken into consideration when interpreting results of behavioral tests such as memory-related or anxiety-related tests [26, 27]. Additionally, a recent study showed that a strong relationship existed between the early stage of decreased motor function and cognitive impairment [28]. To rule out the possibility that the fear conditioning assessment was influenced by the locomotor dysfunction, we performed open-field test to assess spontaneous activity after fear conditioning test. There were no differences in the number of rearings (Fig. 3c) and the number of crossings (Fig. 3d) across four experimental groups.

Pre-existing Higher Circulating TMAO Results in Microglia-Mediated Neuroinflammation After SEV Exposure

Microglia-mediated neuroinflammation in the hippocampus has been suggested to contribute to cognitive dysfunction. We therefore measured microglial activity as well as expression of key proinflammatory cytokines in the hippocampus. One week after SEV exposure, the number of activated microglia (Fig. 4a, b) and the protein levels of IL-1β (Fig. 5a, b) and TNF-α (Fig. 5a, c) were comparable between

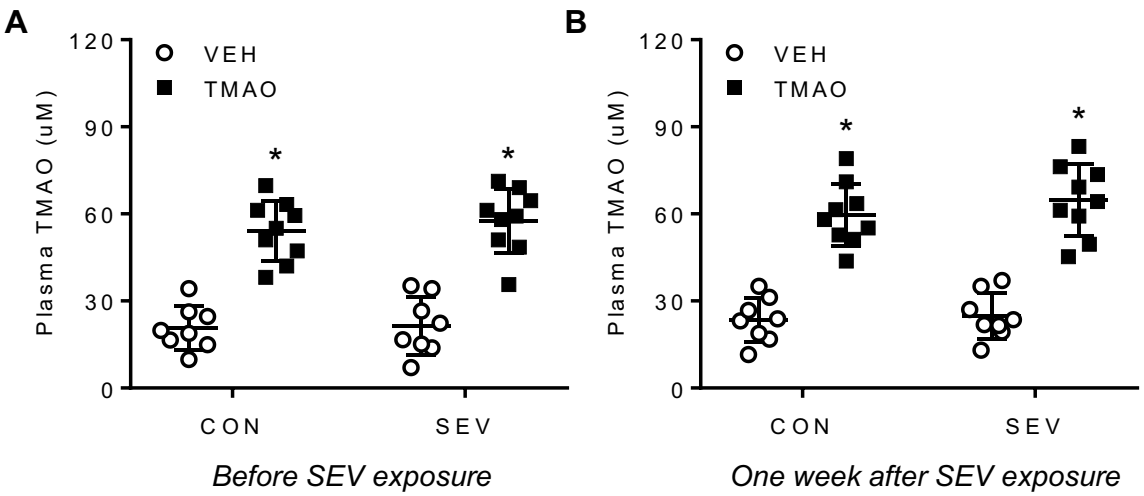

Fig. 2 Effects of TMAO treatment on plasma TMAO levels before (**a**) and 1 week after SEV (**b**) exposure. Data are mean ± SE (n = 8–9 per group). *P < 0.05 versus CON + VEH or SEV + VEH. *CON* control exposure, *SEV* sevoflurane exposure, *VEH* vehicle treatment

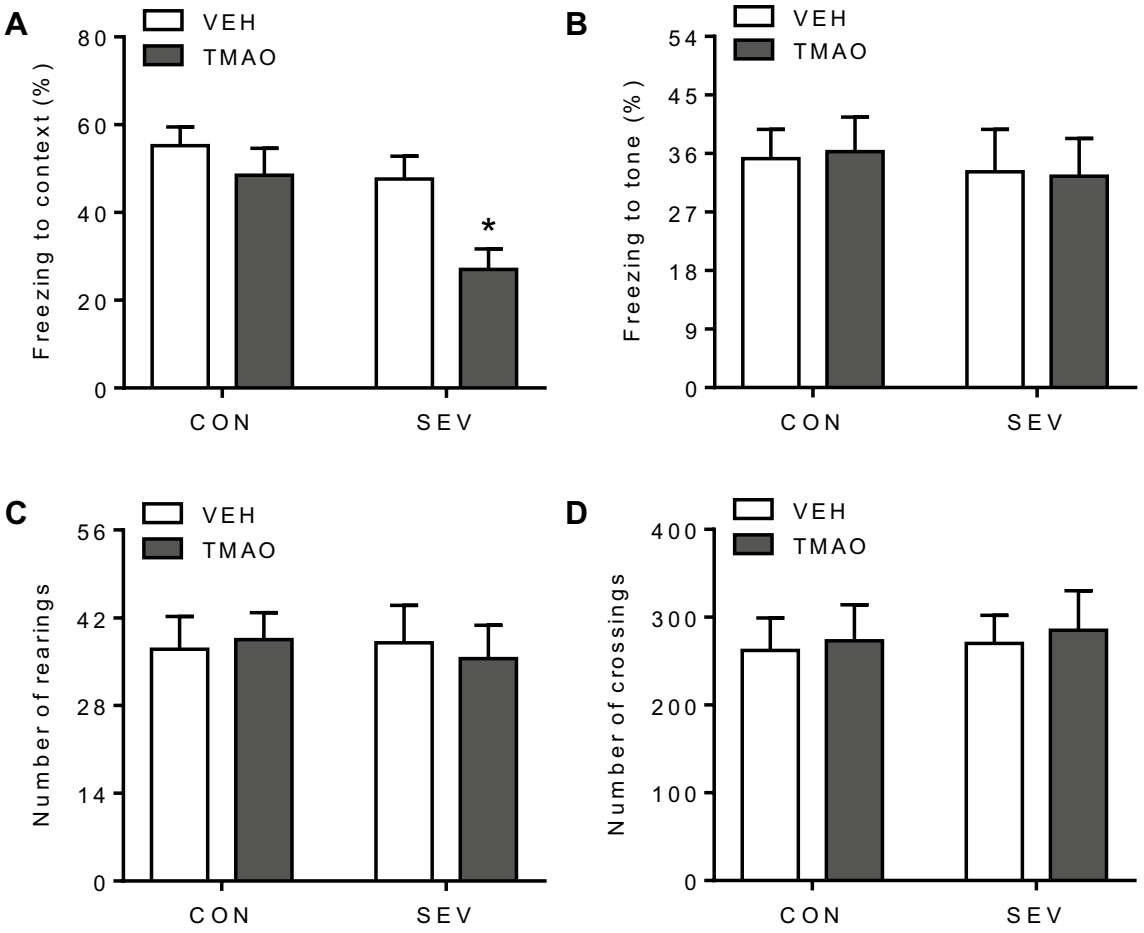

Fig. 3 The influences of TMAO treatment on memory and spontaneous activity in rats 1 week after SEV exposure. **a**, **b** The freezing time to the context (hippocampus-dependent memory) and to the tone (hippocampus-independent memory) as assessed by fear conditioning test; **c**, **d** the number of rearings and crossings as measured by open field test. Data are mean ± SE (n = 8–9 per group). *P < 0.05 versus other groups

CON + VEH rats, CON + TMAO rats and SEV + VEH rats. However, the number of activated microglia and the protein levels of IL-1β and TNF-α were significantly increased in SEV + TMAO rats when compared with all other groups. Additionally, increased protein levels of IL-1β and TNF-α in the hippocampus were negatively correlated with the reduced freezing in the contextual fear conditioning test in SEV + TMAO rats (Fig. 5d, e).

Pre-existing Higher Circulating TMAO Augments NADPH Oxidase-Dependent ROS Production After SEV Exposure

ROS primarily produced via NADPH oxidase play an important role in mediating microglia activity and the release of proinflammatory cytokines [3]. We next assessed NADPH oxidase activity and ROS production in the hippocampus. The NADPH oxidase activity was similar in CON + VEH rats and CON + TMAO rats (Fig. 6a). Compared with the respective CON groups, both SEV + VEH rats and SEV + TMAO rats exhibited significantly increase in NADPH oxidase activity. Notably, the increase in NADPH oxidase activity was greater in SEV + TMAO rats than SEV + VEH rats.

There were no differences in H_2O_2 levels between the two CON groups (Fig. 6b). H_2O_2 levels tended to be higher in SEV + VEH rats as compared to CON + VEH rats, but this trend did not reach statistical significance. H_2O_2 levels were significantly higher in SEV + TMAO rats compared with the other groups.

Pre-existing Higher Circulating TMAO Downregulates MsrA Expression After SEV Exposure

MsrA is a critical antioxidant enzyme that participates in cell and tissue protection [29, 30]. New evidence reveals that microglia-mediated neuroinflammation in the brain can be regulated by MsrA through reducing ROS production [31].

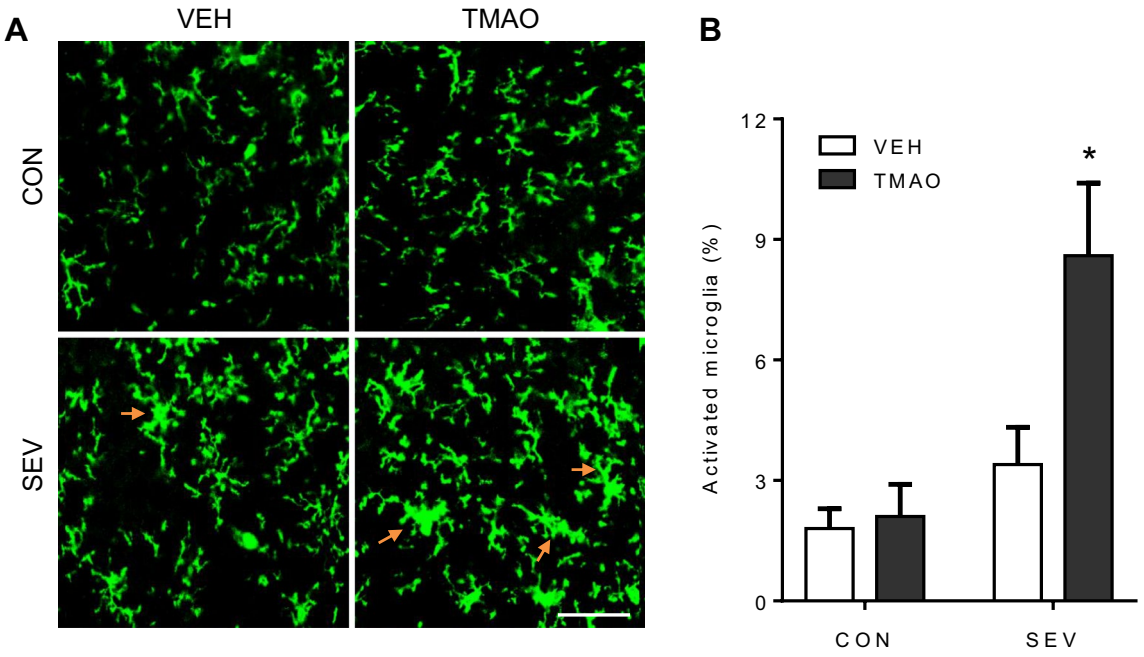

Fig. 4 The influence of TMAO treatment on microglia activation in the hippocampus 1 week after SEV exposure. **a** Representative photomicrographs showing CD11b-immunoreactive microglia in each group. **b** Quantitative analysis of activated microglia in the hippocampus in each group. Arrows indicate activated microglia. Scale bar: 50 μm. Data are mean ± SE (n = 6–7 per group). *P < 0.05 versus other groups

We further measured MsrA expression in the hippocampus. As presented in Fig. 7, MsrA expression was significantly increased in SEV + VEH rats compared with CON + VEH rats at 1 week after SEV exposure (Fig. 7a, b). Compared with the respective VEH-treated groups, CON + TMAO rats and SEV + TMAO rats had significantly reduced MsrA expression. Moreover, reduced MsrA expression was negatively correlated with increased IL-1β and TNF-α expression in the hippocampus in SEV + TMAO rats (Fig. 7c, d).

Discussion

This study investigated the effect of pre-existing higher circulating TMAO on cognitive function in aged rats after a moderate duration of SEV exposure. The main findings are that (1) pre-existing higher circulating TMAO leads to cognitive dysfunction in aged rats after SEV exposure but not CON exposure; (2) pre-existing higher circulating TMAO results in increases in NADPH oxidase-dependent ROS production and microglia-mediated neuroinflammation in the hippocampus in aged rats after SEV exposure; (3) higher circulating TMAO downregulates expression of antioxidant enzyme MsrA in the hippocampus in aged rats exposed to either CON or SEV; (4) Increased hippocampal neuroinflammation was negatively correlated with reduced freezing in the contextual fear conditioning test and decreased hippocampal MsrA expression in TMAO-treated rats exposed to SEV.

Gut microbiota-dependent metabolite TMAO has recently drawn a lot of attention as it is closely associated with the pathogenesis of many human diseases, including obesity, type 2 diabetes mellitus, cardiovascular and renal diseases [11, 13–15]. The production of circulating TMAO occurs via a two-step process. First, gut microbes enzymatically generate trimethylamine (TMA) from the TMA-containing dietary components such as choline and carnitine; TMA then enters the circulation and is converted to TMAO in the liver by flavin-containing monooxygenase (FMO) enzymes, primarily FMO3 [32]. Under normal physiologic conditions, circulating TMAO is rapidly cleared by the kidney [33]. Circulating TMAO levels are dependent on several factors, including the dietary habits, gut microbiota, FMO3 activity and kidney function [34]. Alterations in these factors may lead to increased circulating TMAO [34]. Experimental and clinical studies have shown that increased circulating TMAO promotes inflammatory response and ROS production in the peripheral tissues including aorta, heart, and kidney, contributing to the development of multiple cardiovascular and renal diseases [13–15]. A clinical study recently reported that TMAO levels in cerebrospinal fluid are higher in individuals with mild cognitive impairment and Alzheimer's disease, and higher TMAO levels in cerebrospinal fluid are associated with biomarkers of Alzheimer's disease

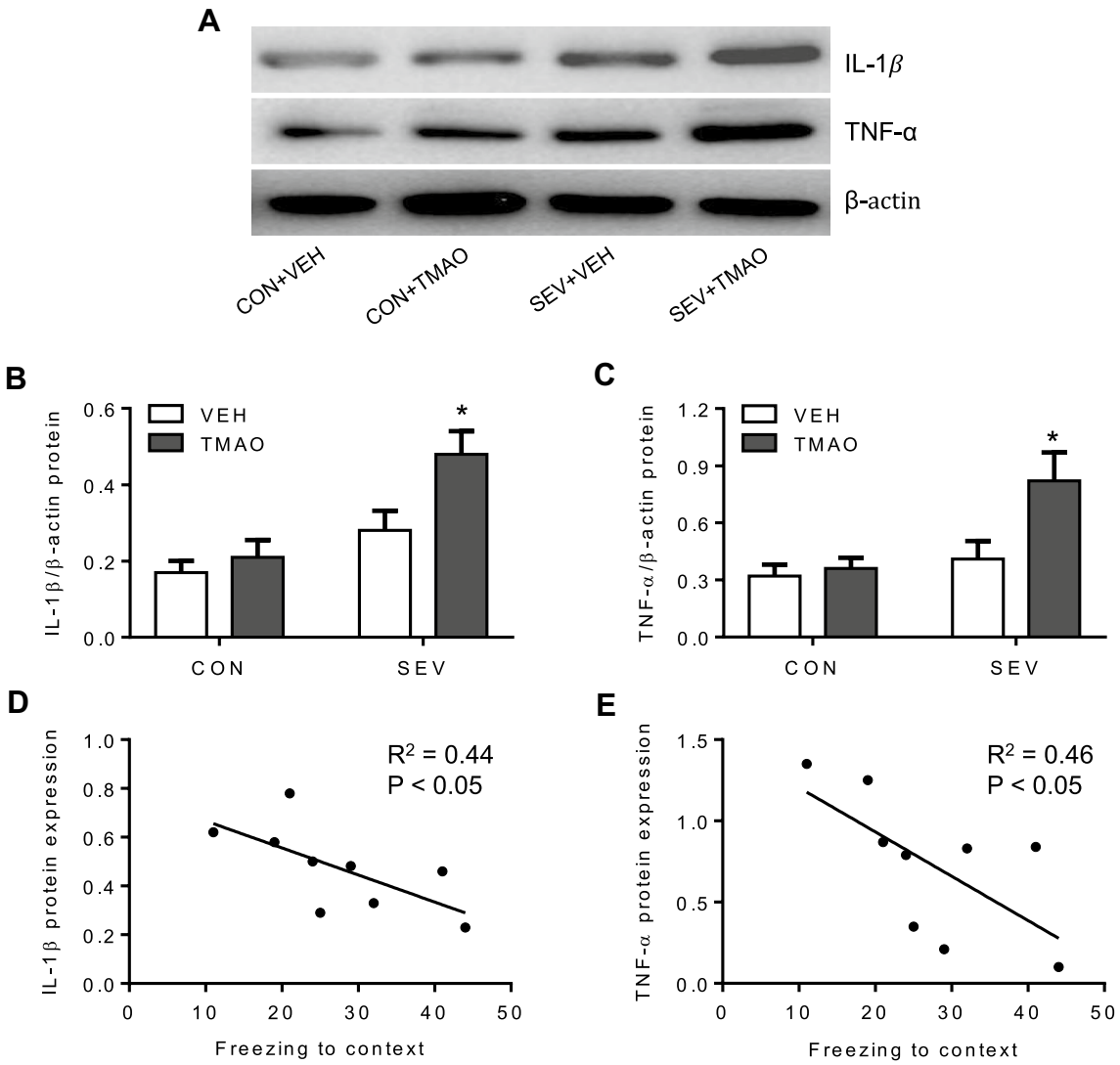

Fig. 5 The influence of TMAO treatment on neuroinflammation in the hippocampus 1 week after SEV exposure, and correlation between hippocampal neuroinflammation and the freezing in the contextual fear conditioning test. **a** Representative Western blots in each group. **b**, **c** Quantitative comparison of protein levels for pro-inflammatory cytokines IL-1β and TNF-α in the hippocampus. **d**, **e** Increased expression of proinflammatory cytokines (IL-1β and TNF-α) was negatively correlated with reduced freezing in the contextual fear conditioning test in SEV + TMAO rats. Data are mean ± SE (n = 8–9 per group). *P < 0.05 versus other groups

pathology and neuronal degeneration [18]. These findings indicate that circulating TMAO is capable of crossing the blood–brain barrier and may therefore be relevant to neurological function or disorders. To date, however, no studies have examined the influences of increased circulating TMAO on anesthetic-induced cognitive dysfunction. In the present study, we found that neither hippocampal-dependent memory function nor hippocampal-independent memory function were impaired in SEV + VEH rats compared with CON + VEH rats. Molecular studies revealed that microglia activity, proinflammatory cytokine expression and ROS production in the hippocampus were comparable between SEV + VEH rats and CON + VEH rats. These results are consistent with previous studies showing that a moderate duration of SEV exposure does not cause cognitive impairment in aged animals under physiological conditions [7, 8]. To examine whether pre-existing higher circulating TMAO influences cognitive function after SEV exposure, we pretreated rats with TMAO and confirmed that circulating TMAO was significantly higher in rats treated with TMAO before and 1 weeks after CON or SEV exposure. Interesting, there were no differences in microglia activity, proinflammatory cytokine expression and ROS production in the hippocampus and cognitive function between CON + TMAO rats and CON + VEH rats. However, SEV + TMAO rats exhibited impairment in hippocampal-dependent memory

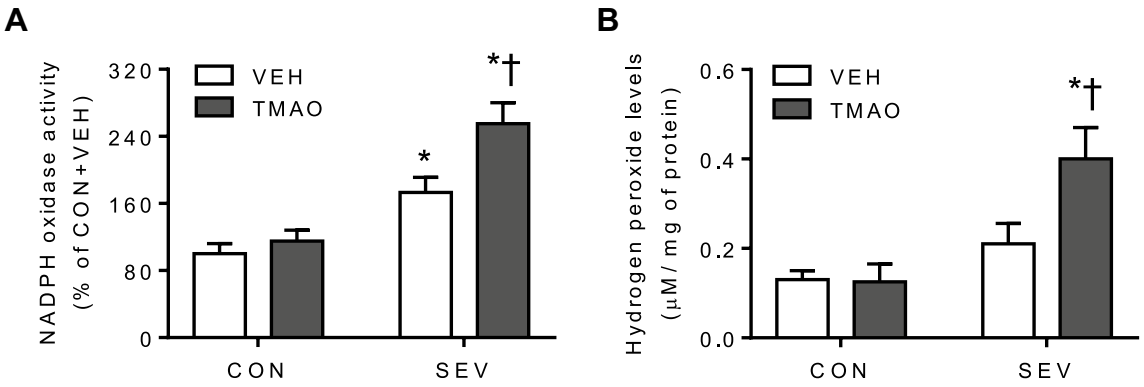

Fig. 6 The influences of TMAO treatment on NADPH oxidase-dependent reactive oxygen species production in the hippocampus 1 week after SEV exposure. **a** NADPH oxidase activity; **b** the levels of hydrogen peroxide, a key endogenous reactive oxygen species. Data are mean ± SE (n = 8–9 per group). *P < 0.05 versus CON + VEH or CON + TMAO; †P < 0.05, SEV + TMAO versus SEV + VEH

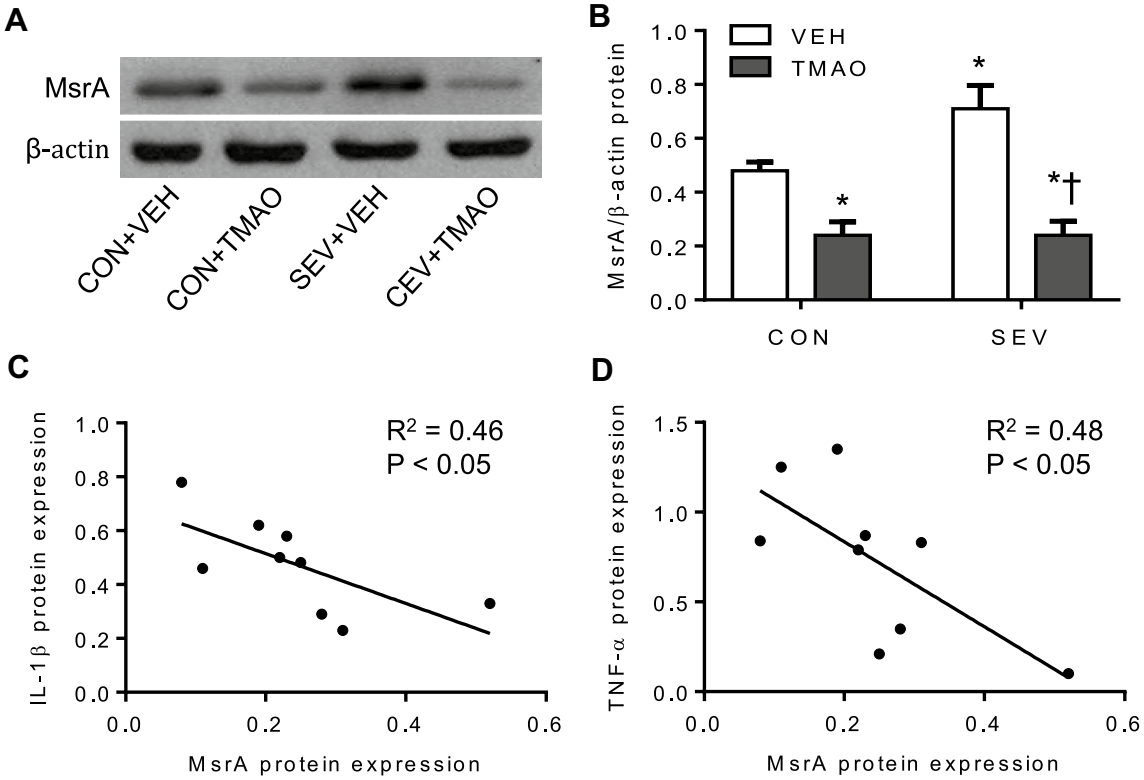

Fig. 7 The influences of TMAO treatment on expression of antioxidant enzyme MsrA in the hippocampus 1 week after SEV exposure, and correlation between expression of MsrA and neuroinflammation in the hippocampus. **a** Representative Western blots in each group. **b** Quantitative comparison of protein levels for antioxidant enzyme MsrA in the hippocampus. **c**, **d** TMAO-induced reduction in MsrA expression was negatively correlated with increased expression of proinflammatory cytokines (IL-1β and TNF-α) in the hippocampus in SEV + TMAO rats. Data are mean ± SE (n = 8–9 per group). *P < 0.05 versus CON + VEH; †P < 0.05, SEV + TMAO versus SEV + VEH

function as indicated by reduced freezing in the contextual fear conditioning test, which was accompanied by increased microglia activity, proinflammatory cytokine expression and ROS production in the hippocampus. Moreover, increased proinflammatory cytokine expression in the hippocampus was negatively correlated with reduced freezing in the contextual fear conditioning test in SEV + TMAO rats. These findings suggest that increased circulating TMAO alone in a

short time period may be insufficient to increase ROS levels and induce neuroinflammation in the hippocampus under physiological conditions. However, pre-existing increased circulating TMAO may enhance the sensitivity and susceptibility of the brain to SEV exposure, leading to increased ROS levels and neuroinflammation in the hippocampus and consequently cognitive impairment.

To further examine the potential mechanisms by which higher circulating TMAO induces sensitization of neuroinflammation and cognitive impairment after SEV exposure, we assessed expression of MsrA, an intracellular enzyme that is responsible for repairing methionine-oxidized proteins and for scavenging ROS [29, 30]. Oxidative stress is a major trigger for upregulating the cellular antioxidant defense system, including MsrA [29]. When either the upregulation of MsrA is inadequate or compromised in response to oxidative stress, a disease process may occur depending on the cellular/organ condition [29]. MsrA is highly expressed in several brain regions including the hippocampus and has been suggested to be associated with learning and memory [35]. Recent evidence reveals that microglia-mediated neuroinflammation can be negatively regulated by MsrA through reducing intracellular ROS accumulation [31]. For example, in vitro studies show that knockout of MsrA leads to exaggeration of lipopolysaccharide-induced microglia activity and proinflammatory mediator expression [31], whereas overexpression of MsrA in microglia attenuates lipopolysaccharide-induced microglia activity and proinflammatory cytokine production by reducing ROS levels [31]. In agreement with the results obtained in in vitro experiments, in vivo study also demonstrates that overexpression of MsrA in the brain reduces microglia activity as well as neuroinflammation in rats receiving lipopolysaccharide infusion [31]. In the present study, we found that MsrA expression was significantly higher in hippocampus in SEV + VEH rats than CON + VEH rats. Because SEV + VEH rats exhibited a significant increase in NADPH oxidase activity and a tendency to increased ROS levels in the hippocampus. We speculate that increased hippocampal MsrA expression in VEH-treated rats exposed to SEV occurs as a compensatory response to increased NADPH oxidase-derived ROS production, which reduces ROS levels in the hippocampus, thus preventing neuroinflammation and cognitive dysfunction after SEV exposure. Importantly, we observed that TMAO treatment significantly reduced hippocampal MsrA expression in rats exposed to both CON and SEV, although the underlying mechanisms were unknown. Reduced hippocampal MsrA expression in rats exposed to SEV but not in rats exposed to CON was associated with increased ROS levels, neuroinflammation and cognitive impairment. Moreover, reduced MsrA expression was negatively correlated with increased IL-1β and TNF-α expression in the hippocampus in SEV + TMAO rats. This finding suggests that reduced hippocampal MsrA expression alone is unable to cause neuroinflammation and cognitive impairment under physiological conditions; however, reduced hippocampal MsrA expression might enhance the susceptibility to SEV-induced oxidative stress, leading to neuroinflammation and cognitive impairment. Indeed, it has been reported that MsrA knockout mouse is more vulnerable to oxidative stress and has several molecular phenotypes that can be associated with age-related diseases including Alzheimer's disease [29].

It should be noted that other antioxidants, except for MsrA, might also be decreased by increased circulating TMAO levels, accounting for sensitization of SEV-induced neuroinflammation and cognitive dysfunction. For example, NF-E2-related transcription factor 2 (Nrf2), a key transcriptional regulator driving antioxidant gene expression and protection from oxidant injury [36], has been shown to regulate SEV-induced neuroinflammation and cognitive dysfunction in animals [37]. Nrf2 activates a number of antioxidant and cytoprotective proteins including thioredoxins, superoxide dismutase-1, glutathione peroxidase, glutathione-S-transferase, glutathione reductase, heme oxygenase-1 and NAD(P)H quinone oxidoreductase-1 [38, 39]. In addition, Nrf2 suppresses hippocampal neuroinflammation by inhibiting NF-kB signaling pathway [40]. A recent study showed that SEV exposure caused a compensatory increase in expression of Nrf2 in the hippocampus in aged rats [37]. Upregulation of Nrf2 in the hippocampus prevents SEV exposure-induced NF-kB activity and proinflammatory cytokine expression in aged rats, whereas knockdown of Nrf2 in the hippocampus enhances SEV exposure-induced NF-kB activity and proinflammatory cytokine expression [37]. These findings suggest that hippocampal Nrf2 also play a role in mediating SEV exposure-induced neuroinflammation. We therefore cannot exclude the possibility that other antioxidants including Nrf2, might also be decreased by increased circulating TMAO levels, partially contributing to sensitization of SEV-induced neuroinflammation and cognitive dysfunction. In addition, the hippocampus is made up of the dentate gyrus (DG/CA4), CA1 and CA2/3 subfields, which perform different functions [41]. Among these subfields, the CA1 region is mostly associated with the formation and consolidation of learning-memory [42], whereas the DG, which contains neural stem cells and neural progenitor cells, is involved in neurogenesis [43]. It has been reported that hippocampal subfields exhibit differential vulnerability to the aging process [44] and SEV exposure [45]. In the present study, the SEV exposure-induced changes in all measured molecular parameters were examined only in the CA1 region of the hippocampus, we could not determine whether there was a particular region within the hippocampus mostly vulnerable to SEV exposure.

One major limitation of this study should be acknowledged. We chose the contextual fear conditioning test to

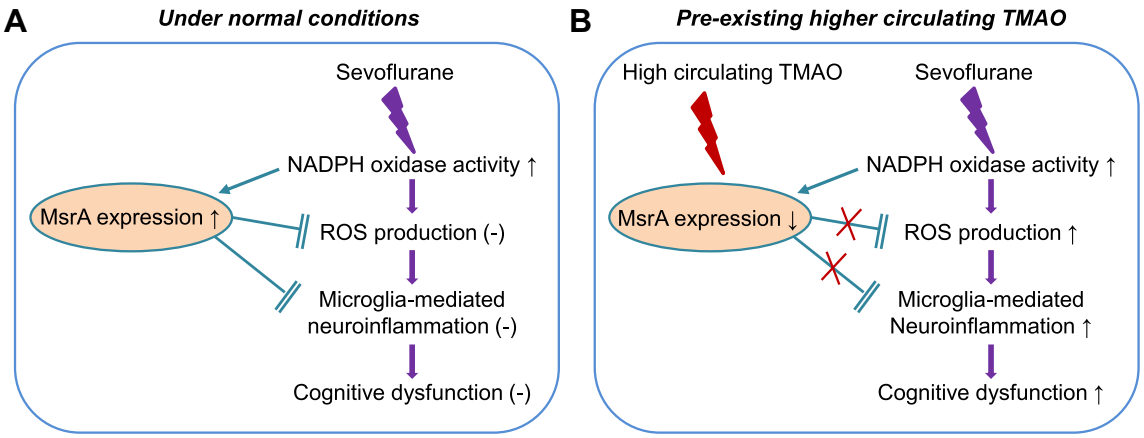

Fig. 8 Schematic diagram showing possible mechanism by which pre-existing higher circulating TMAO leads to increased neuroinflammation in the hippocampus and cognitive dysfunction in rats after SEV exposure. *MsrA* methionine sulfoxide reductase A, *ROS* reactive oxygen species

evaluate cognitive function in aged rats based on previous studies [20, 21]. It should be noted that the contextual fear conditioning test may be influenced by some factors. For example, the animals might overcome contextual fear even in aged subjects simply by increasing the number of training sessions. The Morris water maze test has been suggested to be the gold standard of spatial memory evaluation. Further studies are necessary to perform the Morris water maze test and determine whether the results of the Morris water maze test are consistent with the findings of our study.

In conclusion, the present study demonstrates that pre-existing higher circulating TMAO decreases antioxidant enzyme MsrA expression in the hippocampus, which may enhance the susceptibility to SEV-induced oxidative stress, leading to microglia-mediated neuroinflammation and cognitive impairment in aged rats after SEV exposure (Fig. 8a, b). Higher circulating TMAO has been reported in various cardiovascular and other diseases. Targeting circulating TMAO in the perioperative period may be a novel strategy for the prevention of anesthetic SEV-induced cognitive impairment in elderly patients with higher circulating TMAO.

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Compliance with Ethical Standards

Conflict of interest The authors declare that they have no conflict of interest.

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