Ozone Decreases CRMP2 Phosphorylation by Inhibiting Sema3A to Ameliorate Chronic REM Sleep Deprivation-Induced Cognitive Deficits in Mice

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Abstract

Sleep is essential for proper cognitive functioning. Ozone can delay both the aging process as well as the associated neurodegeneration. An untested hypothesis is that ozone may play a role in reducing the cognitive impairment associated with sleep deprivation. This study was designed to elucidate the mechanism of ozone's effect on chronic REM sleep deprivation induced cognitive dysfunction in mice. Ozone ameliorated cognitive dysfunction in chronic REM sleep deprived mice, increased the number of dendritic spines in the hippocampus region and decreased neuronal loss. Administration of ozone may protect against chronic REM sleep deprivation induced cognitive dysfunction by enhancing the expression ofSema3A and PlexinA1 concentrations as well as P-CRMP2/CRMP2 in the hippocampus. Moreover, ozone was associated with acetylation of α-tubulin, which, in turn, is associated with microtubule network dynamics and modulates ATP synthase activity. In conclusion, ozone may play a neuroprotective role and improve chronic REM sleep deprivation induced spatial recognition and learning memory dysfunction in mice.

Introduction

Sleep is vital to health and has been related to the clearance of metabolic waste products from the brain [1] as well as the promotion of cognitive development [2, 3]. Rapid eye movement sleep is associated with the consolidation of spatial and emotional memory [4, 5]. Epidemiological and experimental data support the association of sleep deprivation (SD) with the possibility of developing cardiovascular and metabolic diseases [6–8], all-cause mortality [9], accidents [10], anxiety [11], and depression [12]. Furthermore, lots of evidences reveal sleep deprivation is linked with neurological disorders [13–16], such as stroke, Alzheimer's disease and cognitive decline.

Ozone, a triatomic molecule with three oxygen atoms, which has been used to treat disease for over 150 years [17], including skin diseases [18], diabetes [19], COVID-19 [20, 21] and neurodegenerative disorders [22–24]. Some studies have shown that ozone has beneficial effects by reducing mitochondrial damage [23] inhibiting oxidative stress and inflammation [25, 26]. Previously we reported that the cognitive decline of APP/PS1 mice was alleviated by ozone therapy. [27]. Recent work has shown that ozone can improve sleep quality in patients with insomnia [28]. The important mechanism of the positive effects of ozone is attributed to up-regulation of cellular antioxidant enzyme activity. When ozone is administrated, it dissolves immediately in the plasma/serum and reacts with PUFA (polyunsaturated fatty acids), leading to the formation of hydrogen peroxide (H_2O_2) as a ROS and 4-hydroxynonenal (4HNE) as a lipid oxidation product (LOP)[29]. Ozonated water (OW), which is ozone gas dissolved in water, is much easier to handle than ozone gas.

The secreted semaphorin-3A (Sema3A) is a chemorepulsive protein, which can promote growth cone collapse and suppress axon formation [30–32]. Our study has demonstrated that mRNA and protein expression of Sema3A were upregulated in the hippocampus after SD and reversed by Ozone treatment[33]. Semaphorins function by binding to their receptors at the cell membrane and activating

particular intracellular signal transmission cascades [34]. PlexinA1 is a receptor for Sema3A [32]. Sema3A binding to plexinA1 activates signaling via downstream molecules such as collapsin response mediator protein (CRMP)-2 [35]. An increasing number of studies indicate that CRMP2 expression level and phosphorylation state have functions beyond synaptic transmission [36], including mitochondrial motility regulation [37], cytoskeletal microtubule system modulation [38] and improvement in cognitive decline [39–41].

Mitochondria convert energy through oxidative phosphorylation (OXPHOS), and ATP synthase, an essential part of OXPHOS, is located in the inner mitochondrial membrane [42]. ATP synthase is a classical rotational molecular motor which can condensate ADP and inorganic phosphate to generate ATP [43]. Previous researches have suggested that the regulation of ATPase can promote the growth of neuronal processes, inhibit apoptosis and promote oxidative metabolic efficiency [43, 44].

Based on these findings, we investigated the possible protective effects of OW on chronic REM sleep deprivation induced cognitive impairment as well as increasing CRMP2 phosphorylation, along with activating Sema3A. Our results showed that OW significantly maintained the synaptic structural plasticity and normalized the microtubule stability induced by chronic REM sleep deprivation. Furthermore, ATPase activity was markedly upregulated in OW treated mice. Taken together, our findings suggested that ozone may be a promising treatment for cognitive impairment caused by sleep deprivation.

Methods

Animals and Treatments

All animal experiments were carried out in compliance with the ARRIVE guidelines and were approved by the Animal Care and Use Committee of Aviation General Hospital of China Medical University Laboratory (HK2022-03). Four to five week-old male ICR mice (Vital River, Beijing, China) weighing 25-27 g were maintained in cages under a 12 h light/12 h dark cycle, with room temperature kept at 23 ± 2 ℃ and kept humidity at 50 ± 5 %. Standard rodent chow and water were provided ad libitum. Investigators assigned randomly animals to four groups (n = 15 per group): the control (Ctrl) group, sleep deprivation (SD) group, low dose (LD) (11.5 μg/ml, i. p.) group, and a high dose (HD) (23 μg/ml, i. p.) group. Except for the control group, all groups had successive 21-day periods of sleep deprivation after 7 days of adaptive feeding (from 10 p.m. to 4 p.m. per day, 4 h for rest). The mice were administered ozone, once a day, from day 14 to day 21 at 4:30 p.m., and the Ctrl and SD groups received physiological saline (0.9% NaCl, 10 ml/kg, i. p.) simultaneously.

Preparation of Ozone Water

We used ozone therapy device to produce ozone water (KASTNER, German). This device can generate OW from O_2 and water while while also can control the concentration of the dissolved O_3. In every trial, OW was administered within 10 min after produced.

Establishment of the Chronic REM Sleep Deprivation Model

The chronic REM sleep deprivation model was established by a modified multi-platform water environment method [45]. Fifteen interconnected platforms (platforms were of 1cm diameter each) are put in the water tank (90 cm × 60 cm × 40 cm). The water reached a depth of 1.5 cm below the platform's base and was maintained at a temperature of 25 ± 2 °C. Therefore, when the animal entered the REM sleep state, it would fall into the water and wake up. The animals had unrestricted access to food and water. Mice of the Ctrl group were placed on larger platforms (7 cm in diameter) surrounded by water with their cage mates. Prior to the chronic REM sleep deprivation, the mice were placed on the platform to adapt to the condition for 1 week (30 minutes per day).

Morris Water Maze Test

MWM is a classic experiment used to assess rodent spatial memory ability [46]. The MWM consists of a black tank which was filled with enough fresh water (22-25 °C). Animals were taught to swim to a platform that was submerged 0.5 cm beneath the water. The spatial acquisition trial and the probing trial are the two components of the MWM trials. For the spatial acquisition experiment, four days of learning and followed by tests of learning. In brief, mice were randomly assigned to one of four different starting positions for four training trials per day. Each mouse was given 60 s to look for the platform in each trial. If the mouse was unable to locate the platform, the experimenter directed it to climb the platform and become acquainted with its surroundings for 30 s. The probe trial was held on the fifth day following the last day of training (Fig. 1). The hidden platform was removed before the probe trial. Mice were placed in the maze at 180° from their original platform position and permitted to swim in the tank for 60 s.

Open Field Test

This test was used to assess the locomotor activity and anxiety-like behavior in rodents [47]. Mice (n=6 per group) were placed in the open field apparatus (cubic black box of 42 cm × 42 cm × 25 cm in size) and allowed to move freely for 5 min. After each test, the apparatus was carefully cleaned with alcohol.

Novel Object Recognition Test

The NOR is a behavioral method that assesses the rodent's recognition and memory ability by utilizing the rodent's inherent tendency to approach and explore novel objects [48]. Two identical objects were presented, and the mice (n=6 per group) were free to explore for 5 min. Next, one of the objects is replaced by a novel one and the experiment is repeated. To eliminate potential odors, alcohol should be sprayed on the test equipment every two test intervals. The discrimination index was calculated as the percentage of time spent in exploring the novel object divided by total amount of time in exploring both objects.

Nissl Staining

Nissl staining revealed changes in the expression of hippocampal neuronal cells in mice. The dehydration and paraffin embedding methods described above were used. Brain slices were placed horizontally with drops of Nissl staining solution (0.5 % toluidine blue) for 10 - 20 minutes before being washed and dehydrated. Finally, xylene was used to clean the slides before the cover was slipped. The number of neurons in the CA1 and CA3 regions were counted by using an ImageJ analysis system (version 1.43, National Institutes of Health, USA).

Golgi-Cox Staining

Golgi-Cox staining of experimental samples to clearly observe the dendritic spines on neurons (n=3 per group). Briefly, under deep anesthesia with 2 % pentobarbital sodium (50 mg/kg), mice were transcardially perfused with 0.9% saline. Then the whole brains of the mice were immersed in Golgi-Cox solution in the dark for 14 days. Every 48 hours, the staining solution was changed. After that, the distilled water were used to wash tissues (three times), immersed overnight in 80% glacial acetic acid, washed again in distilled water, then put in 30% sucrose. Finally, the dried tissue slides (100 μm) were treated with concentrated ammonia (15 mins), distilled water (washed 1 min), acidic firm film fixing solution (15 mins), distilled water (3 mins), dried, and sealed with glycerin gelatin. The Panoramic 250 (3D Histech) digital section scanner was used to capture images, which were then processed with ImageJ software for spine density analysis.

Western Blotting

We used cold PBS to wash tissues twice or three times with before being homogenized in 1% PMSF RIPA lysis buffer (Servicebio, Wuhan, China). Total proteins were quantified using the BCA protein assay kit (Servicebio, Wuhan, China). After activation with methanol, samples (n=3) were separated by SDS PAGE and electro-transferred onto PVDF (0.45 um) membranes. Following that, the membranes were blocked for 1 hour at room temperature. Then, the membranes were incubated with the following primary antibodies overnight at 4 °C: PSD-95 (1:1000, P78352, Cell Signaling Technology, USA), SYN (1:1000, P08247, Cell Signaling Technology, USA), Sema3A (1:1000, ab199475, Abcam, UK), CRMP2(1:10000, ab129082, Abcam, UK), phosphor-Thr514-CRMP2 (1:1000, ab62478, Abcam, UK), Acet-α-Tubulin (1:1000, sc-23950, Santa Cruz Biotechnology, USA) and β-Actin (1:1000, GB11001, Servicebio, China). TBST was applied to wash the membranes five times, each for five minutes. After that, the membranes were incubated in blocking buffer with HRP conjugated Goat Anti-Rabbit IgG (1:3000, GB23303, Servicebio, China) for 1 h before being washed with TBST. For capturing images, the enhanced chemical lighting (ECL) method was applied. The optical density value of the target band was quantified with the ImageJ (n=3 per group).

Immunohistochemistry

After deparaffinizing and rehydrating the paraffin section, the tissue sections were put in a microwave oven which contain with citric acid antigen repair buffer (pH 6.0) for antigen repair. Subsequently, the tissues were incubated in 3 % hydrogen peroxide to block endogenous peroxidase and sealed with 3%

BSA. Then incubated with primary antibodies: Sema3A (1:8000, ab199475, Abcam, UK), PlexinA1(1:200, orb6757, Biorbyt, UK), CRMP2(1:1000, ab129082, Abcam, UK) at 4 °C overnight. The biotinylated HRP goat anti-rabbit (IgG) secondary antibody was added and incubated for 50 minutes at room temperature following washed with PBS. The immune reaction was visualized by incubating with DAB for 5 min. The Image-Pro-Plus software (Media Cybernetics, Silver Spring, USA) was used to calculate the average optical density value.

ATP Synthase Activity

The Mitochondrial Complex V Kit (AIDISHENG, Jiangsu, China) was applied for measuring ATP synthase activity after isolating mitochondria from the hippocampus following the manufacturer's instructions. ATP synthesis results in production of ATP which is accompanied with the reduction of NAD+ to NADH that is then monitored as an increase in absorbance at 340nm. The OD340 was continuously monitored using a Spectra Max M5 microplate reader (Molecular Devices, USA).

Statistical Analysis

All of the data were presented as the mean ± SD. All statistical analyses were carried out using SPSS 26.0 software (IBM Corporation, Armonk, NY, USA). All charts are created by GraphPad Prism 8.4.3 software (GraphPad Software, La Jolla, CA). The difference between two groups was compared using the unpaired t-test. Multigroup comparisons were carried out using one-way ANOVA followed by Tukey post-hoc test. Values of $P < 0.05$ were considered statistically significant. All experiments were repeated at least 3 times.

Results

OW Suppresses Spatial Recognition and Learning Memory Dysfunction in Chronic REM Sleep Deprivation Mice

To verify chronic REM sleep deprivation induced spatial recognition and learning memory dysfunction, mice were evaluated in the Morris water maze and NOR experiment. As shown in Fig. 2A, C and D, no differences were found in escape latency, the number of crossing platforms, swimming speed before chronic REM SD. During the second test period, we reversed the platform. In the navigation test, SD groups spent more time in locating the platform (Fig. 2B) than those in the Ctrl group on the fourth day ($^{*}P < 0.05$). The latency was significantly lower in the LD and HD groups compared with the SD group ($^{\#}P < 0.05$). Meanwhile, the spatial probe test shown the number of crossing platforms were reduced in the SD group (Fig. 2C). Administration of both LD and HD OW reduced the decline in the SD induced platform crossing number.

After SD for 21 consecutive days, we started the NOR experiment 24 hours later. The desire to explore new objects was significantly decreased in the SD groups compared to the Ctrl group ($^{*}P < 0.05$, Fig. 3).

By contrast, the exploration index for new objects was significantly higher in the LD and HD OW treatment groups ($^{\#}P < 0.05$).

Effect of OW in Locomotor Activities in Chronic REM Sleep Deprivation Mice

To evaluate SD induced anxiety behaviors, we performed an open field test. The total distance moved by the mice in each group was not significantly different ($P > 0.05$; Fig. 4A). Therefore, it is unlikely that exercise had an effect on the results. SD decreased the activity distance in the central area compared to the Ctrl group, while LD and HD OW treatment increased the activity distance in the central area ($P < 0.05$; Fig. 4B).

OW Ameliorates Neural Damage in the Hippocampus of Chronic REM Sleep Deprivation Mice

The results of Nissl staining (Fig. 5A) demonstrated that the Nissl vesicles in the neuronal cells were clearly stained and numerous. Statistically, compared to the Ctrl group, chronic REM sleep deprivation reduced the number of hippocampal neurons significantly ($^{*}P < 0.05$; Fig. 5B). Compared with the SD group, the density of normal neurons in the LD and HD groups were clearly increased ($^{\#}P < 0.05$).

OW Alleviated Synaptic Plasticity Impairment in Chronic REM Sleep Deprivation Mice

To investigate whether OW administration interfered with the structural plasticity of dendritic spines, we selected Golgi-Cox staining to analyze the density of dendritic spines in CA1 and CA3 region (Fig. 6A). Consistent with our hypothesis, SD significantly decreased the density of neuronal dendritic spines compared to the Ctrl group in the CA1 and CA3 region ($^{*}P < 0.05$; Fig. 6B). Spine density in the LD and HD group were higher than that in the SD group ($^{\#}P < 0.05$). Furthermore, western blot analyses showed a reduction of SYN ($^{*}P < 0.05$, Fig 6C) and PSD-95 ($^{*}P < 0.05$, Fig 6D) in the hippocampus of the SD group. Treatment with OW significantly attenuated the decline of both SYN and PSD-95 proteins in the hippocampus compared to the SD group ($^{\#}P < 0.05$).

OW Exerts Neuroprotective Effects via Inhibition of the Sema3A Expression to Decreases CRMP2 Phosphorylation

Immunohistochemistry was used to investigate OW's neuroprotective effects by observing changes in Sema3A, PlexinA1, and CRMP2 in the CA1 and CA3 regions (Fig 7A, B). Chronic REM sleep deprivation increased the expression of Sema3A compared with the Ctrl group. Administration of OW decreased the levels of Sema3A in the CA1 and CA3 hippocampal region compared with the SD group ($^{\#}P < 0.05$; Fig. 7C, D). Similar findings were seen with PlexinA1. The expression of CRMP2 in the SD group were decreased compared to the Ctrl group, while the expression levels of CRMP2 in the LD and HD groups rebounded compared to the SD group.

We also employed the Western blot method to measure the expression of the proteins Sema3A, CRMP2, and p-CRMP2. As expected, the levels of Sema3A in SD mice were enhanced compared with those of the

Ctrl group; however, they were inhibited in the OW groups ($^{\#}P < 0.05$; Fig 7E). The ratio of the average optical density of P-CRMP2 to CRMP2 was used as an object of analysis, it was discovered that this ratio increased in the SD group ($^{*}P < 0.05$; Fig 7F), while the same ratio decreased significantly in both LD and HD groups compared with the SD group ($^{\#}P < 0.05$, $^{\#}P < 0.05$).

OW Rescues Microtubule Instability Induced Mitochondrial Dysfunction in Chronic REM Sleep Deprivation Mice

We assessed OW effect on microtubule stability through western blot and measurement of the levels of α-tubulin acetylation. SD significantly decreased the expression of α-tubulin acetylation in the hippocampus compared with the Ctrl group ($^{*}P < 0.05$; Fig 8A). Interestingly, HD OW treatment increased α-tubulin acetylation levels significantly more than the SD group ($^{\#}P < 0.05$; Fig 8B), while LD OW treatment increased α-tubulin acetylation levels. We subsequently analyzed mitochondrial function in the hippocampus by evaluating the ATP synthase activity and mitochondrial membrane potential. The SD group showed a significantly lower ATP synthase activity than the Ctrl group ($^{*}P < 0.05$; Fig 8B). ATPase activity of the OW groups demonstrated an tendency to increase as compared to the SD group ($^{\#}P < 0.05$; Fig 8B).

Discussion

Our present data illustrate that OW is associated with a neuroprotective effect on chronic REM SD induced cognitive deficit and hippocampal neuronal damage in mice. Further, OW was also associated with a significant reduction in SD-induced the synaptic plasticity impairment in the hippocampus as well as the reduced expression of SYN and PSD-95 proteins. Moreover, we observed that OW effectively decreased Sema3A and the ratio of P-CRMP2 to CRMP2 expression compared with the SD group. OW treatment significantly increased α-tubulin acetylation levels and upregulated the activity of ATPase.

Sleep is divided into REM sleep and non-REM sleep. REM sleep can result in a loss of muscle tone (atonia) and twitching [49, 50]. The present study used a modified multi-platform water environment method to induce REM sleep deprivation [51, 52]. Previous studies have shown that chronically restricted and disrupted sleep is a risk factor in a variety of disorders in brain function [53]. Sleep deficiency and disturbance may increase the incidence of dementia [9], result in mood deterioration [53, 54] and cognitive deficits [53, 55, 56]. In this study, we used the MWM experiment to investigate the effects of spatial learning process and memory performance [57] and the NOR test to evaluate recognition memory [48]. The results of the MWM and NOR test supported previous studies in which sleep deprivation not only led to memory defects, but also caused significant impairment of spatial memory function (Fig. 2,3). OW administration was associated with a reversal in SD-induced cognitive dysfunction that suggests a protective effect of OW on cognitive function. Despite the negative impacts on cognition, previous researches have also indicated that SD is generally linked to anxious behavior [58, 59]. Therefore, we carried out the OFT test to evaluate locomotor activity and anxiety-like behavior in mice [47]. OW

ameliorated the increment of spontaneous activity compare with the SD group and confirmed that the administration of different dose of OW exerted anti-anxiety effects (Fig. 4).

The hippocampus play an essential role in cognitive functions [60]. In agreement with the current results [61], mice in the SD group displayed neural damage in the hippocampus, as demonstrated by the reduced number of neurons (Fig. 5). OW in different dosages was shown to attenuate the impairment of neurons in SD mice. In addition, SD may also impair hippocampal function by affecting synaptic structural plasticity [62]. Changes in spine density and resulting alterations in synaptic efficacy are crucial for learning and memory [55, 58, 62]. As was shown by Golgi staining, SD significantly reduced branch arborization and dendritic organization, and these properties were reversed by OW treatment (Fig. 6).

Synaptic functional proteins SYN and PSD-95 are associated with synaptic plasticity and cognitive function [63]. In this study, presynaptic SYN and postsynaptic PSD-95 levels increased significantly after the administration of OW to the SD group mice (Fig. 6). The impairment of synaptic plasticity may be associated with SD-induced decreases in spine density and synaptic protein levels and the deterioration in learning and memory observed in behavioral tests. This also suggests synaptic plasticity impairments may be responsible for cognitive dysfunction caused by SD. Interestingly, in our study, treatment with OW increased dendritic spine density and global dendritic intersections in the hippocampus and prevented the SD-induced decrease of SYN and PSD-95.

Semaphorins are a type of membrane bound proteins which plays an essential role in neurites growth, guidance, and the formation of new synaptic connections during embryonic development [34]. Several studies have reported that Sema3A is one of the best characterized axon guidance molecules, involved in the regulation of axon repulsion, dendritic branching and synaptic development via binding protein neuropilin-1 (NRP1) and the signal transducing protein PlexinA complex [35, 64]. However, some studies have also reported effects of Sema3A different from the previously described functions. Increased expression of Sema3A, for example, is observed in many CNS injury models [65, 66], upregulated Sema3A induced different classes of neuronal cells death [67], in addition, Sema3A negatively regulated axonal regeneration[68, 69]. Sema3A signaling is linked to neurodegenerative diseases [67, 70, 71] such as Amyotrophic Lateral Sclerosis (ALS), Alzheimer's disease and multiple sclerosis. Sema3A interacts with PlexinA1 and induces phosphorylation of CRMP [72]. CRMPs were discovered to be the mediating proteins for the repulsive axon guidance molecule Sema3A. Kawashima T et al. have found that Sema3A regulated dendritic development of cortical pyramidal by Tyr504 phosphorylation of CRMP1 neurons [73]. Activating the Sema3A signaling system also induces CRMP2 phosphorylation. The increment in phosphorylation of CRMP2 at Thr514 occurs concomitantly with decreases in the binding activity of CRMP2 to tubulin [74]. Downregulation of CRMP2 expression and upregulation of phosphorylated CRMP2 expression in the hippocampus significantly decreases the interaction between CRMP2 and α-tubulin and decreases microtubular dynamics [38].

Acetylation of α-tubulin is a marker for stable microtubules and is associated with several neurological disorders [75]. In addition, hyperphosphorylated CRMP2 increases mitochondrial fragmentation and

suppresses mitochondrial motility in neurons [37]. Microtubular stabilization plays a key role in providing tracks for mitochondrial transport and tethering in addition to the stabilization of dendritic mitochondrial compartments [76–78]. Mitochondrial compartments can provide local energy for synaptic translation and newly synthesized proteins. [76].

Memory formation is influenced by the modification of the local synaptic proteome and our previous studies [79] also have shown that cognitive function is related to mitochondria which can regulate glial cell aggregation. ATP synthase of the F-type (FOF1) is found in the mitochondrial inner membrane in eukaryotes. The main function of which is control of ATP synthesis and transmembrane potential [80]. We noted significantly increased Sema3A and PlexinA1 expression was associated with SD, as well as elevations in the P-CRMP2/CRMP2 ratio (Fig. 7). Furthermore, we found SD induced mitochondrial dysfunction, that acted on the mitochondria transport-mediated microtubular instability and lowered ATP synthase activity compared to the C group: administration of OW blocked these SD induced changes (Fig. 8). These results suggests that cognitive performance is associated with the overexpression of Sema3A which induces Thr514 phosphorylation of CRMP2 and subsequent microtubular instability and may be associated with mitochondrial abnormalities and altered ATPase activity. Interestingly, each Sema3 molecule differentially and precisely regulates various cognitive function through its specific receptor complex. A recent study reported that Sema3A-plexinA4 signaling mediated fear learning and memory through decreased phosphorylation of serine 522 of CRMP2 [81]. Future studies are needed to delineate how Sema3A influences CRMP2-mediated cognitive dysfunction.

The most commonly used drugs for sleep disorders are replete with serious side effects, including cognitive impairment, tolerance, abuse, and dependence [82]. Ozone has been used as a powerful antioxidant in medicine and appears to act through inhibition of the mitogen-activated protein kinase phosphatase (MAPK) 1, Nrf2, and rapamycin (mTOR) signaling pathway in addition to overproduction of nitric oxide (NO) [22]. In a recent study Lin et al [27] reported a neuroprotective effect of ozone. The effect alleviated the behavioral and pathological deterioration associated with SD presumably via a reduction in the expression of APP and activation of astrocytes. Administration of OW is a potentially safe and simple alternative to anti-tumor treatments [83]. While emerging studies have pointed out various uses for ozone, more research is needed to determine the mechanism of ozone's effect on memory.

There are some limitations in our research. Firstly, we did not evaluate the stress-oxidant biomarkers and ATP content in the hippocampus and we need to explore changes in mitochondrial structure and membrane potentials to demonstrate that OW can improve sleep deprivation induced learning and memory impairment by improving mitochondrial dysfunction. Secondly, we used microtubular dynamic related proteins to examine the importance of microtubule dynamics in mitochondrial function and this may be simplistic. Thirdly, our experiments did not involve inhibitors, nor did we use a positive drug control group. Although our results suggest that SD impaired hippocampal function is associated with an alteration in synaptic plasticity at the structural level, further study is needed to detect functional plasticity such as LTP to delineate changes in synaptic plasticity.

Conclusions

The present study has provided substantial evidence suggesting that OW significantly attenuates the SD induced deficits in cognition and improves synaptic structural plasticity through modulation of Sema3A signaling. OW therapy could be a useful clinical alternative medical therapy to assist in obviating the adverse effects of insomnia.

Abbreviations

SD	Sleep Deprivation
REM	Rapid eye movement
Sema3A	Semaphorin 3A
OW	Ozonated water
Ctrl	Control group
LD	Low dose
HD	High dose
CRMP2	Collapsin response mediator protein-2
MWM	Morris water maze
NOR	Novel object recognition
OFT	Open field test
SYN	Synaptophysin
PSD-95	Postsynaptic density-95
ATPase	ATP Synthase

Declarations

Ethics approval and consent to participate

All methods were performed in accordance with the relevant guidelines and regulations. Approval was granted by The Animal Care and Use Committee of Aviation General Hospital of China Medical University Laboratory (HK2022-03). The experimental design was taken seriously in reducing animal numbers and suffering.

Consent for publication

Not applicable.

Availability of data and materials

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

Material preparation, data collection and analysis were performed by JRT, LNS and YL. JRT was a major contributor in writing the manuscript. JW revised the manuscript. JXA supervised the study. All authors read and approved the final manuscript.

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Figures

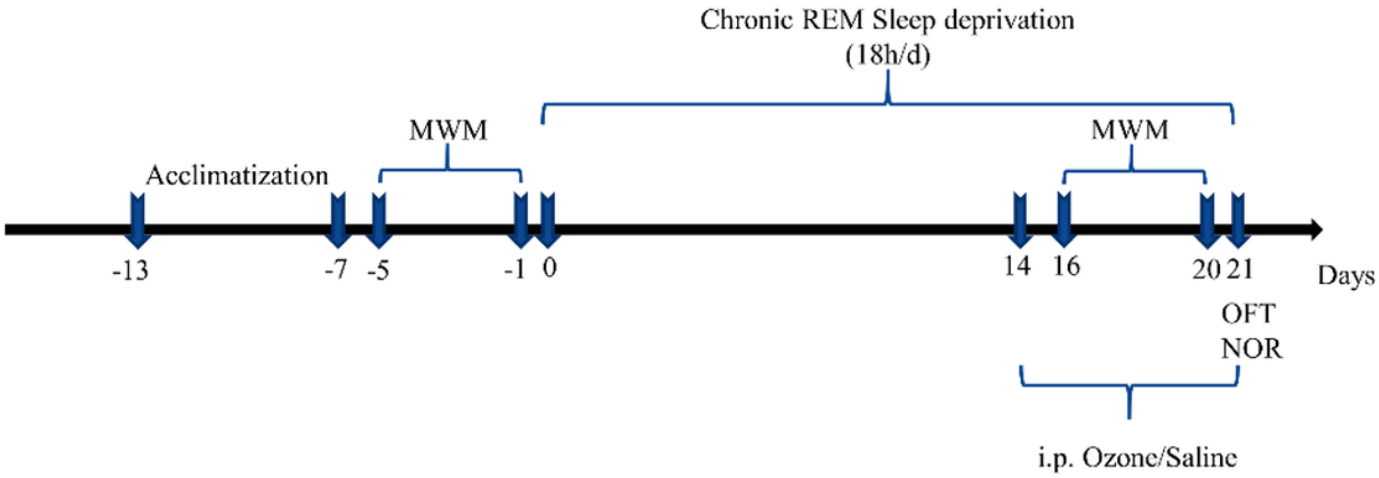

Figure 1

Experimental design

Mice were randomly allocated to the control group (n = 15), the SD group (n = 15), the low dose group (n = 15), and the high dose group (n = 15). Chronic REM SD paradigm was conducted by the modified multiple platform method. Upon the termination of 21 days SD, behavioral tests, histological, or biochemical assessment were performed on all four groups of mice.

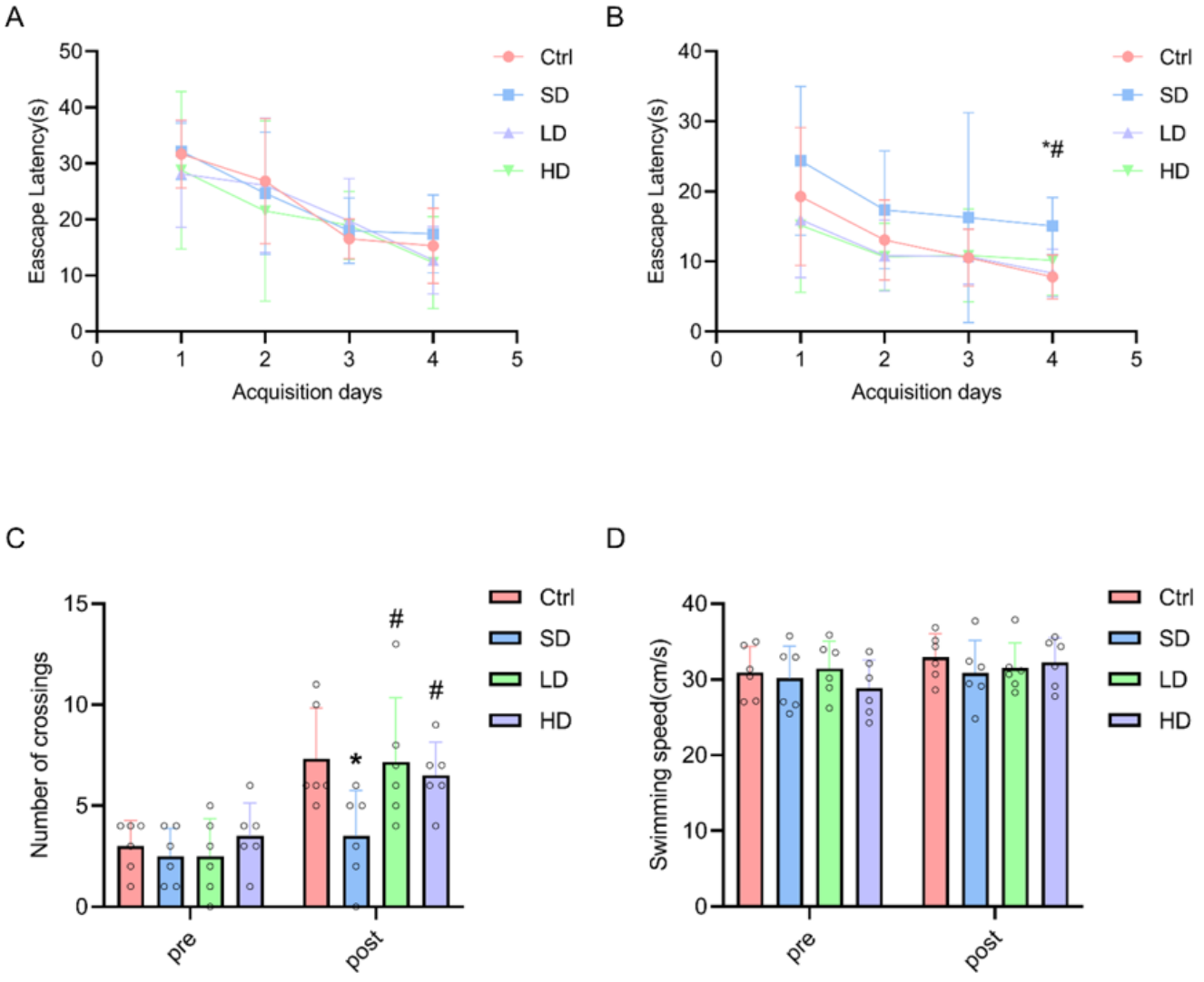

Figure 2

OW prevented sleep deprivation-induced spatial memory deficit in mice tested in Morris Water Maze (MWM).

The Escape latency (A) (B) over 4 consecutive days in the MWM test. There were not significantly different in escape latency (A), number of platform crossings (C) and swimming speed (D) of MWM before sleep deprivation. Mice in the SD group presented fewer platform crossings (C) than the Ctrl group. However, the SD-induced spatial memory deficit was reversed by OW administration (B, C). n = 6 in each group. *$P < 0.05$ vs. Ctrl group; #$P < 0.05$ vs. SD group.

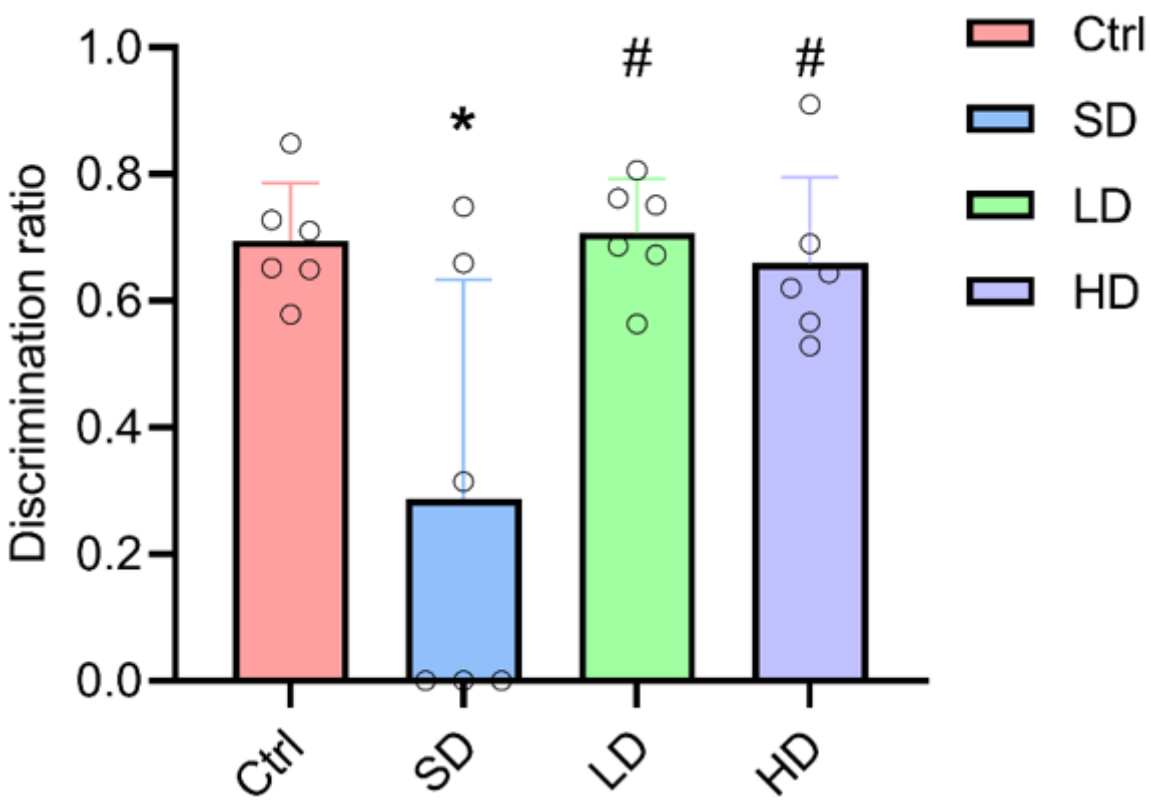

Figure 3

Object recognition memory was assessed in mice using the novel object recognition test (NOR).

Recognition memory was impaired in the SD but rescued in the OW treatment group. n = 6 in each group. *$P < 0.05$ vs. Ctrl group; $^{\#}P < 0.05$ vs. SD group.

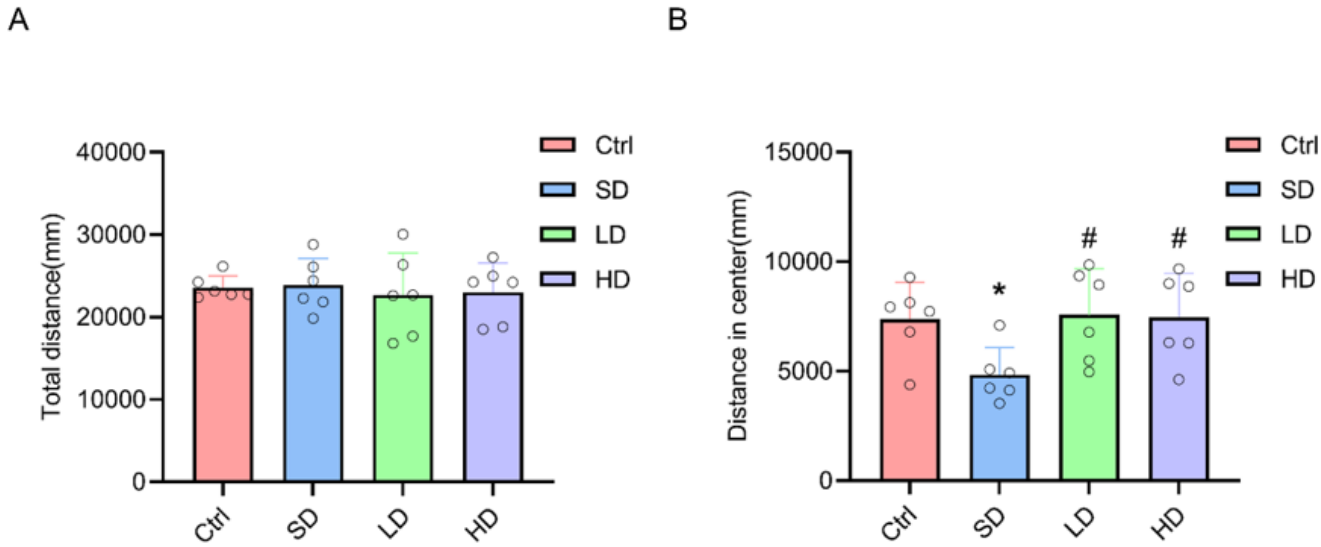

Figure 4

Locomotor activity and anxiety state of the mice were evaluated using the open field test (OFT)

(A) Total distance. (B) Distance in center. n = 6 in each group. *P < 0.05 vs. Ctrl group; #P < 0.05 vs. SD group.

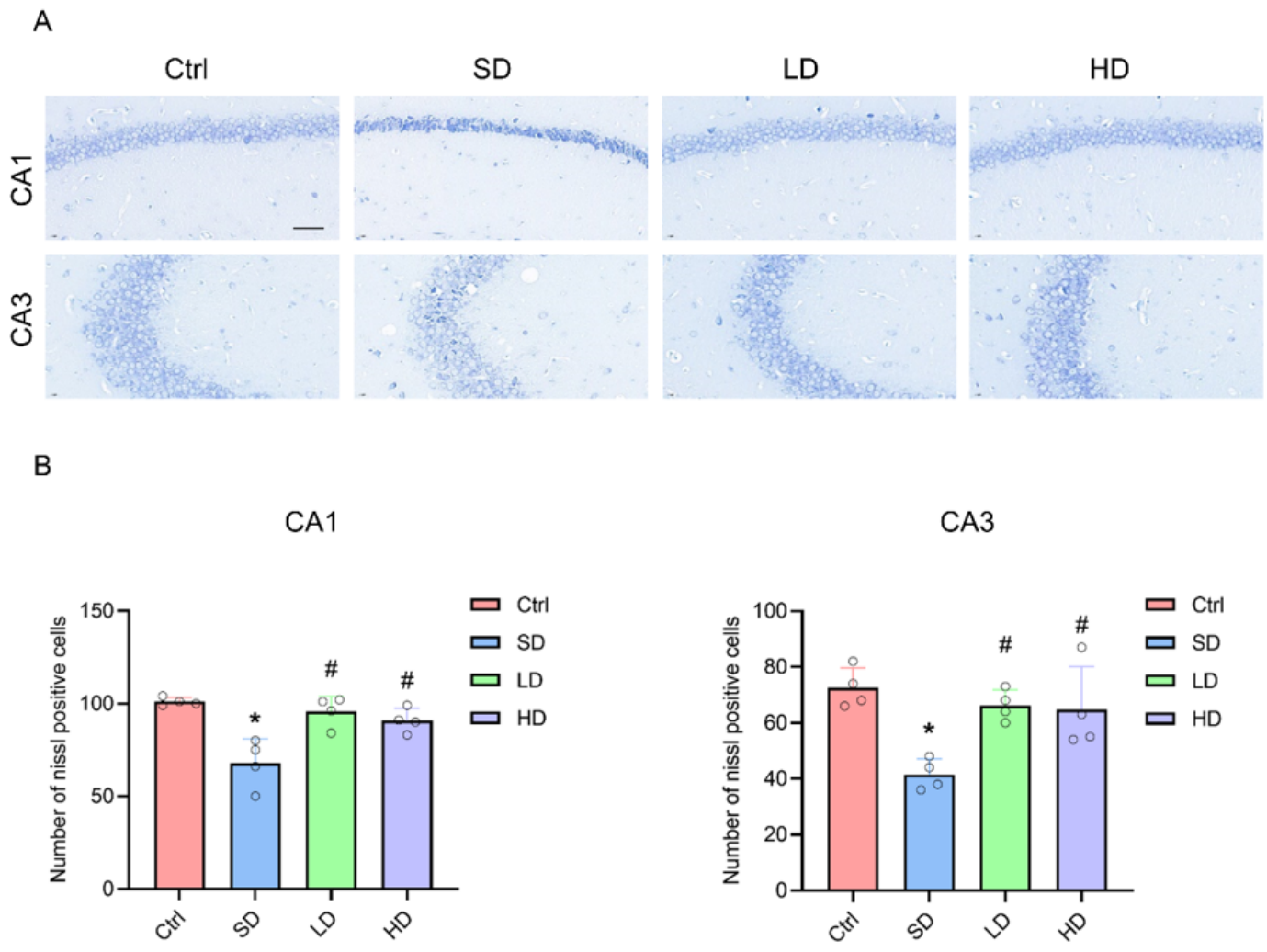

Figure 5

The effects of OW on the hippocampal pathological changes in SD mice.

(A) Representative microphotographs of the Nissl's stained CA1 and CA3 area of hippocampal sections from the four groups at ×40 magnification. Nissl bodies were dyed deep blue, and the cell nuclei were lightly stained. n = 4. (B) Quantification of the number of surviving neurons in the CA1 and CA3 area of hippocampus in all groups. n = 4 per group. Scale bar = 20μm. *P < 0.05 vs. Ctrl group; #P < 0.05 vs. SD group.

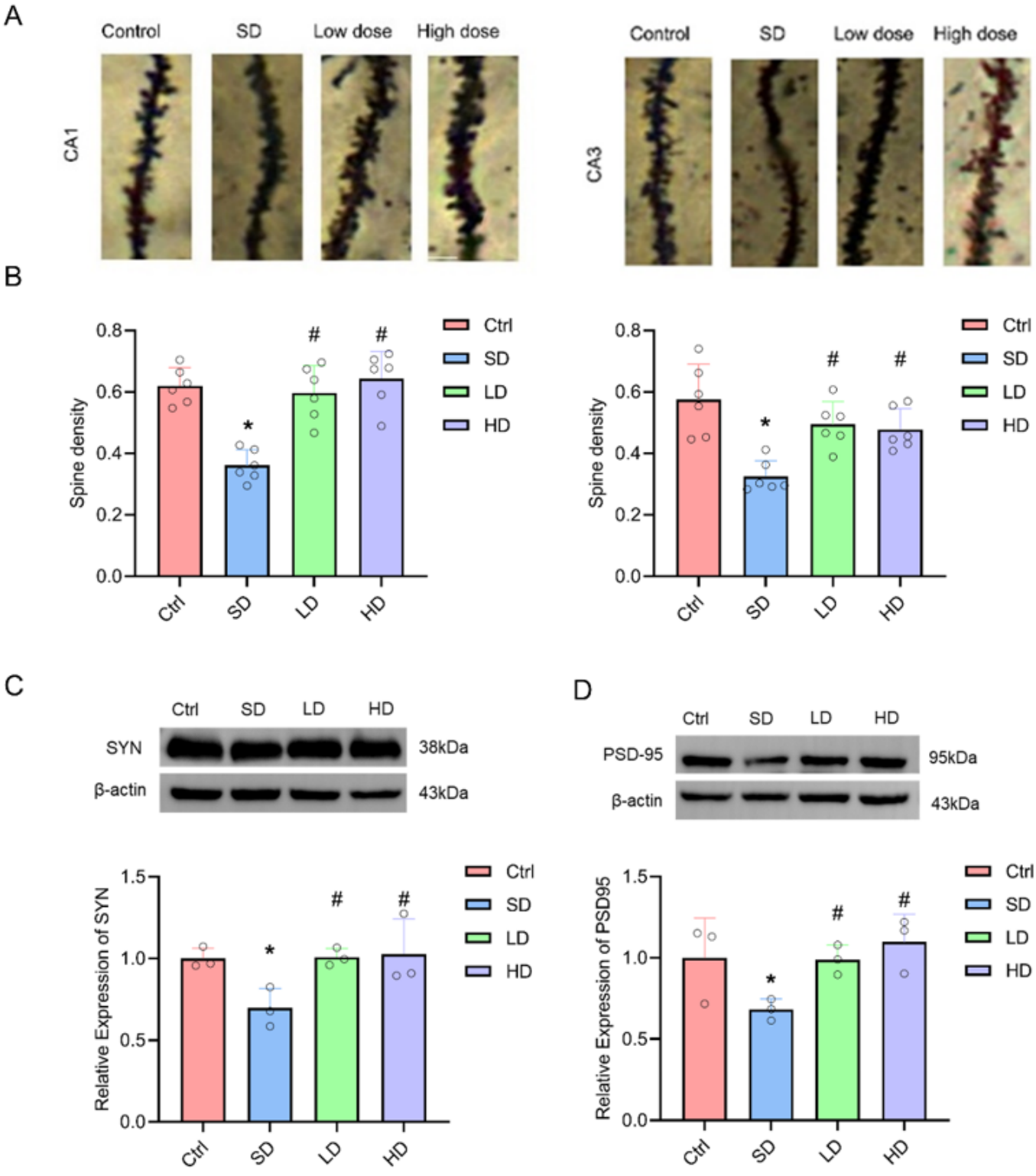

Figure 6

The implications of OW on synaptic plasticity impairment in SD mice

(A) Representative pictures of each group of dendritic spines at ×100 magnification. Scale bar = 5 μm. (B) The density of dendritic spines per 10 μm. (C) Protein levels of SYN were analyzed by western blot assay. (D) Protein levels of PSD-95 were analyzed by western blot assay. The western blot images in (C) and (D)

were cropped. n = 3 per group. *$P < 0.05$ vs. Ctrl group; $^{\#}P < 0.05$ vs. SD group. Full-length blots are presented in Figure S1.

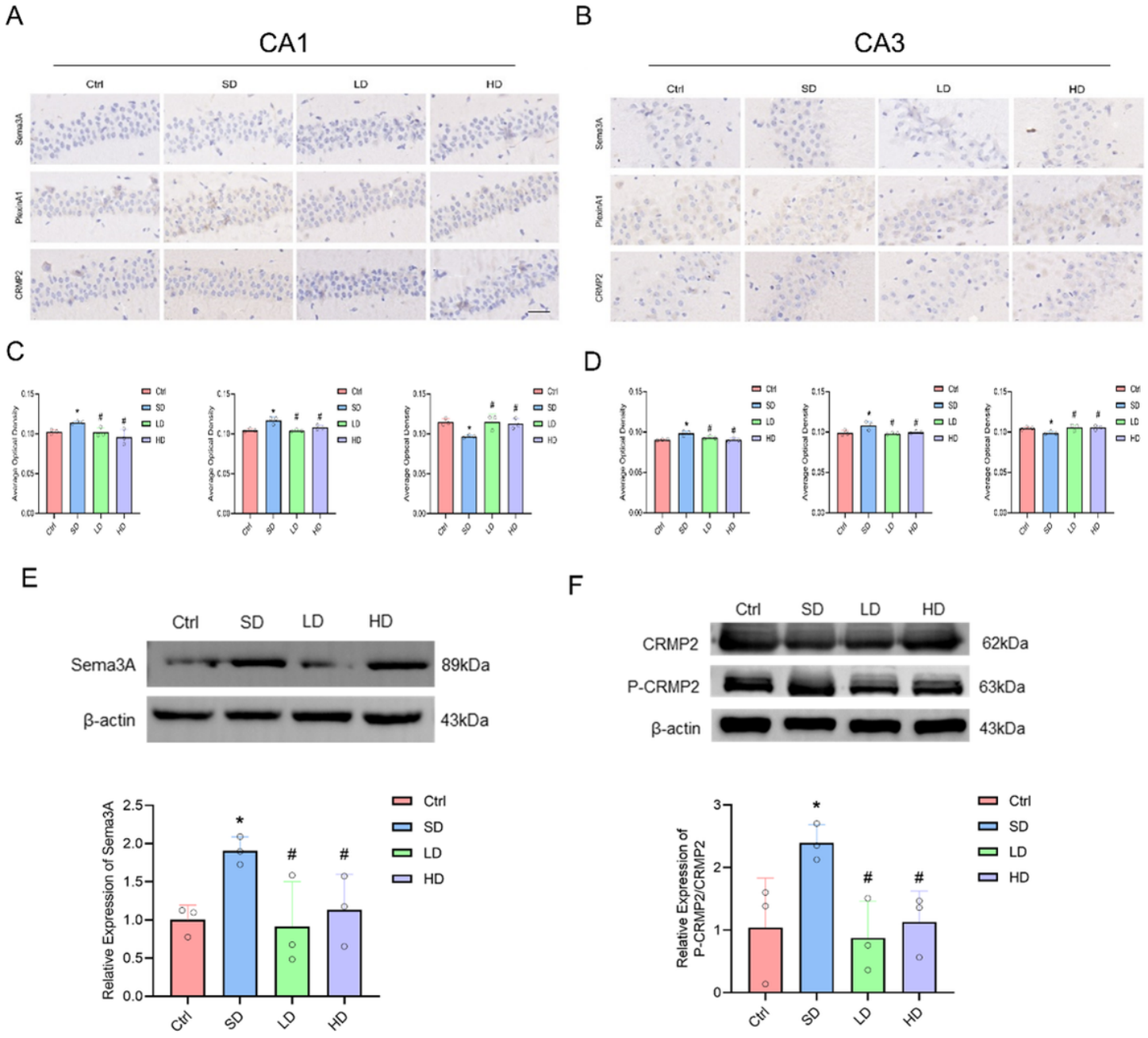

Figure 7

Protein expression in mice CA1 and CA3 area of hippocampus

(A) Representative microphotographs of Sema3A, PlexinA1 and CRMP2 in CA1 area of hippocampal sections at ×100 magnification. Scale bar = 5 μm. (B) Representative microphotographs of Sema3A,

PlexinA1 and CRMP2 in CA3 area of hippocampal sections at ×100 magnification. Scale bar = 5 μm. (C) Sema3A, PlexinA1 and CRMP2 expression in the CA1 hippocampus of mice. (D) Sema3A, PlexinA1 and CRMP2 expression in the CA3 hippocampus of mice. (E) Protein levels of Sema3A were analyzed by western blot assay. n = 3 per group. (F) Protein levels of P-CRMP2/CRMP2 were analyzed by western blot assay. n = 3 per group. The western blot images in (E) and (F) were cropped. *P < 0.05 vs. control group; $^{\#}P$ < 0.05 vs. SD group. Full-length blots are presented in Figure S2.

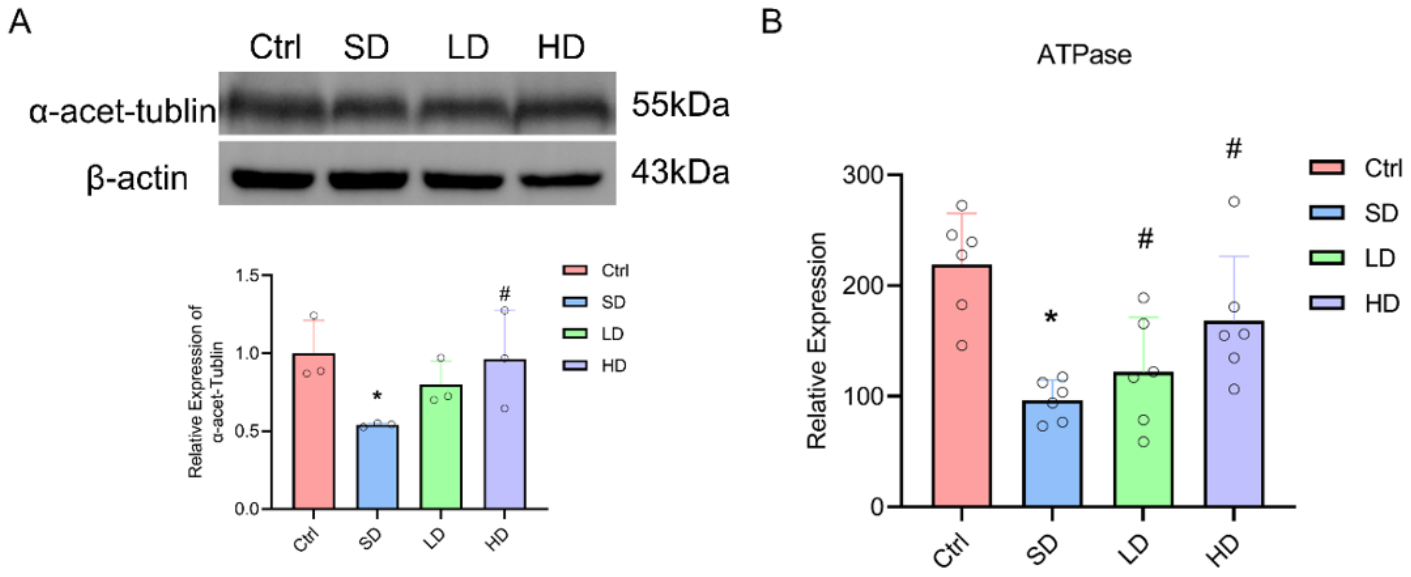

Figure 8

The effects of ozone on microtubule instability induced mitochondrial dysfunction in SD mice

(A) Protein levels of α-acet-Tubulin were analyzed by western blot assay. The western blot images was cropped. n = 3 per group. (B) The activity of ATP synthase. The Y-axis represents the unit of enzyme activity, defined as 1 nmol of NADPH per gram of tissue per minute. n = 6 per group. *P < 0.05 vs. control group; $^{\#}P$ < 0.05 vs. SD group. 'Full-length blots are presented in Figure S3.

Supplementary Files

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