Research paper

Dietary methionine restriction attenuates cognitive dysfunction associated with sleep deprivation by promoting production of hydrogen sulfide, upregulating expression of BDNF and suppressing neuro-inflammation in rats

Keywords
cognitive dysfunction, methionine, H2S, sleep deprivation, neuroinflammation

Abstract

Introduction
In this study, we aimed to study the potential role of H2S and the application of dietary methionine restriction (DMR) in the treatment of cognitive dysfunction in sleep deprived rat models.

Material and methods
The rat groups were established as a Control group, a sleep deprivation (SD) group, a SD + NaSH group and a SD + DMR group. Behavioral data was studied via Morris water maze test. TUNEL assay, real-time PCR, immunohistochemistry (IHC) and enzyme-linked immunosorbent assay (ELISA) were performed to study the differences of cognitive impairment related genes and proteins in different rat groups.

Results
Path length and escape latency were higher in the sleep deprived rats compared with the control rats, which were subsequently recovered by the treatment of NaSH or DMR. Also, DMR most significantly recovered the cognitive impairment and neuron status of sleep deprived rats compared with the administration of NaSH. The elevated level of hippocampal Iba1 and H2S production was recovered by NaSH and DMR, and the expressions of hippocampal phenotypic-related genes including NOS, CD68, CD32 and CD206 mRNA also showed similar trend as hippocampal Iba1. Meanwhile, the increased relative expression of IL-6 and IL-4 were recovered by DMR in sleep deprived rats, while the reduced level of hippocampal brain-derived neurotrophic factor (BDNF) and cystathionine γ-lyase (CSE) were elevated by the treatment of NaSH and MDR, with MDR exhibiting the most significant effect.

Conclusions
The application of DMR could attenuate cognitive dysfunction in sleep deprivation rats by upregulating the production of H2S and BDNF expression and alleviating neuro-inflammation responses.

Dietary methionine restriction attenuates cognitive dysfunction associated with sleep deprivation by promoting production of hydrogen sulfide, upregulating expression of BDNF and suppressing neuro-inflammation in rats

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Abstract

In this study, we aimed to study the potential role of H_2S and the application of dietary methionine restriction (DMR) in the treatment of cognitive dysfunction in ~~sleep deprived~~sleep-deprived rat models. The rat groups were established as a Control~~Sham~~ group, a sleep deprivation (SD) group, a SD + NaSH group and a SD + DMR group. Behavioral data was studied via Morris water maze test. TUNEL assay, real-time PCR, immunohistochemistry (IHC) and enzyme-linked immunosorbent assay (ELISA) were performed to study the differences of cognitive impairment related genes and proteins in different rat groups. Path length and escape latency were higher in the ~~sleep deprived~~sleep-deprived rats compared with the ~~sham-operated~~control rats, which were subsequently recovered by the treatment of NaSH or DMR. Also, DMR most significantly recovered the cognitive impairment and neuron status of ~~sleep deprived~~sleep-deprived rats compared with the administration of NaSH. The elevated level of hippocampal Iba1 and H_2S production was recovered by NaSH and DMR, and the

expressions of hippocampal phenotypic-related genes including NOS, CD68, CD32 and CD206 mRNA also showed similar trend as hippocampal Iba1. Meanwhile, the increased relative expression of IL-6 and IL-4 were recovered by DMR in ~~sleep deprived~~sleep-deprived rats, while the reduced level of hippocampal brain-derived neurotrophic factor (BDNF) and cystathionine γ-lyase (CSE) were elevated by the treatment of NaSH and MDR, with MDR exhibiting the most significant effect. The application of DMR could attenuate cognitive dysfunction in sleep deprivation rats by upregulating the production of H_2S and BDNF expression and alleviating neuro-inflammation responses.

Running title: Dietary methionine restriction attenuates cognitive dysfunction

Keywords: methionine, sleep deprivation, cognitive dysfunction, H_2S, neuro~~n~~-inflammation

Introduction

Sleep is a fundamental physiological process crucial to one's health, and sleep disorder has been listed as one of the important health-related issues worldwide. As previously reported, sleep deprivation ~~not only~~ impairs vigilant attention (1), increases the formation of false memory (2) and weakens the cognitive performance (3)~~, but also damages the immune system and even induce diseases including hypertension and diabetes (4)~~. Moreover, ~~compared with other patient groups,~~ sleep deprivation raised more concerns in menopausal women (4, 5). Therefore, ~~to seek for other~~ treatment methods which ~~may~~ presents few side effects for the management of sleep disturbances are~~is~~ necessary, especially for menopausal women.

Methionine functions as a precursor of homocysteine, a non-protein-forming sulfur amino acid (6). Homocysteine was found to be associated with cognitive impairment as the increased total plasma homocysteine level has been found to induce cognitive decline and white matter damage (7). And the deprivation of methionine not only

induce rapid reduction of body weight by influencing the metabolism mechanisms of obese adults (8, 9), but also exert therapeutic effects in the management of diseases such as glioma (10), hepatosteatosis (11), and age-associated cognitive decline (12-14). For example, in their study which explore the effect of dietary methionine restriction (DMR) on age-related cognitive decline, Ren et al. found that DMR elevated expression of fibroblast growth factor 21 (FGF21), a key mediator for neuro functions and hippocampal mitochondrial biogenesis (12). And by regulating expression of FGF21, DMR also modulates energy balance and adipose tissue remodeling (13). Therefore, DMR is best known as dietary treatment methods which could reduce the incidence age-related disease.

Hydrogen sulfide (H_2S) has been widely acknowledged as a key signaling factor which exhibit positive effects in the management of sleep deprivation. For example, H_2S was found to antagonize hippocampal damage in sleep-deprived rat models (15), and further alleviate cognitive dysfunction in sleep-deprived rat models by suppressing excessive hippocampal autophagy (16). Moreover, H_2S could also prevent the depression and anxiety symptoms caused by sleep deprivation by modulating the signaling pathway of silence information regulating factor-1 (Sirt-1), which plays a key role in neuroprotection (17, 18). Also, multiple investigations demonstrated the anti-oxidative effect, anti-ER stress, and antiapoptotic properties of H_2S in central nervous system (19, 20), the performance of which is strongly associated with the adverse effects caused by sleep deprivation (21).

Among previous publications which reported the effect of H_2S in brain functions, a report by Wei et al. suggested that in SD rats treated with H_2S donor NaHS, the endogenous production of H_2S as well as the expression of brain-derived neurotrophic factor (BDNF) was evidently increased, leading to the attenuated endoplasmic reticulum stress and

neuronal apoptosis in hippocampus (22). Meanwhile, it is also reported that paradoxical sleep deprivation could down-regulate the level of hippocampal BDNF (23, 24). And BDNF has been proved to play key roles in cognition-related functions including the efficacy of exercise on synaptic plasticity n spatial learning (25) and synaptic plasticity (26). In this study, we aimed to study the role of H_2S and the application of ~~dietary methionine restriction~~DMR in the treatment of cognitive dysfunction in ~~sleep deprived~~sleep-deprived rat models.

Methods

Animals

Thirty-two~~32~~ male Sprague Dawley~~SD~~ rats (weight between 220 g to 255 g; 6 weeks old) were enrolled in this study. The rats were divided into four groups with 8 random rats in each group. All animals were housed under a 12-h light-dark cycle with free access to food and water. The housing facility temperature was maintained between 22 °C to 25 °C, with the humidity ranging between 45 % to 60%. All procedures were carried out according to the instructions in the National Research Council's Guide for the Care and Use of Laboratory Animals and the institutional Ethics Committee approved the study (ID: H-HNNHY-190813X01).

Experimental groups

The animal groups were established as a Control~~SHAM~~ group, a sleep deprivation (SD) group, a SD+Sodium hydrosulfide (NaSH) group, a SD+DMR group. The rats assigned to the ~~SHAM~~Control group were kept in the home cages, and the rats subjected to establish SD models were exercised in the water pool designed for the multiple platforms method (MPM) (27). There were 10 columns located 2 cm above the water surface in the pool, and each column was 7 cm in diameter. These columns enabled the rats to move between different platforms. Moreover, once the rapid eye movement sleep (REM) occurs in a rat, the lost muscle tone would result in contact with water, thus achieving

the establishment of SD rats. For rats in the SD+NaSH group, the SD rats were injected with 100 μmol/mL NaHS resolved in PBS. For rats in the SD+DMR group, the rats were fed with diets containing a restricted dosage of 0.15% D,L-methionine for 72 hours (28).

Morris water maze (MWM) test

MWM test was performed with the rat groups to evaluate the effect of DMR on rat spatial learning and memory according to previously published methods (29, 30). The MWM test equipment is a ring-shaped pool divided into for quadrants, with 155 cm in diameter and 70cm in height. The pool was filled with ~~hygienic~~ water with 40 cm in depth. The temperature of water pool is maintained at 23–25°C. Before the grouping of experiment animals, all rats were trained to find an escape platform with 10 cm in diameter placed above the water surface under the guidance of visual cues for at least 3 hours. These visual cues during the training contributed to the spatial learning of rats. Subsequently, after 30 mins of animal group establishment, the behavioral test was carried out by releasing the rats into the pool for the search of unmarked hidden platform with submerged 1 cm below the water surface in the target quadrant. Each experiment phase lasts for 60 seconds for 4 phases for each quadrant. The interval between each phase is 60 seconds. And the WMW test was repeated in 3 quadrants. The path length and escape latency to reach the hidden platform were recorded to indicate the changes of spatial learning in different animal groups.

Additionally, a probe test was performed with the rat groups to evaluate the effect of DMR on rat spatial memory. The hidden platform was removed and the rats were individually released into the pool to swim for 60 seconds. The percentage time and distance in the target quadrant where the platform was submerged before were recorded to indicate the changes of spatial memory in different animal groups.

TUNEL

The neuron apoptosis was evaluated by TUNEL assays. Prior to TUNEL assays, the brain tissues were processed with PBS, and 3% hydrogen peroxide to block the endogenous peroxidase activities. After being dried, the tissues were subjected to TUNEL assays with a TUNEL assay kit (Beyotime Biotechnology, Shanghai, China) according to the kit's instruction. Subsequently, the sections were stained with a DAB substrate (Thermo Fisher Scientific, MA, US) before being observed and assessed under an Olympus light microscope (Olympus Corporation, JP).

RNA isolation and real-time PCR

Total RNA was isolated by from peripheral blood samples utilizing a Trizol reagent (Invitrogen, Carlsbad, CA). And the extracted total RNA was reverse-transcribed into cDNA using a Taqman Reverse Transcription assay kit (Invitrogen, Carlsbad, CA) according to the kit's instructions. Finally, real-time PCR analysis was performed to evaluate the expression level of Iba1 mRNA, iNOS mRNA, CD68 mRNA, CD32 mRNA, CD206 mRNA, BDNF mRNA, cystathionine γ-lyase (CSE) mRNA on a PRISM 7500 real time PCR machine (Applied Biosystems, Foster City, CA). The quantification of relative expression of these genes utilized GAPDH as an internal reference gene following the $2^{-\Delta\Delta Ct}$ calculation method.

IHC

The levels of Iba1, BDNF, CSE in the hippocampal tissues were investigated by IHC assays. Prior to the IHC assay, the collected hippocampus tissues were fixed in 10% formalin solution (pH value = 7.4), paraffin embedded, sliced into 2 mm thick sections and de-paraffined using xylene. Subsequently, the hippocampal sections were blocked with 0.3% hydrogen peroxide for 20 min and blocked with 10% FBS. Finally, the hippocampal sections were incubated with primary antibodies against Iba1 (Catalogue number: ab178846, Abcam, Cambridge, MA), BDNF (Catalogue number: ab178846, Abcam,

Cambridge, MA), CSE (Catalogue number: #30068, Cell Signaling Technology, MA, US) overnight, followed by the incubation with horseradish peroxidase-conjugated secondary antibodies (Abcam, Cambridge, MA) for 60 min. Finally, the sections were counter-stained with a DAB substrate (Sigma-Aldrich, St. Louis, MO) before being observed and assessed under an Olympus light microscope (Olympus, Tokyo, Japan).

ELISA

To evaluate the hippocampal production of IL-6, Il-4, H_2S in the animal models, ELISA assays were performed with ELISA kits of IL-6, Il-4, H_2S (Abcam, Cambridge, UK) according to the kit's instructions. Prior to the ELISA assays, the collected hippocampus tissues were processed with RIPA lysis buffer and centrifugated for the removal cell debris.

Statistical analysis

Data obtained from the above experiments were collected and analyzed with statistical analysis software SPSS v 19.0 (IBM, NY, US). The comparisons between multiple groups were analyzed using one-way ANOVA, and Tukey's test was used as the post-hoc test. All data were expressed as mean ± standard deviation, and the statistical significance level was set as less than $P < 0.05$.

Results

Recovery effect of DMR on the behavioral data of sleep-deprived rats

Compared with the control~~sham-operated~~ rats, the ~~sleep deprived~~sleep-deprived rats travelled longer distance (Fig.1A) and spent more time (Fig.1B) to reach the hidden platform. However, NaSH treatment and DMR both reduced the path length and escape latency during platforms, and DMR exhibited a more significant recovery effect upon these behavioral data. Moreover, when observing the percentage of their swimming distance and escape latencies among the rat groups (Fig.1C), compared with the rats in the ~~SHAM~~Control group, a

significant reduction in the ~~sleep deprived~~sleep-deprived rats was found. Also, the rats in the SD + NaSH and the SD + DMR groups spent less time and travelled less distance in finding the target quadrant than the rats in the SD group. Besides, TUNEL assay upon the apoptosis of neurons (Fig.1D) showed significant recovery of neurons in ~~sleep deprived~~sleep-deprived rats treated with NaSH or DMR, with the recovery effect of DMR being most significant.

Recovery effect of DMR on the expression of hippocampal Iba1

As shown in Figure 2, we measured the level of hippocampal Iba1 in the rat models to study the effect of DMR upon the expression of Iba1 in vivo. IHC assay (Fig.2A and 2B) showed that the number of Iba1 positive cells was evidently higher in the ~~sleep deprived~~sleep-deprived rats compared with the ~~Sham~~Control group, and the administration of NaSH and DMR both reduced the number of of Iba1 positive cells. Specially, the number of Iba1 positive cells in the SD + DMR group is lowest among all ~~sleep deprived~~sleep-deprived rats. Similarly, the relative expression of Iba1 mRNA (Fig.2C) also presented the same trend among the rat groups, indicating the recovery effect of DMR on the expression of hippocampal Iba1.

Recovery effect of DMR on the expressions of phenotypic-related genes in hippocampus

The upregulated expression of hippocampal phenotypic-related genes, including NOS mRNA, CD68 mRNA, CD32 mRNA and CD206 mRNA, commonly characterize the incidence of cognitive impairment. As shown in Figure 3, we observed the expression of NOS mRNA (Fig.3A), CD68 mRNA (Fig.3B), CD32 mRNA (Fig.3C) and CD206 mRNA (Fig.3D) in the hippocampal tissues collected from the rat models. Accordingly, we found that the relative hippocampal expressions of phenotypic-related genes were all evidently increased in ~~sleep deprived~~sleep-deprived rats compared with ~~sham-operated~~control rats, and these highly expressed genes in ~~sleep deprived~~sleep-deprived rats were

significantly recovered by the administration of NaSH or the application of DMR. Specially, the recovery effect of DMR was more significant than that of the NaSH treatment. Also, ELISA assays indicated that the increased relative expression of IL-6 (Fig.3E) and IL-4 (Fig.3F) were also recovered by DMR in ~~sleep deprived~~sleep-deprived rats.

Promotive effect of DMR on the expression of hippocampal BDNF and CSE

In this study, we also evaluated the mRNA level of hippocampal BDNF (Fig.4) and CSE (Fig.5). Accordingly, the numbers of BDNF positive cells (Fig.4A and 4B) or CSE positive cells (Fig.5A and 5B) were both significantly decreased due to the deprivation of sleep in rat models. And compared with the SD group, DMR most significantly promoted the reduced numbers of BDNF and CSE positive cells. Meanwhile, the evidently downregulated hippocampal BDNF mRNA (Fig.4C) expressions were also elevated by the treatment of NaSH and MDR, with MDR exhibiting the most significant effect. Similar results were obtained in respect to the relative expressions of CSE mRNA (Fig.5C) and H_2S production (Fig.5D).

Discussion

~~Sleep deprivation is characterized as a state of inadequate quantity or quality of sleep (31). And sleep deprivation has been reported to induce brain function impairment (1), aggravate oxidative stress and inflammation (32), activate DNA damage response (33), suppresses neuronal proliferation (34), and reduce hippocampal gliogenesis (35). Moreover, epidemiological reports have stated that sleep deprivation, especially chronic sleep deprivation, increased the risk of diseases including coronary artery disease, hypertension, arrhythmias, diabetes and obesity (36). Among all the personal characteristics, aging is a factor which influences the individual's ability of deal with sleep deprivation, supported by the fact that the brain function of an aged individual is generally weaker (37). Besides, sex is also an influencing~~

~~factor. It was reported that women, especially menopausal women, may suffer more from sleep deprivation than man with slower recovery duration (38).~~ In this study, by using indicators including path length, escape latency, cognitive impairment and neuron status, we studied the effect of ~~dietary methionine restriction~~ DMR on sleep deprivation~~,~~ and cognitive dysfunction associated with sleep deprivation. Behavioral data such as path length and escape latency were higher in the ~~sleep deprived~~sleep-deprived rats compared with the ~~sham-operated~~control rats, which were subsequently recovered by the treatment of NaSH or DMR. Also, DMR most significantly recovered the cognitive impairment and neuron status of ~~sleep deprived~~sleep-deprived rats compared with the administration of NaSH.

As a health intervention which is deemed to exert positive effect on the cognitive dysfunctions in postmenopausal women, the method of exercise could also alter the expression of hippocampal BDNF (31). BDNF not only functions as a mediator in CNS, but also provides neurotrophic and neuroprotective support to different subpopulations of neurons (32). The decreased hippocampal BDNF levels were reported to be associated with the impairment in the cognitive function in aged individuals (33). Moreover, sleep deprivation after contextual conditioning has been proved to inhibit the level of BDNF and the according signaling (34). In this study, we found that the mRNA and protein level of hippocampal BDNF and CSE were reduced in ~~sleep deprived~~sleep-deprived rats, which is ~~in~~ consistent with the results of previous studies. And the reduced BDNF levels were elevated by the treatment of NaSH and MDR, with MDR exhibiting the most significant effect.

The hippocampal protective effect of H_2S against cognitive dysfunction induced by sleep deprivation has been validated in sleep-deprived rat models (15, 35). Meanwhile, CSE, which is produced in neurons, is the biosynthetic enzyme for the H_2S (36). In CSE mutant mice, the

suppressive effect of NF-κB upon apoptosis is diminished as well (36). Moreover, NF-κB regulates the immune and inflammatory responses in brain ischemia-reperfusion rats (37, 38). Therefore, H_2S production is also associated with neuro-inflammatory responses. In this study, we found that the elevated level of hippocampal H_2S production was recovered by NaSH and DMR, with DMR exhibiting the most significant recovery effect in ~~sleep deprived~~sleep-deprived rats.

Several molecules have been reported to be associated with cognitive dysfunctions. For example, the up-regulation of CD68 was reported to be correlated with longer latency times of normally aged rats in the MWM test (39). And the combination of CD68 and Iba1, another microglial marker, could function as biomarkers fro the characterization of microglial phenotype in age-associated deep subcortical white matter lesions (40). Moreover, CD32 also functions as a surface protein on brain microglia for the characterization of M2b macrophage in Alzheimer's disease patients (41). IL-6 was reported to be associated with cognitive function (42) while IL-4 could attenuate reference memory impairment by modulating the hippocampal-BDNF signaling pathway (43). In this study, the expressions of hippocampal phenotypic-related genes including NOS mRNA, CD68 mRNA, CD32 mRNA and CD206 mRNA also showed similar trend as hippocampal Iba1. Meanwhile, the increased relative expression of IL-6 and IL-4 were also recovered by DMR in ~~sleep deprived~~sleep-deprived rats.

However, this study is limited as further validation due to the lack of further validation of ~~the relatively small sample size in the animal experiment. Also,~~ the effect of DMR ~~should be further verified~~ in clinical practice in sleep-deprived patients, especially the mechanism of modulating the production of hydrogen sulfide and the level of BDNF. Therefore, in our future studies, larger sample size for the more comprehensive animal study will be exploited. And further observations in clinical trials are also necessary.

Conclusions

In conclusion, we identified that the application of DMR could attenuate cognitive dysfunction in sleep deprivation rat models.

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Competing interests

None.

Data availability statement

The datasets generated in this study is available from the corresponding authors upon reasonable request.

Figure legends

Figure 1

Recovery effect of DMR on the behavioral data of sleep-deprived rats (scale bar = 50 μm)

A: The increased path length of sleep-deprived rats to reach the hidden platform was inhibited by NaSH or DMR;

B: The prolonged escape latency of ~~sleep-deprived~~sleep-deprived rats to find the hidden platform was reduced by NaSH or DMR;

C: The percentage of distance, time and crossing in the target quadrant during the probe phase of ~~sleep-deprived~~sleep-deprived rats was increased by NaSH or DMR;

D: TUNEL assay indicated that NaSH or DMR could alleviated the aggravated neuron apoptosis in ~~sleep-deprived~~sleep-deprived rats.

Figure 2

Recovery effect of DMR on the expression of hippocampal Iba1 mRNA and protein (* P valu < 0.05 vs. ~~SHAM~~Control group; ** P value < 0.05 vs. SD group; # P value < 0.05 vs. SD+NaSH group; scale bar = 50 μm).

A: IHC assay upon Iba1 expression in hippocampal tissues showed that NaSH or DMR could reduce the up-regulated Iba1 expression;

B: The increased number of Iba1 positive microglia in ~~sleep deprived~~sleep-deprived rats was reduced by NaSH or DMR;

C: The relative expression level of Iba1 mRNA was elevated in ~~sleep deprived~~sleep-deprived rats, and NaSH or DMR could restored the elevated Iba1 mRNA expression.

Figure 3

Recovery effect of DMR on the expressions of phenotypic-related genes in hippocampus (* P valu < 0.05 vs. Control~~SHAM~~ group; ** P value < 0.05 vs. SD group; # P value < 0.05 vs. SD+NaSH group).

A: Relative expression of iNOS mRNA was increased in ~~sleep deprived~~sleep-deprived rats, while both NaSH and DMR restored the dysregulation of iNOS mRNA level;

B: Relative expression of CD68 mRNA was increased in ~~sleep deprived~~sleep-deprived rats, while both NaSH and DMR restored the dysregulation of CD68 mRNA level;

C: Relative expression of CD32 mRNA was increased in ~~sleep deprived~~sleep-deprived rats, while both NaSH and DMR restored the dysregulation of CD32 mRNA level;

D: Relative expression of CD206 mRNA was increased in ~~sleep deprived~~sleep-deprived rats, while both NaSH and DMR restored the dysregulation of CD206 mRNA level;

E: Relative expression of IL-6 was increased in ~~sleep deprived~~sleep-deprived rats, which was suppressed by treatment of NaSH and DMR;

F: Relative expression of IL-4 was increased in ~~sleep deprived~~sleep-deprived rats, which was suppressed by treatment of NaSH and DMR.

Figure 4

Promotive effect of DMR on the expression of hippocampal BDNF mRNA and protein (* P valu < 0.05 vs. Control~~SHAM~~ group; ** P value < 0.05 vs. SD group; # P value < 0.05 vs. SD+NaSH group; scale bar = 50 μm).

A: IHC assay showed that hippocampal BDNF expression was reduced in ~~sleep deprived~~sleep-deprived rats, and NaSH or DMR antagonized the changes of BDNF expression;

B: The reduced number of BDNF positive microglia in ~~sleep deprived~~sleep-deprived rats was recovered by NaSH or DMR;

C: The relative expression level of BDNF mRNA was reduced in ~~sleep deprived~~sleep-deprived rats, and NaSH or DMR antagonized the changes of BDNF expression.

Figure 5

Promotive effect of DMR on the expression of hippocampal CSE mRNA and production of H_2S (* P valu < 0.05 vs. Control~~SHAM~~ group; ** P value < 0.05 vs. SD group; # P value < 0.05 vs. SD+NaSH group; scale bar = 50 μm)

A: IHC assay showed that hippocampal CSE expression was reduced in ~~sleep deprived~~sleep-deprived rats, and NaSH or DMR antagonized the changes of BDNF expression;

B: The reduced number of CSE positive microglia in ~~sleep deprived~~sleep-deprived rats was recovered by NaSH or DMR;

C: The relative expression level of CSE mRNA was reduced in ~~sleep deprived~~sleep-deprived rats, and NaSH or DMR antagonized the changes of BDNF expression;

D: H_2S production was lowest in ~~sleep deprived~~sleep-deprived rats, and NaSH or DMR treatment antagonized the reduction of H_2S production.

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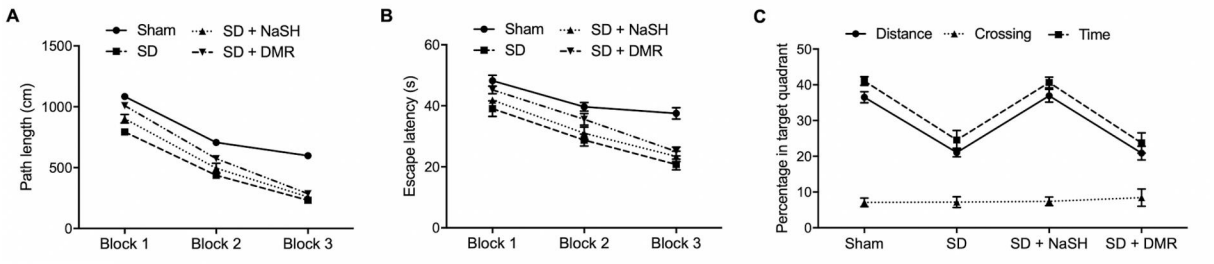
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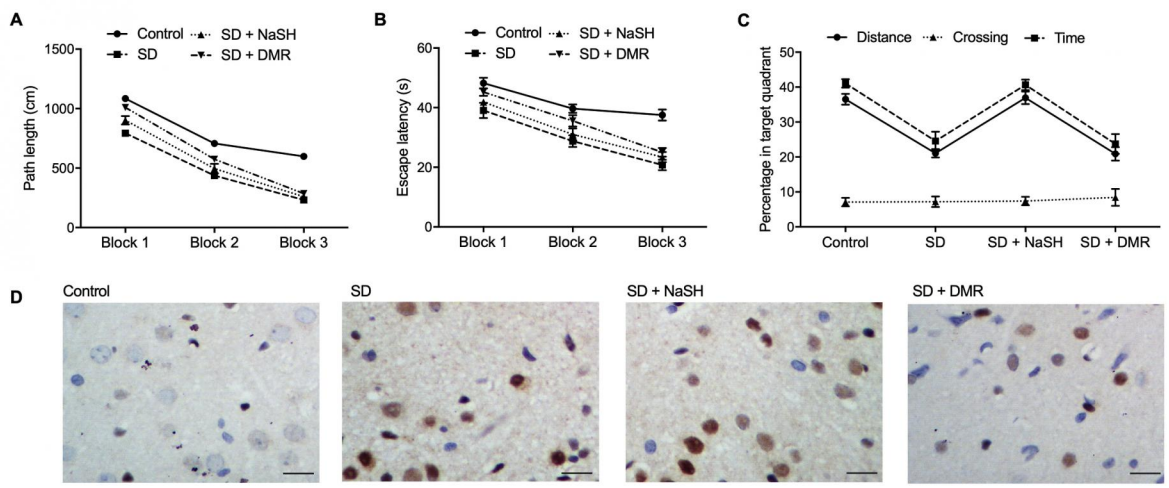

Figure 1
Recovery effect of DMR on the behavioral data of sleep-deprived rats (scale bar = 50 μm)
A: The increased path length of sleep-deprived rats to reach the hidden platform was inhibited by NaSH or DMR;
B: The prolonged escape latency of sleep-deprived rats to find the hidden platform was reduced by NaSH or DMR;
C: The percentage of distance, time and crossing in the target quadrant during the probe phase of sleep-deprived rats was increased by NaSH or DMR;
D: TUNEL assay indicated that NaSH or DMR could alleviated the aggravated neuron apoptosis in sleep-deprived rats.

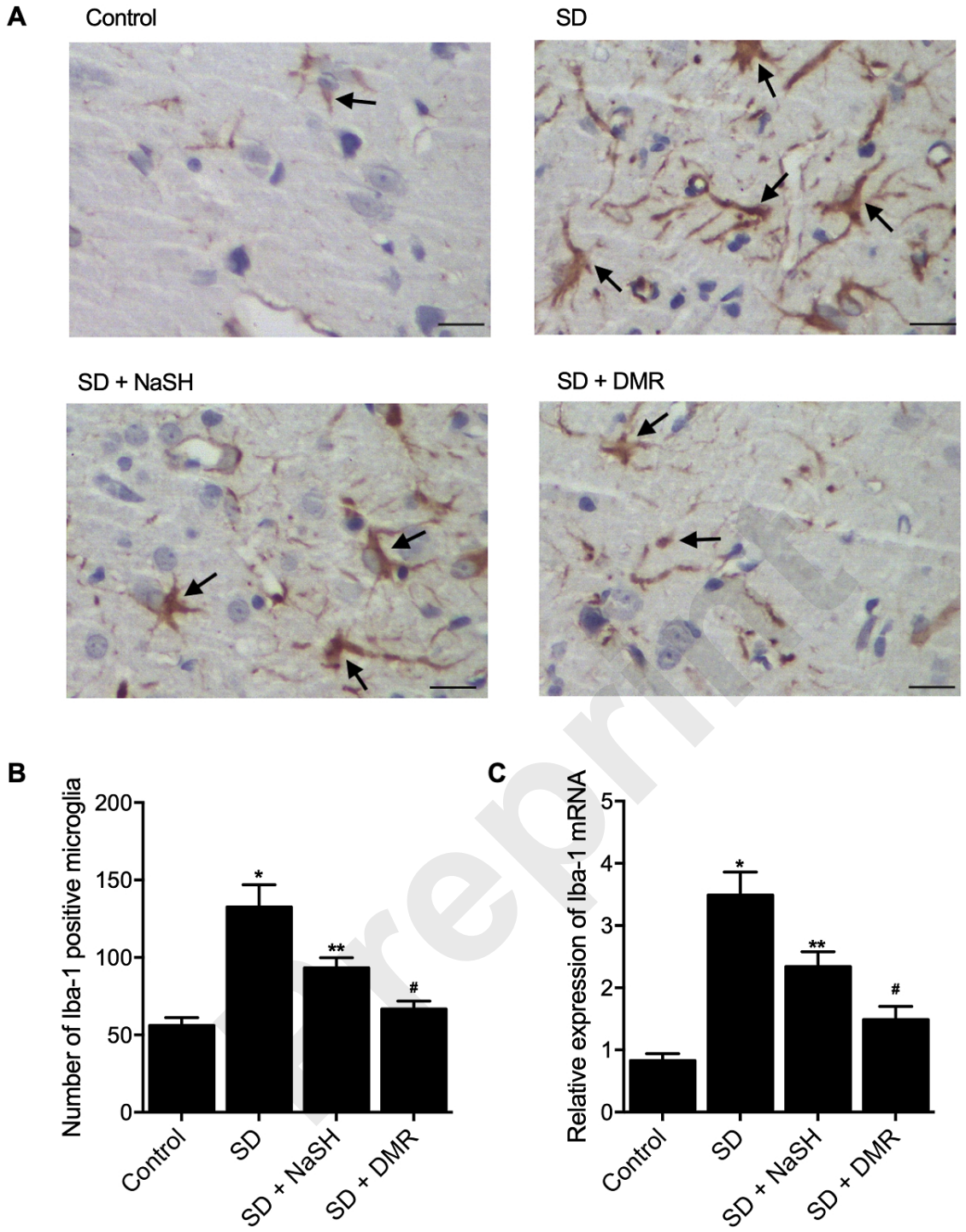

Figure 2
Recovery effect of DMR on the expression of hippocampal Iba1 mRNA and protein (* P valu < 0.05 vs. Control group; ** P value < 0.05 vs. SD group; # P value < 0.05 vs. SD+NaSH group; scale bar = 50 μm).
A: IHC assay upon Iba1 expression in hippocampal tissues showed that NaSH or DMR could reduce the up-regulated Iba1 expression;
B: The increased number of Iba1 positive microglia in sleep-deprived rats was reduced by NaSH or DMR;
C: The relative expression level of Iba1 mRNA was elevated in sleep-deprived rats, and NaSH or DMR could restored the elevated Iba1 mRNA expression.

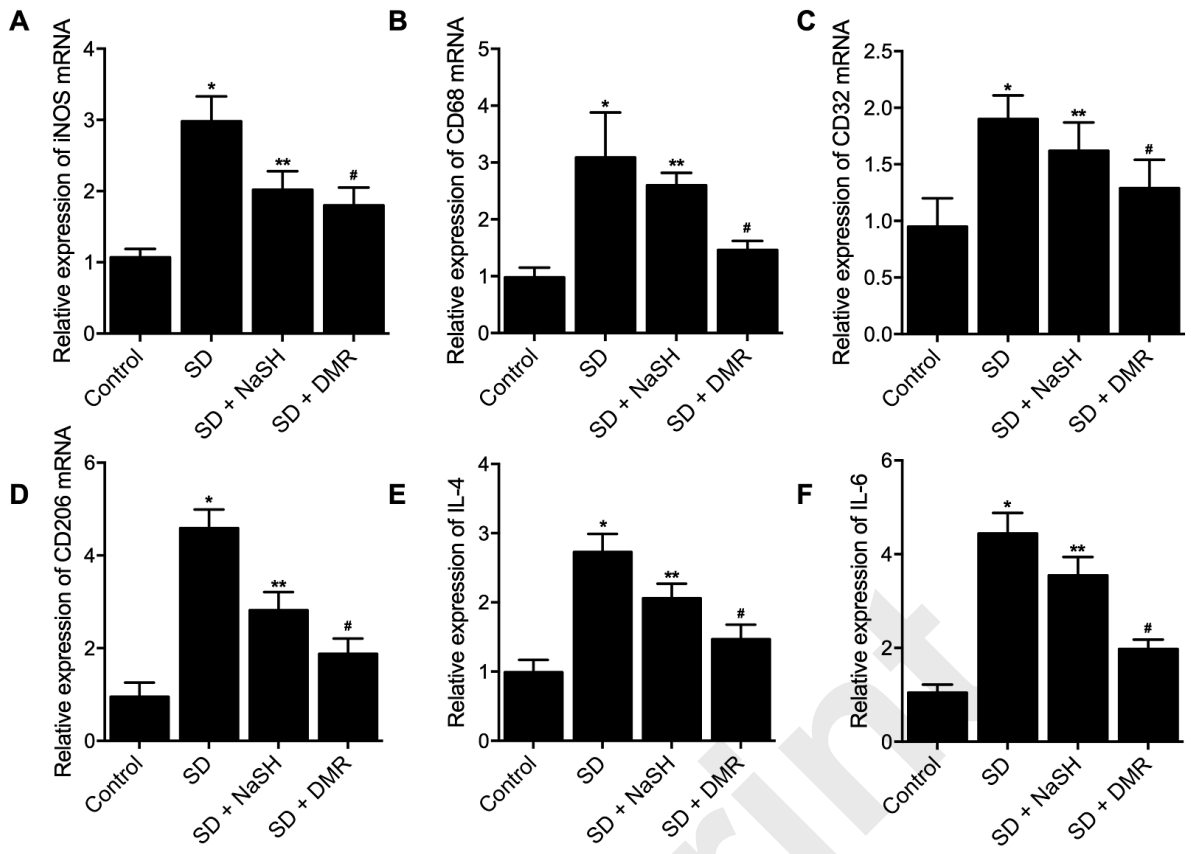

Figure 3
Recovery effect of DMR on the expressions of phenotypic-related genes in hippocampus (* P valu < 0.05 vs. Control group; ** P value < 0.05 vs. SD group; # P value < 0.05 vs. SD+NaSH group).
A: Relative expression of iNOS mRNA was increased in sleep-deprived rats, while both NaSH and DMR restored the dysregulation of iNOS mRNA level;
B: Relative expression of CD68 mRNA was increased in sleep-deprived rats, while both NaSH and DMR restored the dysregulation of CD68 mRNA level;
C: Relative expression of CD32 mRNA was increased in sleep-deprived rats, while both NaSH and DMR restored the dysregulation of CD32 mRNA level;
D: Relative expression of CD206 mRNA was increased in sleep-deprived rats, while both NaSH and DMR restored the dysregulation of CD206 mRNA level;
E: Relative expression of IL-6 was increased in sleep-deprived rats, which was suppressed by treatment of NaSH and DMR;
F: Relative expression of IL-4 was increased in sleep-deprived rats, which was suppressed by treatment of NaSH and DMR.

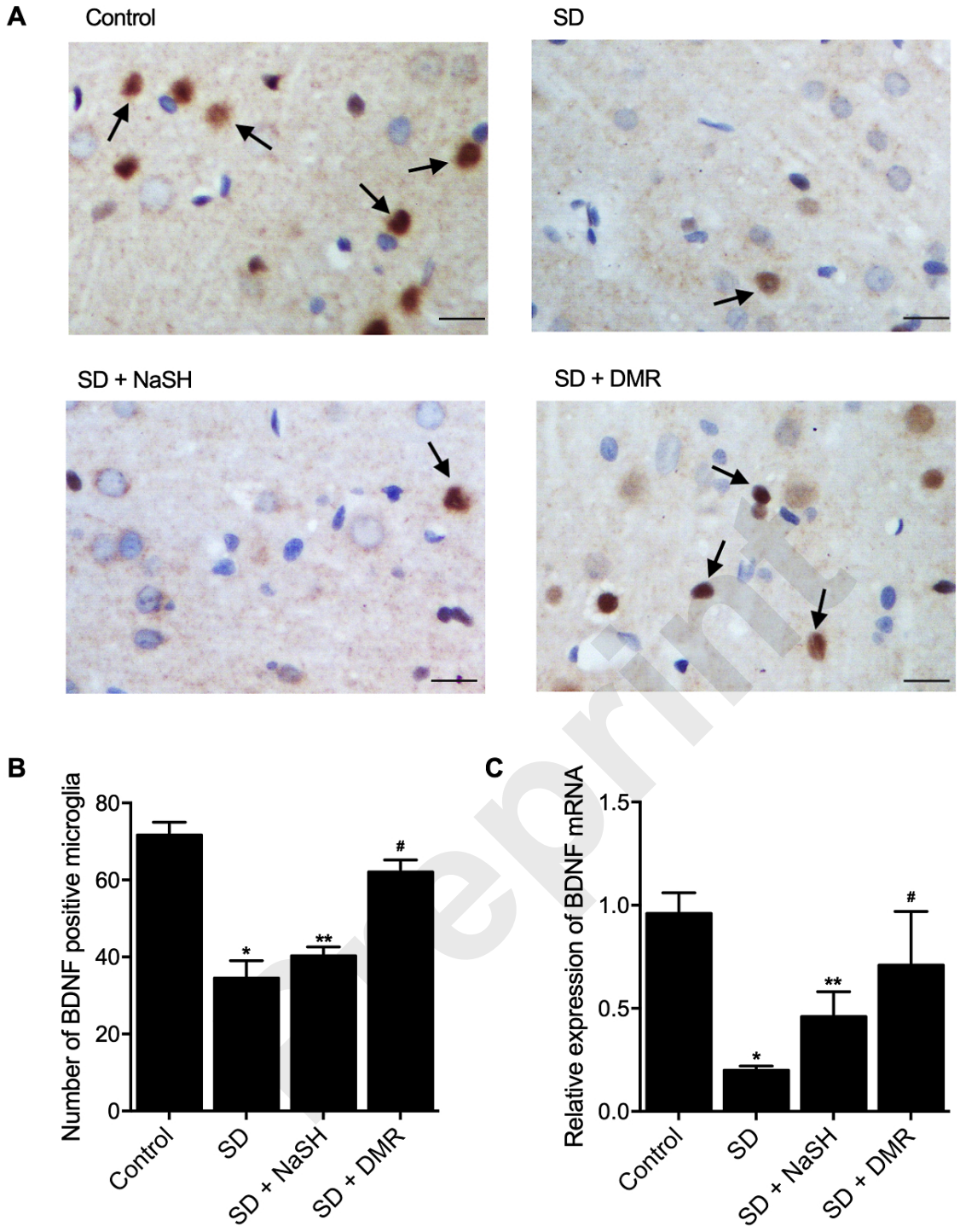

Figure 4
Promotive effect of DMR on the expression of hippocampal BDNF mRNA and protein (* P valu < 0.05 vs. Control group; ** P value < 0.05 vs. SD group; # P value < 0.05 vs. SD+NaSH group; scale bar = 50 μm).
A: IHC assay showed that hippocampal BDNF expression was reduced in sleep-deprived rats, and NaSH or DMR antagonized the changes of BDNF expression;
B: The reduced number of BDNF positive microglia in sleep-deprived rats was recovered by NaSH or DMR;
C: The relative expression level of BDNF mRNA was reduced in sleep-deprived rats, and NaSH or DMR antagonized the changes of BDNF expression.

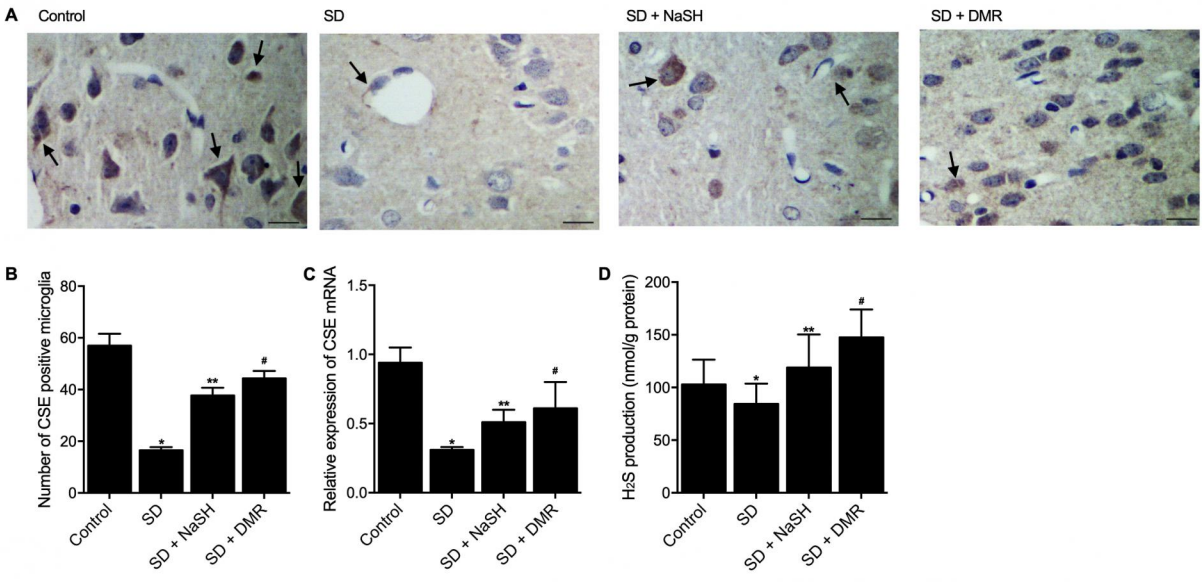

Figure 5
Promotive effect of DMR on the expression of hippocampal CSE mRNA and production of H2S (* P valu < 0.05 vs. Control group; ** P value < 0.05 vs. SD group; # P value < 0.05 vs. SD+NaSH group; scale bar = 50 μm)
A: IHC assay showed that hippocampal CSE expression was reduced in sleep-deprived rats, and NaSH or DMR antagonized the changes of BDNF expression;
B: The reduced number of CSE positive microglia in sleep-deprived rats was recovered by NaSH or DMR;
C: The relative expression level of CSE mRNA was reduced in sleep-deprived rats, and NaSH or DMR antagonized the changes of BDNF expression;
D: H2S production was lowest in sleep-deprived rats, and NaSH or DMR treatment antagonized the reduction of H2S production.