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REGULAR RESEARCH ARTICLE

Hydrogen Sulfide Ameliorates Homocysteine-Induced Cognitive Dysfunction by Inhibition of Reactive Aldehydes Involving Upregulation of ALDH2

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Abstract

Background: Homocysteine, a risk factor for Alzheimer's disease, induces cognitive dysfunction. Reactive aldehydes play an important role in cognitive dysfunction. Aldehyde-dehydrogenase 2 detoxifies reactive aldehydes. Hydrogen sulfide, a novel neuromodulator, has neuroprotective effects and regulates learning and memory. Our previous work confirmed that the disturbance of hydrogen sulfide synthesis is invovled in homocysteine-induced defects in learning and memory. Therefore, the present work was to explore whether hydrogen sulfide ameliorates homocysteine-generated cognitive dysfunction and to investigate whether its underlying mechanism is related to attenuating accumulation of reactive aldehydes by upregulation of aldehyde-dehydrogenase 2.
Methods: The cognitive function of rats was assessed by the Morris water maze test and the novel object recognition test. The levels of malondialdehyde, 4-hydroxynonenal, and glutathione as well as the activity of aldehyde-dehydrogenase 2 were determined by enzyme linked immunosorbent assay; the expression of aldehyde-dehydrogenase 2 was detected by western blot.
Results: The behavior experiments, Morris water maze test and novel objects recognition test, showed that homocysteine induced deficiency in learning and memory in rats, and this deficiency was reversed by treatment of NaHS (a donor of hydrogen sulfide). We demonstrated that NaHS inhibited homocysteine-induced increases in generations of MDA and 4-HNE in the hippocampus of rats and that hydrogen sulfide reversed homocysteine-induced decreases in the level of glutathione as well as the activity and expression of aldehyde-dehydrogenase 2 in the hippocampus of rats.
Conclusion: Hydrogen sulfide ameliorates homocysteine-induced impairment in cognitive function by decreasing accumulation of reactive aldehydes as a result of upregulations of glutathione and aldehyde-dehydrogenase 2.

Keywords: ALDH2, cognitive dysfunction, GSH, homocysteine, hydrogen sulfide, reactive aldehydes

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Significance Statement

Homocysteine (Hcy), an independent risk factor for Alzheimer's disease (AD), induces the deficit in learning and memory. Thus, finding a new strategy to antagonize Hcy-induced cognitive dysfunction has important values in the prevention and treatment of AD. This study is to explore whether hydrogen sulfide (H_2S), the third gasotransmitter, improves the Hcy-generated cognitive dysfunction and the underlying mechanisms. In the present study, we found that H_2S ameliorated Hcy-induced cognitive dysfunction. We also demonstrated that H_2S inhibited reactive aldehydes accumulation, preserved glutathione homeostasis, and upregulated aldehyde-dehydrogenase 2 activity and expression in the hippocampus of Hcy-exposed rats. Our present findings identify that H_2S is a potential target for therapeutic intervention in Hcy-induced cognitive impairment and provide a new strategy in the prevention and treatment of Hcy-induced neurodegenerative diseases.

Introduction

Homocysteine (Hcy), a sulfur-containing amino acid derived from the metabolism of methionine (Prudova et al., 2006; Parkhitko et al., 2016), is an independent risk factor for Alzheimer's disease (AD) (Seshadri et al., 2002; Dwyer et al., 2004; Van Dam and Van Gool, 2009; Miwa et al., 2015; Hu et al., 2016a). Hcy markedly enhances the vulnerability of neuronal cells to excitotoxicity and elicits neuronal cell death in a variety of neuronal types (Hankey and Eikelboom, 1999; Kim et al., 2007). Furthermore, it was reported that treatment of rats with Hcy induces deficits in learning and memory function (Zhang et al., 2009; Li et al., 2014b; Agrawal et al., 2015; Miyazaki et al., 2015), suggesting that Hcy has harmful effects on cognitive function. Thus, finding a new strategy to antagonize Hcy-induced cognitive dysfunction has important value in the prevention and treatment of AD.

Hydrogen sulfide (H_2S), the third endogenous gasotransmitter, is an important neuromodulator and neuroprotectant (Kimura, 2002; Hu et al., 2011; Zhou and Tang, 2011; Kimura et al., 2012). It has been reported that H_2S facilitates the formation of hippocampal LTP (Abe and Kimura, 1996) and attenuates lipopolysaccharide- or beta-amyloid-induced spatial learning and memory impairment (Gong et al., 2011a, 2011b). A recent study suggested that H_2S ameliorates Hcy-induced AD-like pathology, blood-brain barrier disruption (Kamat et al., 2016). The most recent in vitro and in vivo studies by our group have demonstrated that inhibition of endogenous H_2S generation is associated with Hcy-induced neurotoxicity (Tang et al., 2011), while H_2S antagonizes Hcy-induced neurotoxicity in PC12 cells (Tang et al., 2010), and that decrease in endogenous H_2S generation contributes to Hcy-induced deficit in learning and memory of rats (Li et al., 2014b). Therefore, we speculated that H_2S can ameliorate Hcy-induced cognitive dysfunction.

"Aldehyde load" has become a new therapeutic target in neurodegenerative disease (Wood, 2006; Wood et al., 2006, 2007). Reactive aldehydes are the product of lipid peroxidation, including malondialdehyde (MDA) and 4-hydroxynonenal (4-HNE) (Aldini et al., 2007; Mali et al., 2016). MDA and 4-HNE are the most important products of peroxidation of cellular membrane lipids. The accumulation of reactive aldehydes such as MDA and 4-HNE has been observed in the brain tissues in neurodegenerative disorders such as AD and Parkinson's disease (Lovell et al., 1997; Montine et al., 1997; Sayre et al., 1997; Ishrat et al., 2009). It has been demonstrated that the accumulation of reactive aldehydes not only lead to neuronal death (Mark et al., 1997) but also result in a decline in cognitive function (Zarkovic, 2003). Therefore, this work investigated whether H_2S-exerted melioration in Hcy-induced cognitive impairment involves the reduction of reactive aldehydes in hippocampus of Hcy-exposed rats. Aldehyde dehydrogenase 2 (ALDH2) is the principal enzyme involved in detoxifying aldehydes, including MDA, 4-HNE, and other aldehydes, by converting them to less toxic acid products (Ohta et al., 2004; Marchitti et al., 2007; Chen et al., 2014; Doorn et al., 2014). It has been reported that the deficiency of ALDH2 in neuronal cells exhibited increased vulnerability to 4-HNE (D'Souza et al., 2015), while overexpression of ALDH2 protects neuronal survival against 4-HNE (Bai and Mei, 2011; Ma et al., 2011; Lee et al., 2012). Thus, we suggest that clearance of reactive aldehydes via upregulation of ALDH2 may be a new opportunity for intervention in neurodegenerative diseases. Therefore, we evaluated whether H_2S upregulates the activity and expression of ALDH2 in the hippocampus of Hcy-exposed rats.

In the present work, we demonstrated that H_2S ameliorated the cognitive dysfunction, inhibited hippocampal reactive aldehydes generation, and upregulated hippocampal glutathione (GSH) as well as ALDH2 activity and expression in Hcy-exposed rats. Our findings therefore suggest that H_2S has the therapeutic potential to intervent Hcy-induced cognitive impairment.

Materials and Methods

Reagents

Sodium hydrosulfide (NaHS, a donor of H_2S) and Hcy were purchased from Sigma Chemical. Anti-ALDH2 antibody was purchased from Abcam Technology. ALDH2 activity assay kit was obtained from Abcam. MDA enzyme linked immunosorbent assay (ELISA) kit was purchased from Uscn Life Science. 4-HNE and GSH ELISA kits were obtained from Bio-Swamp Life Science.

Animals

Adult male Sprague-Dawley rats (260–300 g) were used for all experiments. Animals were purchased from the SJA Lab Animal Center of Changsha. They were housed individually in a temperature- and humidity-controlled room under standard 12-h-light/-dark cycle and given access to standard laboratory food and water. Before the experiments, all rats were allowed to adapt to their new environment for 7 days. The experiments were conducted in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and were approved by the Animal Use and Protection Committee of University of South China. All efforts were made to minimize animal suffering.

Drugs and Treatments

A total of 1.68 mg or 5.6 mg of NaHS was dissolved in 1 mL of phosphate-buffered saline (PBS) to equal concentrations of NaHS 30 or 100 μmol/mL, respectively. Hcy was diluted in sterilized artificial cerebrospinal fluid/50% DMSO and intracerebroventricularly injected (i.c.v.) at a dose of 0.6 μmol. After adaptation

for 7 days, the rats were pretreated with 30 or 100 μmol/mL NaHS (1 mL/kg/d, i.p.) for 7 days and then cotreated with Hcy (0.6 μmol/d, i.c.v.) for 7 days. Behavioral tests were performed 24 hours after the last injection. One day after the behavioral tests, animals were killed, and the hippocampus region tissues of the brain were rapidly removed on the ice-cold artificial cerebrospinal fluid and stored at -80°C for analysis (Figure 1).

Intracerebroventricular Injection

Animals were anesthetized with sodium pentobarbital (45 mg/kg, i.p.) and placed in a stereotaxic frame for operation. The area around the incision was trimmed. PBS or Hcy (0.6 μmol) in 4 μL was injected into the unilateral ventricle using the following coordinates: AP, -1.4; ML, 1.8; DV, -3.0 (in millimeters with respect to bregma and dura; tooth bar at 3.3 mm), with an injection rate of 0.4 μL/min using a 10-μL Hamilton syringe. To ensure that the entire injection had been delivered, the injection needle should be slowly pulled out halfway and kept in position for an additional 2 minutes before being removed. Rats were kept in a warm environment until they recovered from the anesthesia.

Morris Water Maze (MWM) Test

Before the MWM test, the rats of all groups were kept in a controlled room temperature (20°C -24°C) and humidity (60–80%) for 3 days to adapt to the environment and then submitted to the water maze test. The water maze was mainly composed of a large circular pool(180 cm diameter, 60 cm high) with water at 23 ± 2°C and a hidden platform (12.5 cm diameter, 38 cm high) in the pool where they can escape. The pool was divided into 4 quadrants, and the escape platform was placed at a fixed position in the center of one quadrant as the target quadrant, which was hidden 2 cm below the water surface. The walls of the water maze room were fixed with several landmarks as the distal spatial extra-maze cues for the rats to find the platform. The procedure of the MWM test was recorded by an MT-200 Morris Image Motion System (Chengdu Technology and Market Corp). It was carried out 24 hours after the cessation of Hcy treatment and this test was run 8 consecutive days.

Acquisition Trial

The acquisition trial involves training rats to find the hidden platform at a fixed location in space over a series of trials. It uses 7 training days (days 1–7) and 4 trials per day with a 20-minute intertrial interval. The starting position, which was randomly chosen across the 4 trials each day, was equally spaced along the circumference of the pool. The order of the quadrants that rats were placed into was changed each day such that they were never exposed to a sequence of trials that they had had before. Each rat was allowed to swim for a maximum duration of 120 s in each trial to find the platform. After finding and climbing onto the platform, the rat was allowed to remain there for 20 seconds. If an animal did not find the platform within 120 seconds, it was guided to the platform and remained there for 20 seconds before being returned to its home cage. Rats were kept dry and warm between trials.

Probe Trial

After finishing the place navigation task, the rats were subjected to the probe trial (day 8) to assess spatial memory. The platform was removed from the pool and animals were placed into the pool from another quadrant, which was opposite to the target quadrant. The rats were allowed to swim freely for 120 seconds in the water maze. The time that an animal spent in the target quadrant and the number of time that the same animal crossed the former platform area were recorded for measuring spatial memory maintenance.

Visible Platform Test

After the probe test, each rat proceeded to the visible platform test to rule out the possible deficits (as visual, motor, and motivation skills) in sensorimotor processes. The water surface was 2 cm beneath the height of the platform. The escape platform was moved to a novel quadrant in the pool at a fixed location for the next 4 trials. The latency to reach the platform and the average speed were recorded. After finishing the last trial, rats were gently dried and kept warm before returned to their home cages.

Novel Objects Recognition Test

Novel object recognition test was performed to assess hippocampus-dependent cognitive function (D'Agostino et al., 2012; Tang et al., 2013). Before testing, each rat was individually habituated to the test box (50.0 × 50.0 × 60) for 5 minutes once per day without objects. During the training session, 2 different types of objects used in the current study were 10-cm diameter × 7-cm high red crystal balls and 10-cm white plastic objects. On the day of testing, each rat was allowed to freely explore the 2 identical objects secured to the floor in opposite corners of the test box for 5 minutes (familiarization phase), and the time rats spent exploring each object was recorded with stopwatches. After a 60-minute retention interval, each rat was returned to the test box with one identical and one novel object. Each rat was again allowed to explore the objects for 5 minutes (testing phase), and the time rats spent exploring each object was recorded. Training trials were video-taped and the location of the novel object was varied in random order within groups. Exploratory behavior was defined as sniffing, touching, and direct attention to the object. The nose within 1 cm of the object was considered as exploratory behavior. The rats climbing on or chewing the object was not considered as exploratory behavior. The discrimination index (= (novel object – familiar object) / (novel object + familiar object)) was used to measure the cognitive function of rats.

Tissue Sampling

The brain tissues (hippocampus) of the rats were harvested 24 hours after behavioral tests. Animals were given a lethal dose

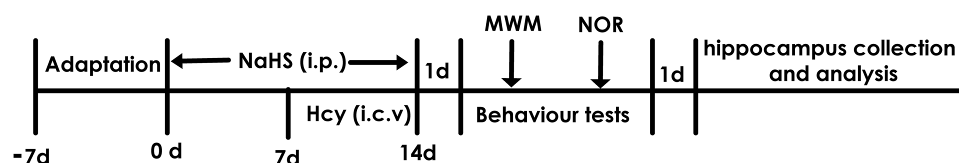

Figure 1. Schematic diagram of the experimental schedule. MWM, Morris water maze test; NOR, novel objects recognition test.

of sodium pentobarbital. The brain was immediately removed. Hippocampus and cortical tissue of the brain were dissected and rapidly frozen in liquid nitrogen. All samples were stored at -80°C until further processing.

The Bicinchoninic Acid (BCA) Assay for Protein Quantitation

Total protein quantification was assessed by BCA assay according to the manufacturer's instructions. Bovine serum albumin (BSA) standard solution was diluted to a concentration range of 0–] to 2 μg/μL with PBS. Already diluted BSA standard solution and protein samples, which was diluted 10 times with PBS, were added to 96-well plates (25 μL/well), respectively. Two parallel wells were made for each sample. Subsequently, 200 μL BCA working solution (BCA reagent A and BCA reagent B by 50:1) was added to each well and incubated at 37°C for 30 minutes. After cooling to room temperature, the absorbance at 562 nm was determined using a microplate reader. The BSA standard curve is used to calculate the relative protein concentration of each sample.

Biochemical Analysis of MDA, 4-HNE, and GSH

The contents of MDA, 4-HNE, and GSH in hippocampus tissue homogenate were measured using MDA, 4-HNE, and GSH ELISA kit, respectively. Briefly, hippocampus tissues were lysed and centrifuged for 10 minutes at 5000 *g*. The supernatants were collected and total protein concentrations were quantified using the BCA protein assay kit. Protein sample (10 μg/mL, 50 μL) was added to the 96-well protein binding plate and incubated at 37°C for 2 hours. After washing 2 times with wash buffer, 100 μL of assay diluent per well was added and incubated for 2 hours on an orbital shaker. Plates were then washed completely with thorough aspiration between each wash, 50 μL of the diluted anti-MDA, anti-4-HNE, or anti-GSHantibody was added to all wells, and incubated for 1 hour. After washing 3 times with wash buffer, 50 μL of the diluted secondary antibody-HRP conjugate was added to each well and incubated for 1 hour on an orbital shaker. Then 50 μL of substrate solution was add to each well and incubated for 15 minutes at room temperature on an orbital shaker. Finally, 50 μL of stop solution was added to terminate the reaction, and the absorbance of each well was recorded using a microplate reader at 450 nm.

ELISA for ALDH2 Activity

The ALDH2 activity in hippocampus tissue homogenate was measured using ALDH2 Activity Assay Kit. Hippocampus tissues were lysed and centrifuged at 5000 *g* for 10 minutes at 4°C. After being quantified using BCA protein assay kit, the equal proteins were performed in a 96-well plate that was coated with an antibody specific for ALDH2. In a nutshell, 100 μL of each diluted sample was added to the 96-well protein binding plate and incubated for 3 hours at room temperature with gentle shaking and then washed 2 times with wash buffer. Next, 200 μL of activity solution (1×Coupler, 25 mM acetaldehyde, 1 mM NAD^+, 1×Reagent Dye) was added to all wells. The absorbance at 450 nm of each well was read on a microplate reader. The concentration of ALDH2 in the samples was then determined by comparing the O.D. of the samples to the standard curve.

Western-Blot Analysis for ALDH2 Expression

The hippocampus tissues were homogenized in lysis buffer (150 mmol/L NaCl, 20 mmol/L Tris-HCl, pH 7.5, 1% Triton X-100, 1 mmol/L phenyl methyl sulfonyl fluoride, 1 mmol/L Na_3VO4, leupeptin, and EDTA) and incubated on ice for 30 minutes. After the samples were centrifuged at 5000 *g* for 10 minutes at 4°C, the supernatant was collected and the protein concentration quantified using a BCA Protein Assay kit (Beyotime). After the supernatant of sample was heated at 100°C for 5 minutes, an equivalent amount of protein extract for each sample was run on sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Subsequently, the protein was transferred to a PVDF membrane and blocked with TBST (50 mmol/L Tris-HCl, pH 7.5, 150 mmol/L NaCl, 0.1% Tween-20) containing 5% BSA (Sigma) for 2 hours at room temperature, and the membranes were incubated with the primary ALDH2 antibodies (Cambridge) diluted 1:1000 and antibody anti-β-actin (Epitomics), which was diluted 1:2000 overnight at 4°C. After washing with TBST buffer 3 times, the membranes were incubated in anti-rabbit secondary antibody conjugated to horseradish peroxidase (1:5000) for 2 hours. Then the membranes were washed with TBST buffer again and developed with an enhanced chemiluminescence system (ECL) followed by apposition of the membranes with autoradiographic films (Kodak). The integrated optical density for the protein band was calculated by Image-J software.

Statistical Analysis

Data are displayed as the mean ± SEM. The significance of intergroup differences was evaluated by 1-way ANOVA. Differences were considered significant at $P < .05$.

Results

H_2S Improves Spatial Learning and Memory of Hcy-Treated Rats in MWM

To investigate the protective role of H_2S in cognitive dysfunction of Hcy-treated rats, after pretreatment with NaHS (the donor of H_2S, 30 or 100 μmol/kg/d, i.p.) for 7 days and cotreatment with Hcy (0.6 μmol/d, i.c.v.) for 7 days, rats were subjected to the MWM task to test spatial learning and memory. The latency traveled to find the platform in the acquisition phase is shown in Figure 2A-C. All 5 groups over the 7 training days exhibited a decrease in the escape latency (Figure 2A-C). Hcy-treated alone rats exhibited significant higher escape latency on days 5, 6, and 7 during training trials compared with control group rats (Figure 2A), implying a significant impairment of spatial learning in Hcy-exposed rats, and this impairment occurred from training day 5 onward. However, treatment with NaHS (30 or 100 μmol/kg/d, i.p.) significantly decreased the escape latency of Hcy-treated alone rats from training day 5 onward (Figure 2B). Figure 2D shows the representative swimming tracks of rats searching for the underwater platform on the 1st and 7th training days. On the 1st training day, there was no difference of the distance in searching for the hidden platform among the 5 groups. On the 7th training day, Hcy-treated alone rats exhibited a significant increase in the distance swam compared with the control group; however, the rats cotreated with NaHS (30 or 100 μmol/kg/d, i.p.) and Hcy showed a significant decrease in the distance swam compared with the Hcy-treated alone group.

In the probe trial, the platform was removed, and the rats were placed into the quadrant opposite to the target quadrant and allowed to swim freely for 120 seconds. Hcy-treated alone rats showed impaired memory, as evident by their significant decreases in the number of times crossing the target quadrant (Figure 3A) and the time spend in the target quadrant (Figure 3B).

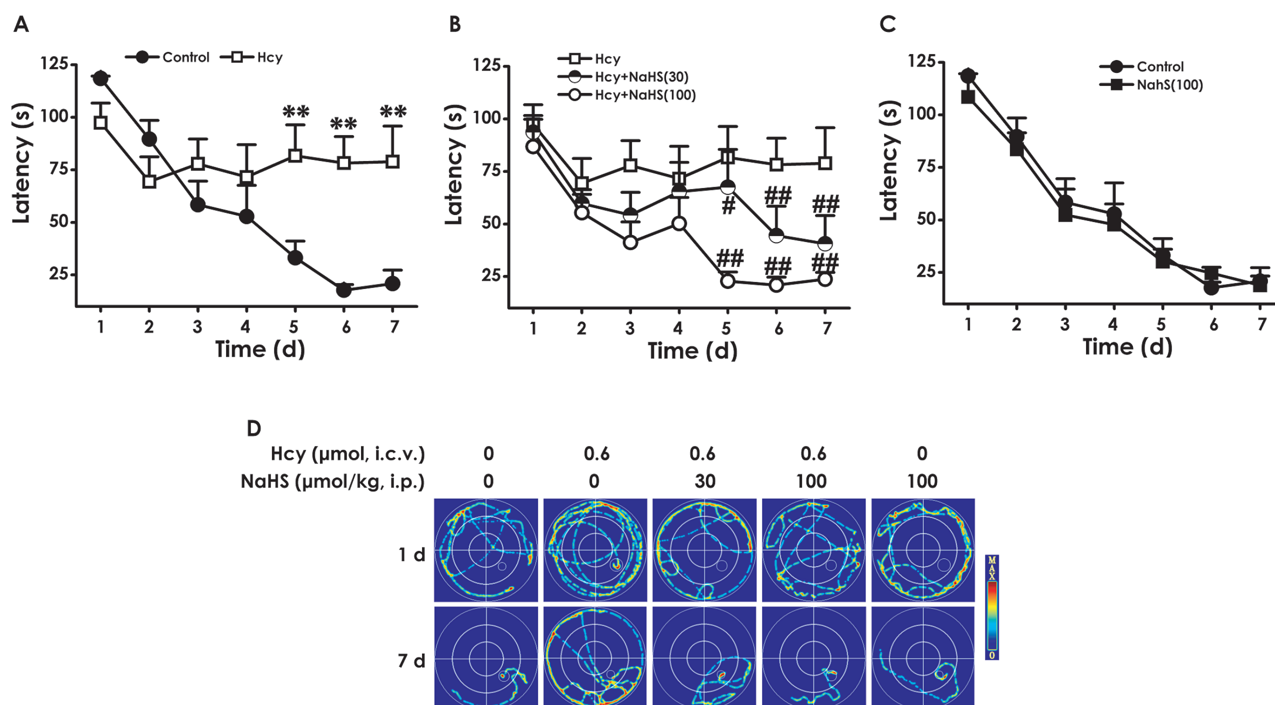

Figure 2. Effects of hydrogen sulfide (H_2S) on homocysteine (Hcy)-induced impairment in learning function of rats in navigation testing of the Morris water maze (MWM). After pretreatment with NaHS (30 or 100 μmol/kg/d, i.p.) for 7 days and then cotreatent with Hcy (0.6 μmol/d, i.c.v.) for 7 days, rats were tested in the MWM task. The latency traveled to find the platform during 7 days in the acquisition phase was recorded in the navigation training (A-C). The swimming tracks of rats searching for the underwater platform at the 1st and 7th training days (D). Values represented as mean ± SEM (n=8). **P < .01, vs control; ##P < .01, vs Hcy-treated alone group.

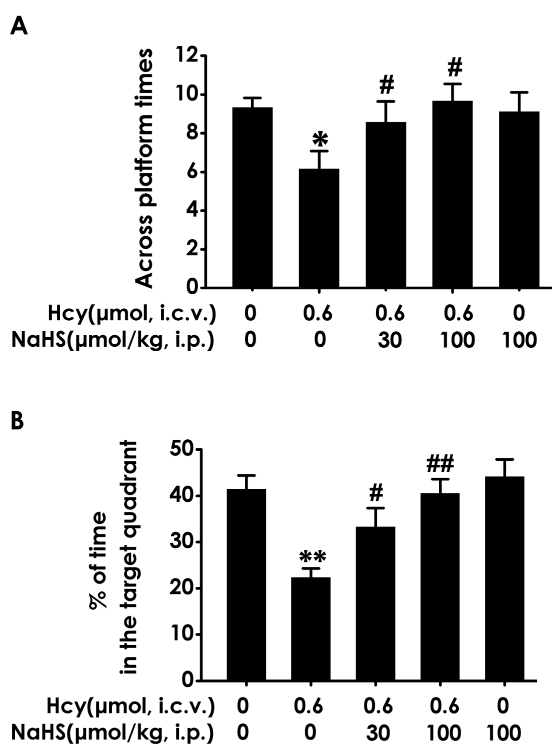

Figure 3. Effects of hydrogen sulfide (H_2S) on homocysteine (Hcy)-induced impairment in memory function of rats in probe trial testing of Morris water maze (MWM). After finishing the navigation task, the platform was removed and the rats were submitted to the probe trail testing. The number of times that the animal crossed the platform area (A) and the percentage of time spent in the target quadrant (B) was analyzed. Values represented as mean ± SEM (n=8), *P < .05, **P < .01, vs control; #P < .05, ##P < .01, vs Hcy-treated alone group.

However, NaHS (30 or 100 μmol/kg/d, i.p.) significantly increased the time that Hcy-treated alone rats spend in the target quadrant (Figure 3A) and the number of times that Hcy-treated alone rats crossed the target quadrant (Figure 3B). Taken together, these data suggest that H_2S reverses the impairment in spatial learning and memory induced by Hcy.

Ruling out the Effects of the Changes of Vision and Motor Ability on Learning and Memory in Rats

To exclude the possibility that the results are due to changes of vision and motor ability in the rats, we examined the escape latency and average swimming speed of rats by performing a visible platform test after finishing the transfer probe trials. There was no statistical difference in the escape latencies (Figure 4A) among all groups in the visible platform test, and there was no significant difference in swimming speed (Figure 4B), which indicated that the alterations of all parameters in the hidden platform tests and probe trials are not due to changes in visual or motor abilities of rats.

H_2S Enhances the Cognitive Function of Hcy-Exposed Rats in Novel Object Recognition Test

To further investigate whether H_2S ameliorates the cognitive dysfunction of Hcy-exposed rats, we also examined the cognitive function of rats using the novel object recognition test. As shown in Figure 5A, the discrimination index in Hcy-exposed alone rats was significantly decreased compared with control; however, treatment with NaHS (30 or 100 μmol/kg/d, i.p.) significantly increased the discrimination index of Hcy-exposed alone rats. In addition, there was no significant difference of the total exploration time among the 5 groups (Fig. 5B). These data

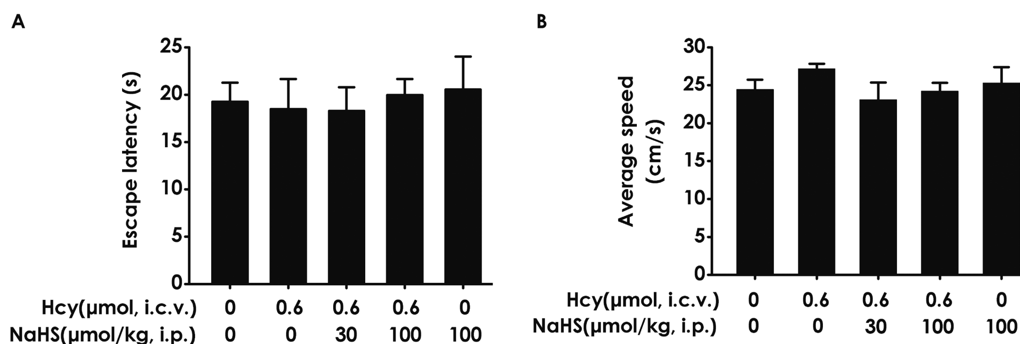

Figure 4. Effects of hydrogen sulfide (H_2S) and homocysteine (Hcy) on the motor function and vision of rats. After finishing the probe test, the rats were submitted to the visible platform test, the latency to reach the platform (A), and the average swimming speeds in probe trials (B) were recorded. Values represented as mean ± SEM, n = 8.

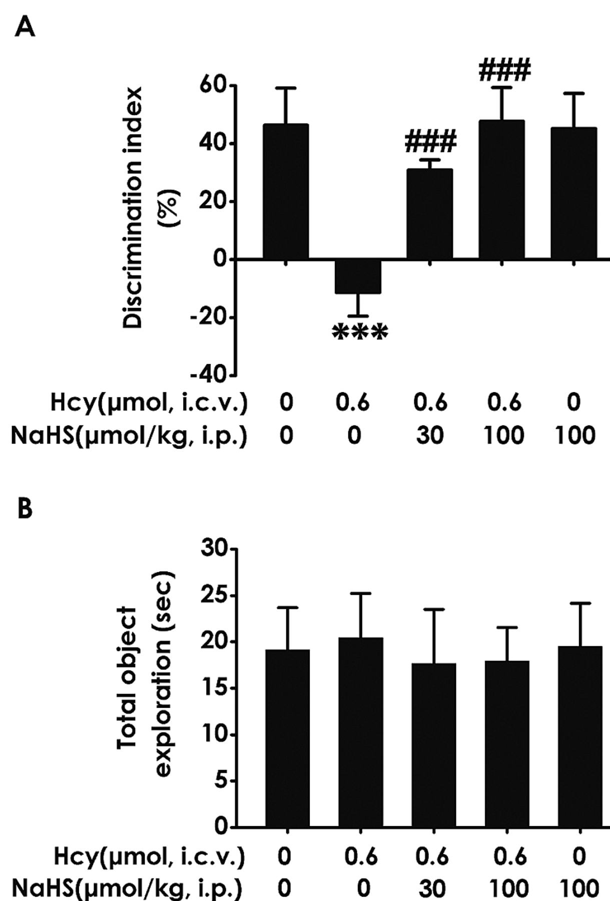

Figure 5. Effects of hydrogen sulfide (H_2S) on homocysteine (Hcy)-induced impairment in the function of cognition of rats determined by novel object discrimination test. After the Morris water maze (MWM) test, the rats were submitted to the novel object recognition test. The discrimination index (A) and the total exploring time of rats in each group (B) were recorded. Values represented as mean ± SEM (n = 8), ***$P < .001$, vs control; ###$P < .001$, vs Hcy-treated alone group.

suggested that H_2S prevents the decline in cognitive function triggered by the exposure of Hcy.

H_2S Inhibits Hcy-Upregulated Levels of the Reactive Aldehydes in the Hippocampus of Rats

To explore the effect of H_2S on Hcy-induced reactive aldehydes in the hippocampus of rats, we measured the levels of MDA and 4-HNE in the hippocampus of rats. As shown in Figure 6, the elevated levels of hippocampal MDA (Figure 6A) and 4-HNE (Figure 6B) by 7-d treatment with Hcy (0.6 μmol, i.c.v.) were markedly reduced by treatment with NaHS (30 or 100 μmol/kg/d, i.p.). NaHS (100 μmol/kg) alone did not affect the levels of MDA (Figure 6A) and 4-HNE (Figure 6B) in control rats. These data indicated that H_2S inhibits Hcy-triggered accumulation of hippocampal reactive aldehydes.

H_2S Increased the Level of GSH in the Hippocampus of Rats Exposed to Hcy

We also investigated the change in the level of GSH in the hippocampus. Hcy (0.6 μmol, i.c.v.) caused a decrease in the level of GSH in the hippocampus of rats, while treatment of NaHS (30 or 100 μmol/kg/d, i.p.) markedly increased the level of GSH in the hippocampus of Hcy-exposed rats (Figure 7), which indicated that H_2S inhibits the decrease in the level of hippocampal GSH induced by Hcy.

H_2S Up-Regulates the Activity and Expression of ALDH2 in the Hippocampus of Rats Exposed to Hcy

It has been certified that ALDH2 has the highest catalytic efficiency for oxidation of reactive aldehydes (Yoval-Sanchez and Rodriguez-Zavala, 2012). To explore whether the inhibitory role of H_2S in Hcy-induced accumulation in hippocampal reactive aldehydes is associated with upregulation of hippocampal ALDH2, we detected the activity and expression of ALDH2 in the hippocampus of Hcy-exposed rats. Hcy (0.6 μmol, i.c.v.) caused decreases in the activity (Figure 8A) and expression (Figure 8B) of ALDH2 in the hippocampus of rats. However, treatment of NaHS (30 or 100 μmol/kg/d, i.p.) markedly increased the activity (Figure 8A) and expression (Figure 8B) of ALDH2 in the hippocampus of Hcy-exposed rats, which indicated the upregulatory role of H_2S in the activity and expression of hippocampal ADLH2.

Discussion

H_2S plays an important role in the regulation of learning and memory (Nagpure and Bian, 2015). The aim of this study was to investigate the protective effects of H_2S against Hcy-induced cognitive dysfunction and its mechanism. Our study showed that (1) H_2S ameliorated the cognitive dysfunction of rats exposed to Hcy; (2) H_2S inhibited Hcy-induced increase in generation of reactive aldehydes in the hippocampus of rats; and (3) H_2S reversed Hcy-induced downregulation in the level of GSH as well as the activity and expression of ALDH2 in the

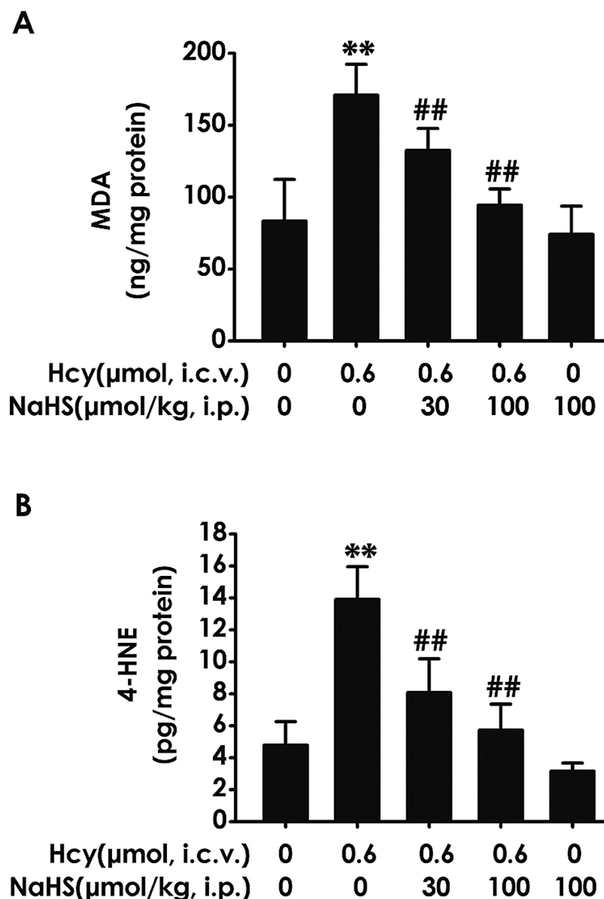

Figure 6. Effects of hydrogen sulfide (H_2S) on homocysteine (Hcy) upregulated the levels of reactive aldehydes in the hippocampus of rats. After finishing the behavioral tests, the hippocampus of rats was homogenized and the levels of reactive aldehydes were detected by measuring the contents of malondialdehyde (MDA) (A) and 4-hydroxynonenal (4-HNE) (B) using an ELISA kit. Values represented as mean ± SEM (n = 3). **P < .01, vs control; ##P < .01, vs Hcy-treated alone group.

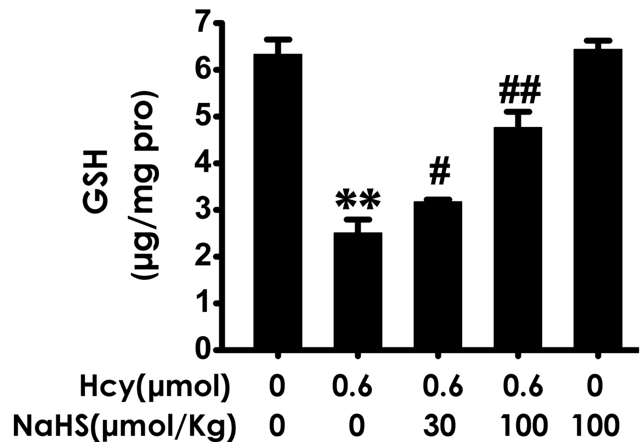

Figure 7. Effects of hydrogen sulfide (H_2S) on homocysteine (Hcy) decreased the level of glutathione (GSH) in the hippocampus of rats. After finishing the behavioral tests, the hippocampus of rats was homogenized and the level of GSH in hippocampus was determined using an ELISA kit. Values represented as mean ± SEM (n = 3). **P < .01, vs control; #P < .05, ##P < .01, vs Hcy-treated alone group.

hippocampus of rats. These data suggest that H_2S ameliorates Hcy-induced impairment in cognitive function by decreasing accumulation of reactive aldehydes, which is involved in upregulation of hippocampal ALDH2.

A moderate elevation of plasma Hcy is a potential risk factor for AD (Seshadri et al., 2002; Dwyer et al., 2004; Van Dam and Van Gool, 2009; Li et al., 2014a; Miwa et al., 2015; Hu et al., 2016a), and Hcy induces a deficit in learning and memory function (Zhang et al., 2009; Li et al., 2014b; Agrawal et al., 2015; Miyazaki et al., 2015). Hcy increases reactive oxygen species (ROS) and stimulates neurotoxicity (Ho et al., 2001; White et al., 2001; Ataie et al., 2012; Sharma et al., 2015). H_2S, a gaseous signaling molecule, scavenges ROS and protects neurons against oxidative stress (Kimura and Kimura, 2004; Whiteman et al., 2004, 2005; Tang et al., 2008; Sakamoto et al., 2014). Furthermore, H_2S plays an important role in regulation of learning and memory (Nagpure and Bian, 2015). Therefore, elucidating the beneficial role of H_2S in Hcy-induced deficit in learning and memory function has an important scientific research value. To investigate the potential treatment effect of H_2S on Hcy-induced deficit in learning and memory, rats were pretreated with NaHS for 7 days and cotreated with Hcy for 7 days, and the functions of learning and memory of rats were tested with the MWM test and the novel object recognition test. The MWM is one of the most frequently used laboratory tools in behavioral neuroscience as a device to investigate spatial learning and memory in laboratory rats (D'Hooge and De Deyn, 2001; Li et al., 2014b). In MWM, the spatial learning of rats is investigated by the hidden-platform acquisition test, and spatial memory is evaluated through the probe trial test. In MWM test, we showed that treatment with NaHS decreases the escape latency in hidden-platform acquisition training and increases the crossing platform times and the percentage of time elapsed in the target quadrant in the probe trail in the Hcy-exposed rats, which indicated that H_2S reverses the impairment in spatial learning and memory induced by Hcy. The novel object recognition test is based on the differential exploration of familiar and new objects (He et al., 2013), and it is used to study short-term declarative memory and attention. In the novel object recognition test, we showed that the discrimination index in Hcy-treated alone rats was significantly increased by treatment with NaHS, which also indicates the harmful role of Hcy in learning and memory can be reversed by H_2S, a toxic gas whose toxicity is associated with concentration. In the present work, NaHS (30 or 100 μmol/kg/d, i.p.) did not lead to a deficit in learning and memory function. Taken together, our present work demonstrates that H_2S prevents Hcy-induced cognitive impairment in rats.

It is known that reactive aldehydes (4-HNE and MDA) generated from lipids peroxides. These aldehydes can form adducts with lipids, proteins, and DNA, leading to their inactivation and damage of living cells (Esterbauer et al., 1991; Hill and Bhatnagar, 2009; Berg et al., 2011; He et al., 2012). It has been shown that the accumulation of aldehydes induces neuronal death and causes synaptic dysfunction (Pedersen et al., 1999) and that reactive aldehydes are increased in brain tissue in neurodegenerative disorders such as AD, amyotrophic lateral sclerosis, and Parkinson's Disease (Zarkovic, 2003; Chiu et al., 2015). It has been reported that Hcy induces learning and memory deficit (Zhang et al., 2009; Li et al., 2014b; Agrawal et al., 2015; Miyazaki et al., 2015). Other studies have demonstrated that Hcy perturbs the methionine cycle (Alternative Medicine ReviewMiller, 2003), increases ROS, and induces oxidative damage to brain tissue (Obeid and Herrmann, 2006), which could be the cause of cognitive decline. Our present work showed that Hcy caused a significant increase in the levels of MDA and 4-HNE in the hippocampus of rats. Therefore, we suggested that accumulation

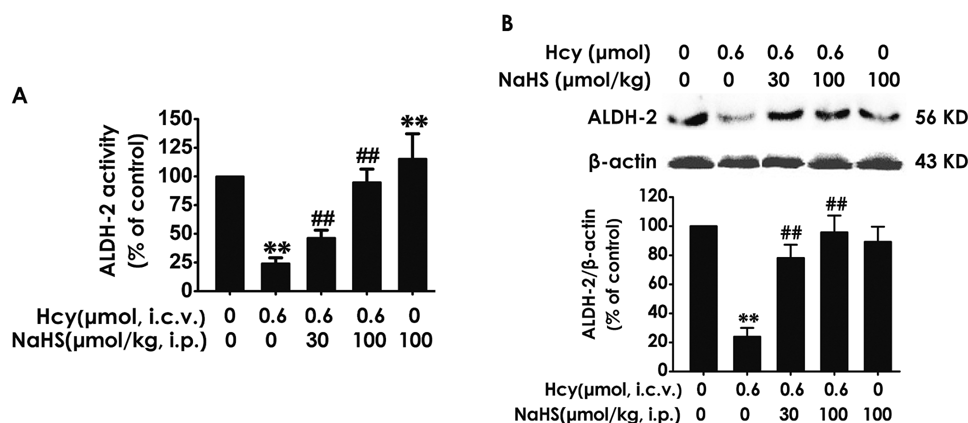

Figure 8. Effects of hydrogen sulfide (H_2S) on homocysteine (Hcy) downregulated the activity and expression of aldehyde-dehydrogenase 2 (ALDH2) in the hippocampus of rats. After finishing the behavioral tests, the hippocampus of rats was homogenized. The activity of ALDH was determined by ALDH2 Activity Assay Kit (A) and the expression of ALDH2 was detected by western blot using an anti-ALDH2 antibody (B). In all blots, β-actin was used as a loading control. Values represented as mean ± SEM (n = 3). **P < .01, vs control; ##P < .01, vs Hcy-treated alone group.

of MDA and 4-HNE in the hippocampus plays a vital role in Hcy-induced cognitive dysfunction. It has been demonstrated that H_2S scavenges ROS and protects neurons against oxidative stress (Kimura and Kimura, 2004; Whiteman et al., 2004, 2005; Tang et al., 2008). We now demonstrated that NaHS markedly reduced the elevated levels of MDA and 4-HNE in Hcy-exposed rats. This finding indicated that the role of H_2S in the clearance of reactive aldehydes is implicated in its protective action against Hcy-induced cognitive function impairment. GSH is an important antioxidant in the brain. The potential HNE conjugations with GSH damages GSH homeostasis. In the present work, we found the level of GSH was decreased in the hippocampus of Hcy-exposed rats, while H_2S reversed the Hcy-induced deficiency in GSH homeostasis. Thus, we envision that restoring GSH homeostasis plays an important role in H_2S-exerted clearance of hippocampal reactive aldehydes and amelioration in cognitive function of Hcy-exposed rats.

ALDH2 is a key enzyme that metabolizes acetaldehyde to acetic acid and also detoxifies ROS-generated aldehyde adducts (Lagranha et al., 2010). In brain, reactive aldehydes are mainly detoxified by ALDHs (Conklin et al., 2007; Ellis, 2007). ALDH2 has the highest catalytic efficiency for oxidation of 4-HNE and MDA (Yoval-Sanchez and Rodriguez-Zavala, 2012). ALDH2 functions as a protector against oxidative stress (Gao et al., 2016; Hu et al., 2016b). Many researchers reported that the levels of MDA and 4-HNE were found to increase in the brain of AD patients (Khan et al., 2012; Zhou et al., 2013) and that a sufficient amount of active ALDH2 is needed to detoxify the increased reactive aldehydes that occur in neurodegenerative diseases (Ohsawa et al., 2008). Numerous studies have demonstrated that deficiency of ALDH2 in neuronal cells exhibited increased vulnerability to 4-HNE (D'Souza et al., 2015). Meanwhile, overexpression of ALDH2 rescues 4-HNE-induced ischemic damage (Bai and Mei, 2011; Ma et al., 2011; Lee et al., 2012) and decrease the levels of MDA and 4-HNE (He et al., 2012). Our results that H_2S can clear reactive aldehydes led us to explore whether H_2S upregulates the activity and expression of ALDH2 in the hippocampus of Hcy-exposed rats. Our present work showed that Hcy caused decreases in the activity and expression of ALDH2 in the hippocampus of rats, while NaSH markedly increased the activity and expression of ALDH2 in the hippocampus of Hcy-exposed rats, which indicated that H_2S has the upregulatory role in hippocampal ALDH2. Taken together, our data suggested that the inhibitory role of H_2S in Hcy-induced cognitive dysfunction may be associated with the upregulation of hippocampal ALDH2, which leads to clearance of reactive aldehydes (4-HNE and MDA) in hippocampus of rats.

In summary, our study elucidates the protective action of H_2S against Hcy-induced impairment in cognitive function, which results from the clearance of hippocampal reactive aldehydes involving maintenance of hippocampal GSH homeostasis and upregulation of hippocampal ALDH2. Our findings signify that H_2S is effective in providing protection against Hcy-induced cognitive dysfunction. To enhance the potential impact and validity of the conclusion, we will in depth explore the underlying mechanisms of the protective action of H_2S in Hcy-generated cognitive dysfunction and investigate a clinically relevant model in our future work.

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Statement of Interest

None potential conflict of interest.

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