Hippocampal ornithine decarboxylase/spermidine pathway mediates H2S-alleviated cognitive impairment in diabetic rats: Involving enhancement of hippocampal autophagic flux

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Abbreviations: H2S, hydrogen sulfide; ODC, ornithine decarboxylase; Spd, spermidine; DFMO, dimethylbiguanide, an inhibitor of ODC.

Introduction: We have previously demonstrated the antagonistic role of hydrogen sulfide (H2S) in the cognitive dysfunction of streptozotocin (STZ)-induced diabetic rats. It has been confirmed that the impaired hippocampal autophagic flux has a key role in the pathogenesis of cognitive impairment and that ornithine decarboxylase (ODC)/spermidine (Spd) pathway plays an important role in the formation of memory by promoting autophagic flux.

Objectives: To investigate the roles of hippocampal ODC/Spd pathway and autophagic flux in H2S-attenuated cognitive impairment in STZ-induced diabetic rats.

Methods: Cognitive function is judged by the novel objective recognition task (NOR), the Y-maze, and the
Diabetes mellitus (DM) leads to many serious and debilitating health complications of multiple organs, such as cardiomyopathy, nephropathy, and retinopathy. Recently, increasing evidence has shown that DM also causes dysfunction of the central nervous system, pathologically characterized by neuron loss and axonal degeneration [1]. Substantial epidemiological evidence supports an association between diabetes and cognitive dysfunction, clinically characterized by chronic progressive varying degrees of cognitive impairment in particular psychomotor slowing and reduced mental flexibility [2]. This phenomenon is termed diabetes-associated cognitive dysfunction (DACD) and has also been confirmed in diabetic animal models. Accumulating evidence has shown that diabetic rodent models presents memory and learning impairments via the impairment of hippocampal neurogenesis [3] and synaptic plasticity [4]. It is therefore of utmost importance to explore novel therapeutic interventions for the treatment of diabetic cognitive dysfunction. Hydrogen sulfide (H$_2$S), a novel gasotransmitter [5,6], works as a neuroprotectant and neuromodulator [7]. Our previous study has demonstrated that H$_2$S ameliorates the cognitive dysfunction in STZ-induced diabetic rats [8], opening up a new perspective for the therapeutic intervention of diabetic cognitive dysfunction. However, the underlying mechanisms are still elusive.

Autophagic flux is defined as the flow of autophagosomes from formation to fusion with lysosomes [9]. Previous studies have demonstrated that the autophagic flux in hippocampus of diabetic rats is impaired [10] and that impaired autophagic flux in hippocampus lead to cognitive impairment [11]. Therefore, developing novel therapeutic strategies to enhance hippocampal autophagic flux plays an important role in treatment for the diabetic cognitive impairment. Interestingly, increasing evidence confirms that restoration of autophagic flux contributes to the neuroprotective and cardioprotective effects of H$_2$S [12,13]. To understand the mechanisms underlying H$_2$S-attenuated diabetic cognitive dysfunction, we explored whether H$_2$S enhances the autophagic flux in the hippocampus of diabetic rats.

Polyamines (PAs) play a key role in brain tissues during the early development of the brain and have neuroprotective effects [14–16]. PAs consist of putrescine (Put), spermidine (Spd) and spermine (Spm). The classical polyamines biosynthesis pathway begins with the formation of Put through amino acids ornithine decarboxylase by ornithine decarboxylase (ODC) [17]. Spd and Spm are then formed by adding aminopropyl groups to Put. ODC is the key enzyme of PA biosynthesis [18]. Thus, inhibition of ODC suppresses the production of Spd [17,19]. It has been confirmed that Spd improves memory impairment via enhancing autophagic flux [16,20,21]. Thus, we explored whether ODC/Spd pathway mediates H$_2$S-ameliorated impairments in cognitive function and hippocampal autophagic flux in STZ-induced diabetic rats.

Our present work showed that H$_2$S enhanced autophagic flux and up-regulated ODC/Spd pathway in the hippocampus of STZ-induced diabetic rats, and that inhibition of ODC/Spd pathway reversed the improving roles of H$_2$S in hippocampal autophagic flux and cognitive function in STZ-induced diabetic rats. We, for the first time, demonstrate that hippocampal ODC/Spd pathway through improving hippocampal autophagic flux mediates H$_2$S-attenuated cognitive impairment in STZ-induced diabetic rats.

Materials and methods

Reagents

NaHS, STZ, and DFMO were obtained from Sigma (Sigma, St. Louis, MO, USA). Specific monoclonal antibody for detecting ODC was supplied by Santa Cruz Biotechnology Inc (Catalog no. : sc-398116, Host: mouse, Delaware Ave Santa Cruz, CA, USA). Specific monoclonal antibodies for detecting Beclin-1 (Catalog no. : 3495 s, Host: rabbit), P62 (Catalog no. : 5114 s, Host: rabbit), and LC3-II (Catalog no. : 4108 s, Host: rabbit) were bought from Cell Signaling Technology (Beverly, MA, USA).

Animals

Sprague-Dawley rats (male, 5-week-old, 260–280 g, n = 121) were purchased from the SJA Lab Animal Center of Changsha (Changsha, Hunan, China). The approval number for experimentation with animals is SYXK (Xiang) 2015–0001. The rats were divided into individual cages, housed in temperature at (24 ± 2) °C, with controlled suitable humidity and ventilation. The room was kept in a 12-hours light/dark circle every day. Rats were given ad libitum access to food and water. Experiments were approved by the Animal Use and Protection Committee of University of South China. All efforts were made to minimize injuries.

Drug treatments and experimental schedule

Rats were randomly divided into seven groups: control group (n = 13), daily intraperitoneal (i.p.) injection phosphate-buffered saline (PBS, 1 ml/Kg); STZ-treated alone group (n = 20), which were singly injected with STZ (55 mg/kg, i.p.) and PBS for 30d (1 ml/kg/i.p.); the co-treatment with STZ and 30 μmol/kg/d NaHS group (n = 20), which received a single injection of STZ (55 mg/kg, i.p.) and NaHS (30 μmol/kg/d, i.p.) for 30 d; the co-treatment with STZ and
100 μmol/kg/d NaHS group (n = 20), which received a single injection of STZ (55 mg/kg, i.p.) and NaHS (100 μmol/kg/d, i.p.) for 30 d; the co-treated with STZ, 100 μmol/kg/d NaHS, and DFMO group (n = 20), which received a single injection of STZ (55 mg/kg, i.p.) and 30-d infusion of NaHS (100 μmol/kg/d, i.p.) as well as intracerebroventricular injection (i.c.v) of DFMO (5 μg/d); 100 μmol/kg/d NaHS-treated alone group (n = 13), which received an intraperitoneal infusion of NaHS (100 μmol/kg/d) for 30 d; and 5 μg/d DFMO-treated alone group (n = 15), which received an intracerebroventricular injection of DFMO (5 μg/d) for 30 d. Behavioral assays to evaluate cognitive level were performed 24 h after the last injection of NaHS and DFMO and within one day after the behavioral tests, rats were sacrificed and the brain tissues were rapidly removed to be stored at −80 °C for analysis (Fig. 1). Meanwhile, the same rats passed all tests during the whole experiment.

**Experimental induction of diabetes**

Diabetes was induced in rats according to the method described previously [22]. STZ was dissolved in 0.1 M sodium citrate buffer, pH 4.4 and administered at the dose of 55 mg/kg through i.p. route. Four days later, nonfasting blood glucose in a tail-vein sample was determined by a glucose analyzer; a value > 16.7 mmol/L was accepted as a successfully created diabetic model. In the drug administration period, the fasting blood glucose levels were examined every 7 days to make sure fasting blood glucose levels > 16.7 mmol/L.

**DFMO administration**

DFMO is a dose- and time-dependently enzyme-activated irreversible inhibitor of ODC. DFMO was administered by intracerebroventricular (i.c.v.) injection for 30 days to inhibit hippocampal ODC/Spd activity. In the drug administration period, the fasting blood glucose levels were examined every 7 days to make sure fasting blood glucose levels > 16.7 mmol/L.

**Behavioral procedures**

**Novel object recognition test**

The novel object recognition (NOR) test was used to assess the ability of rats to recognize a novel object in a familiar environment. It included three phases: adaptive (2 days), training and testing phase. In the adaptive phase, every rat was placed in an empty box (50.0 × 50.0 × 60 cm) to be adaptive to the environment for 30 min. Then each rat was placed in one arm and allowed to move freely in the three arms within 8 min. We recorded the orders and time of rats entering into each arm. If the rats entered three diverse arms in turn, it was regarded as a right alternating sequence. If the rats repeatedly entered the identical arm in three consecutive chances, it was considered as three stainless steel screws. DFMO (5 μg in 2.5 μL) was injected into the ventricle at speed of 0.5 μL/min by using a 5 μL microinjection. Before and after the injection, the needle of microinjection was remained for 1 min in cannula to accommodate injection.

**Y-maze test**

Y-maze test was also performed to assess the function of learning and memory in rats [23]. Y maze (90 cm length × 90 cm width × 76 cm height) consisted of three black-painted arms (A, B and C) at 120° angles to each other. The rats were habituated in the room for Y-maze test for 30 min. Then each rat was placed in one arm and allowed to move freely in the three arms within 8 min. We recorded the orders and time of rats entering into each arm. If the rats entered three diverse arms in turn, it was regarded as a right alternating sequence. If the rats repeatedly entered the identical arm in three consecutive chances, it was considered as
an incorrect alternating sequence. The total time length of rats entering each arm and the frequency of rats entering correct alternating sequence were recorded to assess the spatial orientation learning ability and activity of the rats. To avoid the effects of smell on the experiment, arms and bottoms were cleaned by 75% alcohol before the experiment.

Morris water maze test

Morris water maze (MWM) test is used to assess the spatial learning and memory and the working memory [24]. The maze is consisted of a 160 cm diameter circular water tank, and equally divided into four quadrants (named 1–4 quadrants). The tank was filled with water up to 30 cm, and the water was rendered opaque by adding milk powder. A transparent escape platform (12-cm diameter, 28-cm height) was placed in the 1st maze quadrants (objective quadrant). Swimming track was recorded by a camera capture, and analyzed by MT-200 Morris image motion system (Chengdu Technology and Market Corp, Chengdu, China). The MWM consisted of training trail, probe trail, and visible platform test. Before the training trial, the rats were habituated in the pool without the platform to assess basal swim speed and spatial bias. In the training trail (the 1st-5th days), each rat was randomly placed to different start locations of the pool for every experiment with the hidden platform being maintained in the same quadrant. Swim track and latency to locate the platform were recorded. Every rat received four training periods per day. In probe trail (the 5th day), the escape platform was removed, and each rat swam freely in the pool for 120 s. The time of the rats staying in the target quadrant and the frequency of the rats crossing target quadrant were recorded and calculated. Visible platform test was used to assess visual and sensorimotor skills after the probe test. In visible platform test, the platform was raised 2 cm above the water surface and moved to novel quadrant. The rats were placed in the opposite side of the platform, and the latency to find the platform as well as the average speed were recorded for calculation.

Spermidine Assay

Hippocampus tissues (weighed 60 g) was homogenized with 500 μL MeOH: H₂O (V : V = 4 : 1) and 20 μL 2-Cl-phenylalanine (0.03 mg/ml) for 2 min using a Tissuelyser-192 homogenizer (Shanghai, China). This homogenate of hippocampus tissue was immediately capped in Vials for extraction at –20 °C for 20 min. The extracted samples were then centrifuged at 14,000 g at 4 °C for 10 min and 400 μL of supernatant was transferred to separate vial for GC–MS analysis. The supernatant was evaporated to dryness using a SpeedVac Concentrator System, then subjected to Pyridine hydrochloride solution (15 mg/ml) and vigorously vortex-mixed for 2 min. The supernatant was then collected, subsequently quantified by using BCA™ Protein Assay Kit (Beyotime, Shanghai, China). Equal quantities of total protein (50 μg/lane) were electrophoresed through 12% sodium dodecyl sulfate–polyacrylamide gel, then wet electro-transferred to polyvinylidene fluoride (PVDF). The membranes were blocked with 5% milk in tris-buffered saline (TBS-T,150 mM NaCl, 0.1% Tween 20, 20 mM tris, pH 7.4) at room temperature for 2 h, then incubated with primary antibodies including monoclonal antibody for LC3 I/II, Beclin-1, P62, and ODC (diluted 1:1,000) or β-actin (1:2,000) at 4 °C overnight. After being washed with TBS-T for three times, the membranes were incubated in corresponding secondary antibody horseradish peroxidase (HRP)-conjugated Goat anti-rabbit or Goat anti-mouse (1:5,000) in TBS-T with 5% milk for 2 h at room temperature. The membranes were then washed again and visualized by an enhanced chemiluminescence kit (Millipore Corporation, Billerica, MA, USA). Images were captured by chemiluminescent imaging system (Tanon 5200 Multi, Shanghai, China) and the integrated optical density for the protein band was analyzed by Image-J software.

Statistical analysis

Data were expressed as mean ± S.E.M. Between-group effects on escape latency in the MWM task was analyzed by repeated measures and multivariate analysis of variance (ANOVA). The significance of differences between other parameters was assessed by one-way ANOVA, LSD-t was applied to the analysis of variance and multiple comparisons between groups. Differences were considered statistically significant at P < 0.05.

Result

NaHS attenuates hippocampal impaired autophagic flux in STZ-induced diabetic rats

To explore whether enhancing hippocampal autophagic flux is involved in H₂S-improved cognitive function of STZ-induced diabetic rats, we investigated the effect of H₂S on the autophagic flux in the hippocampus of STZ-induced diabetic rats. As shown in Fig. 2A, the number of autophagosomes (F (4, 10) = 14.790, P = 0.0003) was markedly elevated in the hippocampus of STZ-induced diabetic rats, which significantly decreased by treatment with NaHS (100 μmol/Kg). Furthermore, the expressions of LC3-I/II (F (4, 10) = 5.571, P = 0.0127) (Fig. 2B), Beclin-1 (F (4, 10) = 10.190, P = 0.0015) (Fig. 2C), and p62 (F (4, 10) = 8.058, P = 0.0036) (Fig. 2D) were significantly up-regulated in the hip-
pocampus of STZ-induced diabetic rats, which also were significantly reversed by treatment with NaHS (100 μmol/Kg). These data indicated that the autophagic flux was blocked in the hippocampus of STZ-induced diabetic rats, which was reversed by treatment with NaHS and suggested the involvement of enhancing hippocampal autophagic flux in the improving role of H2S in the cognitive function of STZ-induced diabetic rats.

NaHS upregulates hippocampal ODC/Spd pathway in the STZ-induced diabetic rats

To explore the mediatory role of hippocampal ODC/Spd pathway in H2S-improved cognitive function of STZ-induced diabetic rats, we first investigated the effect of NaHS on the hippocampal ODC/Spd pathway in STZ-induced diabetic rats. The expression of
ODC (F (4, 10) = 4.738, P = 0.021) (Fig. 3A) and the level of Spd (F (4, 10) = 14.019, P = 0.0004) (Fig. 3B) were decreased in the hippocampus of STZ-induced diabetic rats. However, after treatment with NaHS (100 μmol/Kg), the expression of ODC (F (4, 10) = 4.738, P = 0.021) (Fig. 3A) and the level of Spd (F (4, 10) = 14.019, P = 0.0004) (Fig. 3B) in the hippocampus of STZ-induced diabetic rats were increased. Our data indicate that NaHS up-regulates the ODC/Spd pathway in the hippocampus of STZ-induced diabetic rats.

**DFMO blocks NaHS-increased hippocampal spermidine (Spd) in the STZ-induced diabetic rats**

We tested whether DFMO, the inhibitor of ODC, blocks NaHS-increased hippocampal Spd in STZ-exposed rats. As shown in Fig. 4, DFMO (5 μg) markedly decreased the level of Spd (F (4, 10) = 15.369, P = 0.0002) in the hippocampus of rats cotreated with NaHS and STZ.

NaHS and STZ, which indicate that DFMO blocks the ODC/Spd pathway in the hippocampus of rats cotreated with NaHS and STZ.

**DFMO reverses NaHS-attenuated cognitive impairment in STZ-induced diabetic rats**

To investigate the mediaty role of hippocampal ODC/Spd pathway in NaHS-attenuated cognitive impairment in STZ-induced diabetic rats, we further explored whether DFMO alters the improving role of H2S in the cognitive function of STZ-exposed rats using the NOR, Y-maze, and MWM tests.

In the NOR test, NaHS (100 μmol/Kg) significantly increased the discrimination index of STZ-induced diabetic rats; however, DFMO (5 μg) significantly decreased the discrimination index of rats cotreated with NaHS and STZ (F (4, 36) = 11.030, P < 0.0001) (Fig. 5A). Furthermore, there was no difference in the total amounts of exploration time among the five groups (F(4, 36) = 1.773, P = 0.1558) (Fig. 5B). These findings revealed that inhibited hippocampal ODC/Spd pathway reverses NaHS-attenuated cognitive impairment in STZ-induced diabetic rats.

In Y-maze test, treatment with NaHS (100 μmol/Kg) ascended the correct rate in STZ-exposed rats, which was reversed by cotreatment with DFMO (5 μg) (F (4, 37) = 7.185, P = 0.0002) (Fig. 5C), while the total number of entries had no difference among the five groups (F(4, 37) = 0.465, P = 0.761) (Fig. 5D). These data also indicated that inhibited hippocampal ODC/Spd pathway reverses NaHS-improved working memory in STZ-induced diabetic rats.

We further explored the effect of DFMO on the improving role of NaHS in the cognitive function of STZ-treated rats using the MWM test. In the 5th day of acquisition phase, NaHS (100 μmol/Kg) simplified the swimming routes (Fig. 5E) and shortened the escape latency to the hidden platform in STZ-exposed rats (F (4, 28) = 3.053, P = 0.033) (Fig. 5F), which were significantly reversed by cotreatment with DFMO (5 μg), indicating that DFMO prevents NaHS-improved spatial learning in STZ-exposed rats. In the probe trial, NaHS (100 μmol/Kg) increased the proportionality of swimming time in target quadrant (F (4, 35) = 5.368, P = 0.0018) (Fig. 5G) and the times of crossing platform (F (4, 35) = 4.303, P = 0.0062) (Fig. 5H) in STZ-treated rats, which were markedly reversed by cotreatment with DFMO (5 μg), indicating that DFMO abolishes NaHS-improved spatial memory of STZ-exposed rats. In order to rule out the possibility of the alterations of vision and

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**Fig. 3. Effects of NaHS on the ODC/Spd pathway in the hippocampus of STZ-exposed rats.** A, Expression of ODC was tested by western blot using anti-ODC antibody. B, The level of Spd in the hippocampus was detected by GC–MS. Values are means ± SEM. n = 3. ***P < 0.001, ##P < 0.01, vs control group; *P < 0.05, vs STZ group.

**Fig. 4. Effects of DFMO on NaHS-increased the level of Spd in the hippocampus of STZ-exposed rats.** The level of Spd in the hippocampus was detected by GC–MS. Values are means ± SEM, n = 3. ***P < 0.001, vs control group; ###P < 0.001, vs STZ group; &&P < 0.01, vs NaHS and STZ cotreated group.
Fig. 5. Effect of DFMO on NaHS-improved cognitive function in STZ-induced diabetic rats. After once injected with STZ (55 mg/kg, i.p.), the rats were treated with NaHS (100 μmol/kg/d, i.p.) and DFMO (5 μg/d, i.p.) for 30 days. A-B, in the Novel object recognition test, the discrimination index (A) and total object exploration (B) were recorded. C-D, in the Y-maze test, the correct rate (C) and total entries number (D) were recorded. E-J, Morris water maze test was used to detect the ability of spatial learning and memory. E-F, in the acquisition phase, the swimming tracks of rats finding for the underwater platform at the 1st and 5th training days (E), the latency to find the underwater platform during 5 days (F) were recorded. G-H, in the probe trail test, the percentage of time in target quadrant (G) and the number of times that the rats crossed the platform (H) were recorded. I-J, in the visible platform test, the latency to reach the platform (I) and the average speed of rats (J) were recorded. Values are means ± SEM, (n = 6–9). ***P < 0.001, *P < 0.05, vs control group; ###P < 0.001, #P < 0.05, vs STZ group; &&P < 0.01, &P < 0.05 vs STZ and NaHS co-treated group.
motor ability, we performed a visible platform test. All groups showed no difference in the latency to the platform \( F (4, 35) = 0.572, P = 0.6849 \) (Fig. 5I) and the average speed \( F (4, 35) = 1.209, P = 0.324 \) (Fig. 5J), which indicated no visual perception and swimming capability change in these experiments. Together, these data from the MWM test also indicated that inhibited hippocampal ODC/Spd pathway reverses NaHS-attenuated cognitive impairment in STZ-induced diabetic rats.

Next, we examined the effect of DFMO on NaHS-improved autophagic flux in the hippocampus of STZ-induced diabetic rats. After treatment with DFMO (5 µg), the number of hippocampal autophagosomes \( F (4, 10) = 12.100, P = 0.0008 \) (Fig. 6A) was increased as well as the expressions of LC3-II/I \( F (4, 10) = 12.724 \), Beclin-1 \( F (4, 10) = 12.090 \), and P62 \( F (4, 10) = 12.890 \) (Fig. 6B-D). Values are means ± SEM, \( n = 3 \). ***\( P < 0.001 \), **\( P < 0.01 \), vs control group; ###\( P < 0.001 \), ##\( P < 0.01 \), &\( P < 0.05 \), vs STZ group; &&&\( P < 0.001 \), &&\( P < 0.01 \), &\( P < 0.05 \) vs STZ and NaHS cotreated group.

**DFMO prevents the improving effect of NaHS on autophagic flux in the hippocampus of STZ-induced diabetic rats**
Based on the regulatory role of H2S in autophagic flux [26,27], the hippocampus of STZ-induced diabetic rats treated with NaHS (100 μmol/Kg), which indicated that inhibited hippocampal ODC/Spd pathway reverses H2S-improved autophagic flux in STZ-induced diabetic rats.

**Discussion**

It has been confirmed that ODC/Spd pathway plays a key role in diabetic cognitive impairment [25]. We have previously demonstrated the improving role of H2S in the cognitive function of STZ-induced diabetic rats [8]. Based on the regulatory role of H2S in autophagic flux [26,27], the present work was to investigate the involvement of enhancing hippocampal autophagic flux in H2S-improved cognitive function in the STZ-exposed rats and the mediatory role of hippocampal ODC/Spd pathway. Our findings are as follows: (1) NaHS up-regulated hippocampal ODC/Spd pathway and enhanced the hippocampal autophagic flux in STZ-induced diabetic rats; (2) Blocked hippocampal ODC/Spd pathway reversed the improving roles of H2S in the hippocampal autophagic flux and cognitive function of STZ-induced diabetic rats. These results indicated that the improving role of H2S in the cognitive function of STZ-induced diabetic rats involves enhancement in hippocampal autophagic flux, as a result of up-regulation of hippocampal ODC/Spd pathway.

The rate of diabetes doubled over the past three decades worldwide, and diabetic cognitive impairment increased accordingly [28,29]. Our previous study found that H2S inhibits the cognitive dysfunction in STZ-induced diabetic rats [8]. A better understanding of why H2S produces an inhibitory role in diabetic cognitive dysfunction may provide novel information critical for the development of strategy for treatment of diabetic cognitive dysfunction based on H2S. Diabetes is known to be associated with the damage of hippocampus, which is often believed to be the cause of learning and memory deficits [30,31]. The damage of hippocampus in diabetes is attributed to the impairment in autophagic flux [10,11,32]. Therefore, the present work explored the changes in the hippocampal autophagic flux to reveal the mechanisms underlying the improving role of H2S in the cognitive function of STZ-induced diabetic rats. Impaired autophagic flux leads to autophagosome accumulation [33–35]. The rising amount of LC3-II/I provides a good index of accumulation of autophagosomes [9]. Beclin-1 is a protein required for the initiation of autophagosome formation and is frequently used as a marker for autophagy [36,37]. P62 is an autophagic cargo protein, which is degraded by lysosomal enzymes and used to determine whether the increase in autophagosomes is due to activation of autophagy or blockade of lysosomal degradation [37,38]. In the present work, we found that the number of autophagosomes corresponded with the levels of LC3-II/I, Beclin-1, and P62 were increased in the hippocampus of STZ-induced diabetic rats, which implied that the fusion of the autophagosomes and lysosomes in the hippocampus of STZ-induced diabetic rats is blocked, meaning that the hippocampal autophagic flux is impaired in the STZ-induced diabetic rats. Notably, treatment with NaHS (100 μmol/Kg) markedly decreased the number of autophagosomes as well as the expressions of LC3-II/I, Beclin-1, and P62 in the hippocampus of STZ-exposed rats, which indicated the improving role of NaHS in the hippocampal autophagic flux of STZ-induced diabetic rats. It has been confirmed that impaired autophagic flux contributes to the pathophysiologic cascade relating with cognitive impairment, while the cognitive impairment is reversed when autophagic flux is restored [25]. Therefore, we suggest that H2S-improved hippocampal autophagic flux contributes to its inhibitory role in the cognitive dysfunction of STZ-induced diabetic rats.

Spd plays an important role in accelerating the autophagic flux [39,40], resulting in the improvement of learning and memory [41]. Therefore, we explored whether hippocampal ODC/Spd pathway mediates H2S-improved hippocampal autophagic flux and cognitive function in STZ-exposed rats. We found that the expression of ODC and the level of Spd were decreased in STZ-induced diabetic rats and that treatment of NaHS increased the expression of ODC and the level of Spd in STZ-induced diabetic rats, which indicated that NaHS up-regulates the pathway of ODC/Spd in the hippocampus of STZ-induced diabetic rats. DFMO is an irreversible inhibitor of ODC [18], and we found that DFMO prevented NaHS-increased the level of Spd in the hippocampus of STZ-exposed rats, validating that DFMO prevents the up-regulatory role of NaHS in the hippocampal ODC/Spd pathway of STZ-exposed rats. Furthermore, we also found that DFMO increased the number of autophagosomes as well as the levels of LC3-II, Beclin-1, and P62 in the hippocampus of rats cotreated with NaHS and STZ. These results revealed that the hippocampal ODC/Spd pathway mediates the improving role of H2S in the hippocampal autophagic flux of STZ-exposed rats.

To investigate whether the ODC/Spd pathway is the underlying mechanism of NaHS-enhanced autophagic flux to inhibit cognitive impairment in the STZ-exposed rats, we explored the effect of DFMO on the improving role of H2S in the cognitive function of STZ-exposed rats. In present study, the NOR, Y-maze, and MWM tests demonstrate that DFMO prevents NaHS from attenuating the cognitive impairment in the STZ-exposed rats, which indicates that ODC/Spd pathway mediates the improving role of H2S in the cognitive function of STZ-induced diabetic rats. It has been confirmed that Spd improves learning and memory by enhancement in autophagic flux [41]. Therefore, these results suggest that the improving role of H2S in the cognitive function of STZ-induced diabetic rats is mediated by upregulation of hippocampal ODC/Spd pathway, which in turn improves the hippocampal autophagic flux.

**Conclusion**

In conclusion, we demonstrated that NaHS restored the autophagic flux and up-regulated the ODC/Spd pathway in the hippocampus of STZ-induced diabetic rats. Furthermore, blocking hippocampal ODC/Spd pathway reversed the protective roles of NaHS against the hippocampal autophagic flux impairment and the cognitive dysfunction in STZ-induced diabetic rats. Our data revealed that up-regulation of hippocampal ODC/Spd pathway, which in turn enhances hippocampal autophagic flux, plays a critical role in the improving effect of H2S on the cognitive function of STZ-induced diabetic rats. These findings offer novel insights into the mechanisms underlying the inhibitory role of H2S in the cognitive impairment of STZ-induced diabetic rats and highlight a promising therapeutic strategy for the diabetic cognitive dysfunction.

**Compliance with Ethics Requirements**

All Institutional and National Guidelines for the care and use of animals (fisheries) were followed.

**Declaration of Competing Interest**

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.
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