Hydrogen Sulfide Promotes Surface Insertion of Hippocampal AMPA Receptor GluR1 Subunit via Phosphorylating at Serine-831/Serine-845 Sites Through a Sulfhydration-Dependent Mechanism

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Keywords

α -Amino-3-hydroxy-5-methylisoxazole-4-propionic acid receptor; GluR1; Hydrogen sulfide; Protein kinases; Protein phosphatase type 2A; Sulfhydration.

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SUMMARY

Aims: Hydrogen sulfide (H_2S) has been widely accepted as a gas neuromodulator to regulate synaptic function. Herein, we set out to determine the effect of H_2S on α -amino-3-hydroxy-5-methylisoxazole-4-propionic acid receptor (AMPA) and its mechanism. **Methods:** BS³ protein cross-linking, Western blot, patch clamp, and biotin-switch assay. **Results:** Bath application of H_2S donor NaHS (50 and 100 μM) rapidly promoted surface insertion of hippocampal AMPAR GluR1 subunit. This effect can be abolished by dithiothreitol (DTT) and mimicked by Na_2S_4 , indicating that a sulfhydration-dependent mechanism may be involved. NaHS increased APMAR-mediated EPSC and led to an elevation of GluR2-lacking AMPAR content. Notably, NaHS did not increase the sulfhydration of AMPAR subunits, but it significantly increased the phosphorylation of GluR1 at serine-831 and serine-845 sites. Postsynaptic signal pathways that control GluR1 phosphorylation, such as protein kinase A (PKA), protein kinase C, and calcium/calmodulin-dependent protein kinases II (CaMKII), were sulfhydrated, activated by NaHS, and these effects can be occluded by DTT. H_2S increased S-sulfhydration of protein phosphatase type 2A (PP2A), which may be partially involved in the activation of signal pathways. **Conclusion:** Our data suggest that H_2S promotes surface insertion of AMPARs via phosphorylation of GluR1, which depends on a sulfhydration-mediated mechanism.

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Introduction

Hydrogen sulfide (H_2S), the third “gasotransmitter” along with nitric oxide (NO) and carbon monoxide, presents at relatively high levels in brain [1,2] and regulates a variety of functions [3–10]. Kimura et al. [9,10] reported that exogenous H_2S increases N-methyl-D-aspartate receptor (NMDAR) responses and facilitates the induction of long-term potentiation (LTP) in the hippocampus, a synaptic model of memory. Although the potential benefits of H_2S for memory have been widely accepted [11–16], the precise mechanism underlying this phenomenon was poorly understood. α -amino-3-hydroxy-5-methylisoxazole-4-propionic acid receptor (AMPA) mediates the majority of fast excitatory synaptic transmission and its dynamic regulation is one of the key elements that allow the nervous system to adapt to environment stimulations. Our laboratory recently showed that H_2S increased the amplitude of evoked excitatory postsynaptic currents

(eEPSCs) mediated by AMPAR in entorhinal cortex [17]. However, the mechanism for H_2S -mediated regulation of AMPARs remains to be established.

Physically, phosphorylation of AMPAR subunits in neuron occurs as an imperative regulatory mechanism that controls their functions [18,19]. Indeed, like many other proteins, phosphorylation of AMPAR is under tight modifications by a large number of protein kinases. Two major phosphorylation sites on the serine residues of the GluR1 C-terminus have been identified: serine-831 and serine-845. Phosphorylation at position 831 (ser831) by protein kinase C (PKC) or calcium/calmodulin-dependent protein kinases II (CaMKII) has long been shown to potentiate the GluR1 current [20–22]. Phosphorylation at position 845 (ser845) by protein kinase A (PKA) has been found to augment the AMPAR currents [23,24]. Although the evidence of the ability of H_2S to activate protein kinases is accumulating [25–30], little is known about the effect of H_2S on the AMPAR phosphorylation.

An interesting question is that how H₂S activates cellular signaling pathways including various protein kinases. Recently, it has been revealed that a novel posttranslational modification on cysteine residues named sulfhydration (SHY, i.e., converts cysteine -SH groups to -SSH) [31–33], which is controlled by H₂S and its oxydates such as hydropersulfides and polysulfides, regulates the function of a wide range of cellular proteins [31–38]. Exogenous supplement of H₂S sulfhydrates various targets and regulates their activity. For instance, H₂S sulfhydrates and inactivates protein tyrosine phosphatase 1B [39], which may result in phosphorylation of protein kinases such as protein kinase-like endoplasmic reticulum kinase. Phosphatases such as protein phosphatase 1 α (PP1 α) and protein phosphatase type 2A (PP2A) control the phosphorylation of postsynaptic kinases [40,41]. Furthermore, the synaptic kinases, including PKA, PKC, and CaMKII could serve as the direct substrates of H₂S. Here, we found a sulfhydration-dependent mechanism that regulates the phosphatases and activates postsynaptic signaling pathways to phosphorylate GluR1 subunit of AMPAR.

Materials and Methods

Animals

All experimental protocols were approved by the Review Committee for the Use of Human or Animal Subjects of Huazhong University of Science and Technology. All the research was conducted in accordance with the *Guide for Care and Use of Laboratory Animals* as adopted and promulgated by the United National Institutes of Health. Male Sprague Dawley rats (220–250 g, 8–9 weeks) were maintained on a controlled 12-h:12-h light/dark cycle at a constant temperature (22 $\pm$ 2°C) and humidity with food and water provided *ad libitum*.

Agents

Sodium hydrogen sulfide (NaHS, 161527), neocuproine (N1501), methyl methanethiosulfonate (MMTS, 64306), dithiothreitol (DTT, 43815), and streptavidin–agarose (S1638) were purchased from Sigma (St. Louis, MO, USA). Biotin-HPDP (21341), horseradish peroxidase (HRP, 31490), and streptavidin–HRP (21140) were from Thermo Fisher Scientific (Waltham, MA, USA). NASPM trihydrochloride (sc-361272) was from Santa Cruz Biotechnology (Dallas, TX, USA) and Na₂S₄ was from Huaxia Reagents (Chengdu, China). Other general agents were purchased from commercial suppliers. NaHS solution was prepared before use and saline was used as the responsive vehicle.

Slice Preparation

Hippocampal slices were prepared as described previously with minor modifications [17]. In brief, the rat brain was removed quickly after the rat was anaesthetized and decapitated, and then transverse hippocampal slices (400 μ m) were made with a Vibratome tissue slicer (VT 1000S; Leica, Solms, Germany) in the slice cutting solution contained 220 mM sucrose, 2.5 mM KCl, 1.3 mM CaCl₂, 2.5 mM MgSO₄, 1 mM NaH₂PO₄, 26 mM NaHCO₃, and 10 mM glucose, pH 7.4, whereas the aCSF contained 129 mM NaCl,

3 mM KCl, 1.25 mM NaH₂PO₄, 1.8 mM MgSO₄, 1.6 mM CaCl₂, 21 mM NaHCO₃, and 10 mM glucose, pH 7.4. After at least 1 h of recovery at room temperature (25 $\pm$ 1°C), an individual slice was transferred to a submerged recording chamber and continuously superfused with aCSF at room temperature (25 $\pm$ 1°C) at a rate of 3–4 mL/min. The NaHS solution was added into the chamber for 30 min. All solutions were saturated with 95% O₂/5% CO₂ (vol/vol). Usually, six to eight fresh dissected hippocampal slices from each rat were used in the study to perform electrophysiological recordings or BS³-cross-linking or biotin-switch assay.

Electrophysiological Recording

Whole-cell patch-clamp recordings were performed according to our previous reports [17]. CA1 neurons were visualized with infrared optics using an upright microscope equipped with a 40 $\times$ water-immersion lens (BX51WI; Olympus, Tokyo, Japan) and infrared-sensitive CCD camera. Excitatory postsynaptic currents (EPSCs) were evoked with a concentric bipolar electrode by electrical stimulation in the presence of γ -aminobutyric acid (GABA)_A receptor antagonist picrotoxin (100 μ M), and for pharmacologically isolated AMPAR-mediated EPSCs, we blocked NMDAR with D-AP5 (100 μ M). For pharmacologically blocked GluR2-lacking AMPAR currents, NASPM (100 μ M) was added.

Surface Receptor Cross-Linking with BS³

Protein cross-linking was performed according to our previous reports [42]. Hippocampal slices were added to Eppendorf tubes containing ice-cold aCSF, which were spiked with 2 mM bis (sulfosuccinimidyl) suberate (BS³; Pierce Biotechnology, Waltham, MA, USA) immediately after addition of the tissue, and were cross-linked for 60 min at 4°C with gentle agitation. This reaction was terminated by quenching with 100 mM glycine (15 min at 4°C) and the slices were pelleted by brief centrifugation, resuspended in ice-cold lysis buffer containing protease and phosphatase inhibitors, homogenized rapidly by sonicating for 5 s, and centrifuged (12,000 g for 15 min at 4°C). The supernatant fraction was aliquoted and stored at –80°C before Western blot. The protein levels of surface (S) and intracellular (I) were transformed into the ratio of S/I. The total proteins were detected without BS³ cross-linking by Western blot using specific antibody and normalized to a loading control (β -actin).

S-Sulfhydration (Biotin-Switch) Assay

Detection of S-sulfhydration by biotin-switch assay was according to the method described in previous study with modifications [31,32]. Briefly, the brain slices of rats with or without NaHS treatment (100 μ M, 30 min) were homogenized in HEN buffer (250 mM Hepes–NaOH pH 7.7, 1 mM EDTA, and 0.1 mM neocuproine) supplemented with 100 μ M deferoxamine, 1% Nonidet P-40, protease inhibitor cocktail, and 1 mM PMSF, sonicated, and then centrifuged (12,000 g, 4°C, 15 min). Tissue lysates were added to HENS buffer (HEN buffer plus 25% v/v SDS, 9:1 v/v) adjusted to 20 mM MMTS at 50°C for 20 min with frequent vortexing. The MMTS was then removed by acetone and proteins precipitated at –20°C for 20 min. After acetone removal, the

proteins were resuspended in HENS buffer and 4 mM biotin-HPDP. After incubation (25°C, 3 h), total biotinylated proteins were detected by using streptavidin–HRP and biotinylated proteins were precipitated by streptavidin–agarose beads for specific protein. The biotinylated proteins were eluted and detected by using specific antibody. For quantitation of protein sulfhydrylation, samples were subjected to blots with total lysates as “loads.”

Western Blotting

The slices treated with NaHS or vehicle were used for performing Western blotting and all the samples were heated for 5 min at 95°C in loading buffer. The procedures were processed according to our previous protocols [42]. Anti- β -actin (1:3000), anti-phospho-PKA-R11 (serine-96, 1:800), anti-PKA (1:1000), anti-GluR1 (1:500), anti-GluR2 (1:500), anti-phospho-GluR1 (serine-831, 1:500), anti-phospho-GluR1 (serine-845, 1:500), anti-PP2A (1:400), and anti-PP1 α (1:300) were purchased from Millipore (Billerica, MA, USA); anti-phospho-CaMKII (1:500), anti-CaMKII (1:300), anti-phospho-PKC (1:800), anti-PKC (1:1000), and anti-phospho-GluR2 (Tyr-869/873/876, 1:1000) were obtained from Cell Signaling Company (San Francisco, CA, USA); anti-ERK1/2 (1:500) and anti-phospho-extracellular signal-regulated kinase (ERK) 1/2 (1:500) were purchased from Santa Cruz Biotechnology (Waltham, MA, USA). ImageJ software (NIH, Bethesda, MD, USA) was used to quantify the detected bands and the results were normalized to the control group. All the samples in each Western blotting experiment were independent from 4 to 7 rats, and the assays were performed two or three replicates for each sample.

Data Analysis

Values were presented as mean $\pm$ SEM. A two-sided unpaired Student's *t*-test and one- or two-way analysis of variance (ANOVA) followed by *post hoc* LSD test were used for the statistical analysis by employing SPSS 10.0 software (SPSS, Chicago, IL, USA). Differences at the $P < 0.05$ level were considered statistically significant.

Results

NaHS Rapidly Promotes Surface Insertion of GluR1 and Leads to a Larger Ratio of GluR2-Lacking AMPAR Current in the Hippocampal Slices

Firstly, we conducted the experiments to determine the effect of NaHS on the hippocampal slices. Hippocampal slices were incubated with vehicle or NaHS (50, 100 μ M) for 30 min. Different samples were assayed to determine levels of total, surface (S), and intracellular (I) GluR1/GluR2. We observed that NaHS (50, 100 μ M) had no effect on total GluR1 expression ($F_{(2,9)} = 0.003$, $P = 0.997$ for factor NaHS; control, 100% $\pm$ 16.78%; 50 μ M, 101.55% $\pm$ 11.82%, $P = 0.940$ vs. control; 100 μ M, 100.98% $\pm$ 13.15%, $P = 0.962$ vs. control; one-way ANOVA with *post hoc* LSD test; Figure 1A) or total GluR2 expression ($F_{(2,9)} = 0.050$, $P = 0.952$ for factor NaHS; control, 100% $\pm$ 6.64%; 50 μ M, 101.76% $\pm$ 9.08%, $P = 0.884$ vs. control; 100 μ M, 103.70% $\pm$

8.94%, $P = 0.760$ vs. control; one-way ANOVA with *post hoc* LSD test; Figure 1B). As shown in Figure 1C, NaHS (50, 100 μ M) significantly increased the surface expression of GluR1 ($F_{(2,9)} = 8.624$, $P = 0.008$ for factor NaHS; control, 100% $\pm$ 18.11%; 50 μ M, 176.11% $\pm$ 10.87%, $P = 0.006$ vs. control; 100 μ M, 176.70% $\pm$ 15.19%, $P = 0.006$ vs. control; one-way ANOVA with *post hoc* LSD test). Conversely, NaHS (50, 100 μ M) failed to increase the surface expression of GluR2 ($F_{(2,12)} = 1.067$, $P = 0.375$ for factor NaHS; control, 100% $\pm$ 7.52%; 50 μ M, 85.28% $\pm$ 8.40%, $P = 0.170$ vs. control; 100 μ M, 92.28% $\pm$ 4.97%, $P = 0.459$ vs. control; one-way ANOVA with *post hoc* LSD test; Figure 1D).

Considering there was no more surface insertion of GluR2 after NaHS treatment, we hypothesized that H₂S may lead to an increase of GluR2-lacking AMPAR. Thus, we observed the effect of H₂S on the ratio of GluR2-lacking AMPAR. In control group, we found that NASPM (100 μ M), a selective antagonist of GluR2-lacking AMPAR, decreased the AMPAR currents to 70.47% $\pm$ 2.66% (post/pre, Figure 1E). After NaHS (100 μ M) incubation, NASPM decreased the AMPAR currents to 61.19% $\pm$ 2.66% (post/pre, $P = 0.025$ vs. control; unpaired Student's *t*-test; Figure 1E), indicating that H₂S may lead to a larger ratio of GluR2-lacking AMPAR-mediated current.

NaHS Promotes Surface Insertion of GluR1 and Increases AMPAR-Mediated EPSCs via a Sulfhydrylation-Dependent Manner

To gain further insight into the effect of NaHS on AMPARs, we explored the possible mechanisms. DTT was used as a main tool to block sulfhydrylation [31]. Biotin-switch assay has revealed that NaHS (100 μ M) treatment significantly increased protein sulfhydrylation and DTT (100 μ M) abolished the NaHS-induced S-sulfhydrylation (control, 100% $\pm$ 7.14%; DTT, 87.48% $\pm$ 6.87%; NaHS, 184.32% $\pm$ 7.87%; NaHS + DTT, 107.77% $\pm$ 9.11%; $F_{(1,12)} = 32.634$, $P = 0.001$ for factor DTT; $F_{(1,12)} = 45.008$, $P = 0.001$ for factor NaHS; and $F_{(1,12)} = 16.861$, $P = 0.001$ for an interaction between these two factors; two-way ANOVA with *post hoc* LSD test; Figure 2A). The surface expression of GluR1 seemed upregulated in DTT group, which may be due to the effect of DTT on the redox status and NMDAR function in the hippocampus. Under the conditions of DTT existence (100 μ M), NaHS (100 μ M) failed to increase the surface expression of GluR1 (control, 100% $\pm$ 11.98%; DTT, 125.09% $\pm$ 18.17%; NaHS, 246.99% $\pm$ 33.13%; NaHS + DTT, 96.50% $\pm$ 10.03%; $F_{(1,20)} = 9.402$, $P = 0.006$ for factor DTT; $F_{(1,20)} = 8.382$, $P = 0.009$ for factor NaHS; and $F_{(1,20)} = 18.430$, $P = 0.001$ for an interaction between these two factors; two-way ANOVA with *post hoc* LSD test; Figure 2B), indicating that NaHS-induced upregulation of GluR1 surface level may ascribe to protein sulfhydrylation. Recent findings indicate that H₂S-derived polysulfide directly mediates the sulfhydrylation of proteins [43]. Considering DTT may not provide sufficient evidence of the proposed sulfhydrylation-dependent mechanism, we asked that whether polysulfide mimics the effect of H₂S on surface level of AMPARs. Na₂S₄ was used as a donor of polysulfide. Na₂S₄ (25 μ M) significantly increased the surface level of hippocampal GluR1 (170.72% $\pm$ 17.69%, $P = 0.003$ vs. control; unpaired Student's *t*-test; Figure 2C), indicating that sulfhydrylation signal directly increased the surface level of GluR1.

Figure 1 NaHS increases the surface expression of GluR1 and leads to a larger ratio of GluR2-lacking AMPAR current in the hippocampus. **(A)** Representative Western blots and statistical histogram showing NaHS (50, 100 μ M) did not affect the total expression of GluR1 ($n = 4$ rats for each group). **(B)** Representative Western blots and statistical histogram showing NaHS (50, 100 μ M) did not affect the total expression of GluR2 ($n = 4$ rats for each group). **(C)** Representative Western blots and statistical histogram showing NaHS (50, 100 μ M) increased the surface expression of GluR1 ($n = 4$ rats for each group, $**P < 0.01$ vs. control). **(D)** Representative Western blots and statistical histogram showing NaHS (50, 100 μ M) did not affect the surface expression of GluR2 ($n = 5$ rats for each group). **(E)** Representative AMPAR currents recorded from the pyramidal neurons in the hippocampal CA1 region (left). Statistical histogram (right) showing with or without NaHS (100 μ M), NASPM decreased the AMPAR currents differently ($n = 4$ –5 neurons from 4 rats, post/pre, $*P < 0.05$ vs. NASPM group). Data are normalized to the untreated control group and presented as mean $\pm$ SEM. One-way ANOVA and post hoc tests (LSD) were used. GluR1(S): surface GluR1; GluR1(I): intracellular GluR1; GluR2(S): surface GluR2; GluR2(I): intracellular GluR2.

Then, we asked that whether the effect of sulfhydrylation on the surface level of AMPARs regulates synaptic function. The effect of protein sulfhydrylation on AMPAR activity was observed. As shown in the Figure 2D, statistical histogram showed an increase in the amplitude of AMPAR currents (post/pre, $161.64\% \pm 7.96\%$). DTT (100 μ M) was pretreated with slices for 15 min. Under the conditions of DTT (100 μ M) existence, NaHS (100 μ M) perfusion failed to increase AMPAR-mediated EPSCs (post/pre, $103.88\% \pm 3.31\%$, $P = 0.001$ vs. control; unpaired Student's t -test; Figure 2D), indicating that NaHS-induced upregulation of AMPAR activity may ascribe to protein sulfhydrylation. Thus, these results clearly demonstrated that protein sulfhydrylation is mainly responsible for NaHS-induced regulation of AMPAR.

NaHS Increases the Phosphorylation, Not the Sulfhydrylation, of the GluR1 Subunit in the Hippocampus

Then, we asked whether NaHS sulfhydrates AMPAR subunit directly. Surprisingly, as shown in Figure 3A,B, NaHS (100 μ M) did not influence S-sulfhydrylation level of GluR1 (control, $100\% \pm 5.05\%$; NaHS, $105.65\% \pm 5.87\%$; $P = 0.482$ vs. control; unpaired Student's t -test) and GluR2 (control, $100\% \pm 5.26\%$; NaHS, $105.14\% \pm 5.19\%$; $P = 0.503$ vs. control; unpaired Student's t -test). Thus, other mechanisms to mediate the potentiating effect of NaHS on AMPAR function must exist. Then, we investigated the effect of NaHS on

Figure 2 NaHS increases the surface expression of GluR1 subunit and AMPAR function via a sulfhydrylation-dependent manner. **(A)** Biotin-switch assay showing NaHS (100 μ M) increased the level of protein S-sulfhydrylation in the hippocampal CA1 region and dithiothreitol (DTT) (100 μ M) abolished the NaHS-induced S-sulfhydrylation ($n = 4$ rats for each group). **(B)** Representative Western blots and statistical histogram showing DTT (100 μ M) prevented the NaHS-induced surface expression of GluR1 ($n = 6$ rats for each group). **(C)** Representative Western blots and statistical histogram showing Na₂S₄ (25 μ M) increased the surface expression of GluR1 ($n = 7$ rats for each group). **(D)** Representative AMPAR currents recorded from the pyramidal neurons in the hippocampal CA1 region (left). Statistical histogram (right) showing NaHS increased the amplitude of AMPAR currents ($n = 11$ neurons from 7 rats) and DTT (100 μ M) prevented the NaHS-induced enhancement of AMPAR currents ($n = 6$ neurons from 5 rats, post/pre). ** $P < 0.01$ versus control and ## $P < 0.01$ versus NaHS group. Data are normalized to the untreated control group and presented as mean $\pm$ SEM.

phosphorylation of the AMPAR GluR1 subunit in the hippocampus. After incubation for 30 min, NaHS (50, 100 μ M) increased phosphorylation of GluR1-ser845 ($F_{(2,18)} = 3.566$, $P = 0.050$ for factor NaHS; control, $100\% \pm 11.37\%$; 50 μ M, $161.40\% \pm 19.07\%$, $P = 0.027$ vs. control; 100 μ M, $156.25\% \pm 21.91\%$, $P = 0.041$ vs. control; one-way ANOVA with *post hoc* LSD test; Figure 3C) and phosphorylation of GluR1-ser831 ($F_{(2,18)} = 13.757$, $P = 0.001$ for factor NaHS; control, $100\% \pm 10.46\%$; 50 μ M, $124.81\% \pm 22.06\%$, $P = 0.261$ vs. control; 100 μ M, $207.09\% \pm 9.34\%$, $P = 0.001$ vs. control; one-way ANOVA with *post hoc* LSD test; Figure 3D). Phosphorylation of GluR2 was not changed after NaHS (100 μ M) treatment (control, $100\% \pm 5.06\%$; 100 μ M, $101.33\% \pm 6.13\%$, $P = 0.792$ vs. control; unpaired Student's *t*-test; Figure 3E).

NaHS Activates PKA, PKC, and CaMKII in the Hippocampus

Postsynaptic signaling pathways such as PKA, PKC, CaMKII, and ERK are known to act as critical regulators of synaptic AMPAR phosphorylation. As shown in Figure 4A–C, after incubation for 30 min, NaHS (50, 100 μ M) increased phosphorylation of PKA ($F_{(2,15)} = 25.853$, $P = 0.001$ for factor NaHS; control, $100\% \pm 7.22\%$; 50 μ M, $137.14\% \pm 6.53\%$, $P = 0.001$ vs. control; 100 μ M, $161.29\% \pm 3.96\%$, $P = 0.001$ vs. control; one-way ANOVA with *post hoc* LSD test), PKC ($F_{(2,15)} = 7.856$, $P = 0.005$ for factor NaHS; control, $100\% \pm 16.81\%$; 50 μ M, $172.69\% \pm 16.14\%$, $P = 0.013$ vs. control; 100 μ M, $198.53\% \pm 21.29\%$, $P = 0.002$ vs. control; one-way ANOVA with *post hoc* LSD test),

Figure 3 NaHS does not change the S-sulfhydrylation of AMPAR but increases the phosphorylation of GluR1. **(A)** Representative Western blots and statistical histogram showing NaHS (100 μ M) did not influence the S-sulfhydrylation level of GluR1 ($n = 6$ rats for each group). **(B)** Representative Western blots and statistical histogram showing NaHS (100 μ M) did not influence the S-sulfhydrylation level of GluR2 ($n = 6$ rats for each group). **(C)** Representative Western blots and statistical histogram showing NaHS (50, 100 μ M) increased the phosphorylation of GluR1-Ser845 ($n = 7$ rats for each group). **(D)** Representative Western blots and statistical histogram showing NaHS (100 μ M) increased the phosphorylation of GluR1-Ser831 ($n = 7$ rats for each group). **(E)** Representative Western blots and statistical histogram showing NaHS (100 μ M) did not influence the phosphorylation of GluR2-Tyr869/873/876 ($n = 4$ rats for each group). * $P < 0.05$, ** $P < 0.01$ versus control. Data are normalized to the untreated control group and presented as mean $\pm$ SEM.

and CaMKII ($F_{(2,15)} = 6.918$, $P = 0.007$ for factor NaHS; control, $100\% \pm 8.75\%$; 50 μ M, $183.83\% \pm 22.08\%$, $P = 0.050$ vs. control; 100 μ M, $245.96\% \pm 41.97\%$, $P = 0.002$ vs. control; one-way ANOVA with *post hoc* LSD test). However, little effect of NaHS on ERK1/2 phosphorylation was observed ($F_{(2,12)} = 0.543$, $P = 0.595$ for factor NaHS; control, $100\% \pm 5.59\%$; 50 μ M, $110.08\% \pm 6.13\%$, $P = 0.338$ vs. control; 100 μ M, $107.68\% \pm 9.15\%$, $P = 0.462$ vs. control; one-way ANOVA with *post hoc* LSD test; Figure 4D). These data indicate that activation of postsynaptic signaling pathways that control AMPAR phosphorylation may mediate the effect of NaHS on the AMPAR.

DTT Abolishes NaHS-Mediated Activation of PKA, PKC, and CaMKII in the Hippocampus

Then, we further asked whether sulfhydrylation mediates NaHS-induced activation of postsynaptic signaling pathways. In the hippocampal slices, incubation with NaHS (100 μ M) significantly increased phosphorylation of PKA, PKC, and CaMKII, but these effects were largely abolished by DTT, as shown in Figure 5A–C. DTT (100 μ M) prevented the NaHS-induced phosphorylation of PKA (control, $100\% \pm 7.13\%$; DTT, $88.25\% \pm 7.75\%$; NaHS, $150.79\% \pm 9.30\%$; NaHS + DTT, $109.08\% \pm 9.58\%$; $F_{(1,12)} =$

Figure 4 Influences of NaHS on the activation of synaptic kinases in the hippocampus. **(A)** Representative Western blots and statistical histogram showing NaHS (50, 100 μ M) increased the phosphorylation of PKA ($n = 6$ rats for each group). **(B)** Representative Western blots and statistical histogram showing NaHS (50, 100 μ M) increased the phosphorylation of PKC ($n = 6$ rats for each group). **(C)** Representative Western blots and statistical histogram showing NaHS (50, 100 μ M) increased the phosphorylation of CaMKII ($n = 6$ rats for each group). **(D)** Representative Western blots and statistical histogram showing NaHS (50, 100 μ M) did not change the phosphorylation of ERK ($n = 5$ rats for each group). * $P < 0.05$, ** $P < 0.01$ versus control. Data are normalized to the untreated control group and presented as mean $\pm$ SEM.

9.885, $P = 0.008$ for factor DTT; $F_{(1,12)} = 17.744$, $P = 0.001$ for factor NaHS; and $F_{(1,12)} = 3.106$, $P = 0.103$ for an interaction between these two factors; two-way ANOVA with *post hoc* LSD test), PKC (control, 100% $\pm$ 8.58%; DTT, 97.25% $\pm$ 5.14%; NaHS, 141.48% $\pm$ 9.98%; NaHS + DTT, 110.59% $\pm$ 7.23%; $F_{(1,16)} = 4.477$, $P = 0.050$ for factor DTT; $F_{(1,16)} = 11.886$, $P = 0.003$ for factor NaHS; and $F_{(1,16)} = 3.132$, $P = 0.096$ for an interaction between these two factors; two-way ANOVA with *post hoc* LSD test), and CaMKII (control, 100% $\pm$ 15.60%; DTT, 97.78% $\pm$ 19.13%; NaHS, 174.63% $\pm$ 27.13%; NaHS + DTT, 86.90% $\pm$ 10.12%; $F_{(1,20)} = 31.300$, $P = 0.001$ for factor DTT; $F_{(1,20)} = 26.551$, $P = 0.001$ for factor NaHS; and $F_{(1,20)} = 20.671$, $P = 0.001$ for an interaction between these two factors; two-way ANOVA with *post hoc* LSD test). These data indicate that NaHS-linked sulphydration underlies its regulation on postsynaptic signaling pathways.

Then, we asked whether synaptic kinases, including PKA, PKC, and CaMKII, served as the direct substrates of H₂S. As shown in Figure 5D–F, it was found that incubation with NaHS (100 μ M) significantly increased sulphydration of PKA (control, 100% $\pm$ 11.19%; DTT, 98.88% $\pm$ 9.38%; NaHS, 215.51% $\pm$ 22.33%; NaHS + DTT, 121.00% $\pm$ 21.22%; $F_{(1,20)} = 10.624$, $P = 0.004$ for factor DTT; $F_{(1,20)} = 22.007$, $P = 0.001$ for factor NaHS; and $F_{(1,20)} = 10.132$, $P = 0.005$ for an interaction between these two factors; two-way ANOVA with *post hoc* LSD test), PKC (control, 100% $\pm$ 8.41%; DTT, 78.71% $\pm$ 8.45%; NaHS, 175.53% $\pm$ 9.16%; NaHS + DTT, 96.60% $\pm$ 11.17%; $F_{(1,16)} = 23.862$,

$P = 0.001$ for factor DTT; $F_{(1,16)} = 20.732$, $P = 0.001$ for factor NaHS; and $F_{(1,16)} = 7.895$, $P = 0.013$ for an interaction between these two factors; two-way ANOVA with *post hoc* LSD test), and CaMKII (control, 100% $\pm$ 8.10%; DTT, 100.07% $\pm$ 10.05%; NaHS, 173.64% $\pm$ 9.70%; NaHS + DTT, 88.94% $\pm$ 13.97%; $F_{(1,20)} = 15.716$, $P = 0.001$ for factor DTT; $F_{(1,20)} = 8.572$, $P = 0.008$ for factor NaHS; and $F_{(1,20)} = 15.768$, $P = 0.001$ for an interaction between these two factors; two-way ANOVA with *post hoc* LSD test). These data strongly supported that H₂S may trigger kinase phosphorylation via sulphydration of themselves.

NaHS Increases S-Sulphydration of PP2A in the Hippocampus

Phosphatases such as PP1 α and PP2A negatively regulate the phosphorylation of postsynaptic kinases. Thus, the effect of NaHS on the sulphydration of PP2A and PP1 α was tested. NaHS (100 μ M) did not affect the S-sulphydration level of PP1 α in the hippocampal CA1 region (control, 100% $\pm$ 5.89%; NaHS, 109.62% $\pm$ 18.57%; $P = 0.517$ vs. control; unpaired Student's *t*-test; Figure 6A). Conversely, NaHS (100 μ M) increased the S-sulphydration level of PP2A in the hippocampal CA1 region (control, 100% $\pm$ 3.49%; NaHS, 150.61% $\pm$ 10.95%; $P = 0.002$ vs. control; unpaired Student's *t*-test; Figure 6B). Thus, H₂S-linked sulphydration of PP2A may be partially involved in the regulation of phosphorylation of kinases that control AMPAR activity (Figure 6C).

Figure 5 S-sulfhydration mediates NaHS-induced activation of synaptic kinases. (A) Representative Western blots and statistical histogram showing dithiothreitol (DTT) (100 μ M) prevented the NaHS-induced phosphorylation of PKA ($n = 4$ rats for each group). (B) Representative Western blots and statistical histogram showing DTT (100 μ M) prevented the NaHS-induced phosphorylation of PKC ($n = 5$ rats for each group). (C) Representative Western blots and statistical histogram showing DTT (100 μ M) prevented the NaHS-induced phosphorylation of CaMKII ($n = 6$ rats for each group). (D) Representative Western blots and statistical histogram showing NaHS-induced S-sulfhydration of PKA ($n = 6$ rats for each group). (E) Representative Western blots and statistical histogram showing NaHS-induced S-sulfhydration of PKC ($n = 5$ rats for each group). (F) Representative Western blots and statistical histogram showing NaHS-induced S-sulfhydration of CaMKII ($n = 6$ rats for each group). ** $P < 0.01$ versus control. * $P < 0.05$, ## $P < 0.01$ versus NaHS. Data are normalized to the untreated control group and presented as mean $\pm$ SEM.

Figure 6 NaHS increases the S-sulfhydration level of PP2A but not PP1 α in the hippocampus. (A) Representative Western blots and statistical histogram showing NaHS (100 μ M) did not affect the S-sulfhydration level of PP1 α in the hippocampal CA1 region ($n = 11$ rats for control, $n = 4$ rats for NaHS). (B) Representative Western blots and statistical histogram showing NaHS (100 μ M) increased the S-sulfhydration level of PP2A in the hippocampal CA1 region ($n = 5$ rats for each group). ** $P < 0.01$ versus control. Data are normalized to the untreated control group and presented as mean $\pm$ SEM. (C) Hypothesis presentation indicating the underlying mechanism for H₂S-induced surface insertion of AMPAR in the hippocampal CA1 region.

Discussion

This study demonstrates a sulfhydration-dependent mechanism that regulates the AMPAR surface expression and activity. H₂S

sulfhydrates protein kinases and phosphatases, activates protein kinases including PKA, PKC, and CaMKII, phosphorylates AMPA GluR1 subunit, and promotes its surface delivery (shown in Figure 6C).

In the present study, we discovered that NaHS (50 and 100 μ M) rapidly promoted surface insertion of AMPAR GluR1 subunit in the hippocampal slices. This finding was consistent with our previous study that demonstrated the regulation of H₂S on the neuronal excitability in the cortex [17]. In the brain, H₂S presents at relatively high levels (50–160 μ M) [1,2] and plays important roles under various physiological and pathophysiological conditions [3–11,15,33,44–48]. The enhancement of AMPAR function and its surface delivery may underlie the potentiating effect of H₂S on hippocampal LTP. The calcium permeability GluR2-lacking AMPARs contribute to pathophysiology of ischemic brain injury [49,50]. We found that NaHS selectively increased the surface expression of GluR1, which led to an elevation of GluR2-lacking AMPAR content. Thus, our observations may provide new information for further understanding the pathophysiological functions of H₂S in the stroke.

Advances in redox biology have revealed that H₂S initiates S-sulfhydration of Cys residues via forming persulfide and alters protein function. A large proportion of key proteins can be S-sulfhydrated by H₂S [32–39] and we hypothesize that H₂S-linked S-sulfhydration mediates its regulation on AMPAR activity. In our experimental settings, DTT (100 μ M) was preincubated with slices for 15 min and the effect of NaHS (100 μ M) on AMPAR-mediated EPSCs was observed under the conditions of existence of DTT. In this condition, NaHS (100 μ M) perfusion failed to increase AMPAR-mediated EPSCs, indicating that H₂S works through a DTT-sensitive manner. However, biotin assay has revealed that NaHS did not increase S-sulfhydration of GluR1 and GluR2 subunits, but increased phosphorylation of GluR1-ser845 and GluR1-ser831, which controls the surface delivery of GluR1. The GluR1-ser831 was phosphorylated by PKC or CaMKII and the GluR1-ser845 was phosphorylated by PKA. We also observed that NaHS (100 μ M) activated postsynaptic signal pathways including PKA, PKC, and CaMKII, via a DTT-sensitive manner. Recent study has reported that NaHS sulfhydrates and inactivates protein tyrosine phosphatases [39]. In our present study, it was observed that NaHS (100 μ M) significantly increased the S-sulfhydrated protein level of protein kinases including PKA, PKC, CaMKII, and protein

tyrosine phosphatase, PP2A. Although the mechanism underlay sulfhydration-dependent control of kinase activity remains unmasked, H₂S may trigger phosphorylation of kinases via sulfhydration of themselves.

Fundamental chemical principle of sulfhydration seems confused because H₂S is referred to as a reducing agent and the redox potential of H₂S makes it hard to react with free thiols. One possibility is that H₂S-induced persulfide generation is mediated by polysulfides, an oxidized impurity in NaHS solutions [43,51]. This reaction may be responsible for various biological actions of H₂S. Another possibility is that H₂S reacts with oxidized thiols in biological systems to give persulfides [52]. Na₂S₄ was used as a donor of polysulfide and we found that Na₂S₄ (25 μ M) significantly increased the surface level of hippocampal GluR1. We hypothesize that H₂S forms polysulfides and then sulfhydrates PKA, PKC, CaMKII, PP2A to increase the activation of protein kinases. In the next study, the role of polysulfides in the synaptic function should be further asked.

In summary, our studies provide evidence that H₂S-linked S-sulfhydration regulates the AMPAR surface expression and activity, possibly by regulating protein kinase and phosphatase activity. These results also suggest that the rise of brain H₂S level may affect the AMPAR function under various physiological and pathophysiological conditions.

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Conflict of Interest

No competing financial interests exist.

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