Hydrogen Sulfide Induced Disruption of Na\(^+\) Homeostasis in the Cortex

Dongman Chao,*‡ Xiaozhou He,‡ Yilin Yang,‡ Gianfranco Balboni,‡ Severo Salvadori,¶ Dong H. Kim,* and Ying Xia*‡†

*The Vivan L. Smith Department of Neurosurgery, The University of Texas Medical School at Houston, Houston, Texas 77030; ‡Department of Pediatrics, Yale University School of Medicine, New Haven, Connecticut 06511; ¶Research Institute of Modern Medicine, The Third Medical College of Soochow University, Changzhou 213003, China; Department of Life and Environment Sciences, University of Cagliari, I-09124 Cagliari, Italy; and ¶Department of Pharmaceutical Sciences, University of Ferrara, I-44100 Ferrara, Italy

†To whom correspondence should be addressed at The Vivan L. Smith Department of Neurosurgery, The University of Texas Medical School at Houston, 6431 Fannin Street, MSE R444, Houston, TX 77030. Fax: (713) 500-7787. E-mail: ying.xia@uth.tmc.edu.

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Maintenance of ionic balance is essential for neuronal functioning. Hydrogen sulfide (H\(_2\)S), a known toxic environmental gaseous pollutant, has been recently recognized as a gasotransmitter involved in numerous biological processes and is believed to play an important role in the neural activities under both physiological and pathological conditions. However, it is unclear if it plays any role in maintenance of ionic homeostasis in the brain under physiological/pathophysiological conditions. Here, we report by directly measuring Na\(^+\) activity using Na\(^+\) selective electrodes in mouse cortical slices that H\(_2\)S donor sodium hydrosulfide (NaHS) increased Na\(^+\) influx in a concentration-dependent manner. This effect could be partially blocked by either Na\(^+\) channel blocker or N-methyl-D-aspartate receptor (NMDAR) blocker alone or almost completely abolished by coapplication of both blockers but not by non-NMDAR blocker. These data suggest that increased H\(_2\)S in pathophysiological conditions, e.g., hypoxia/ischemia, potentially causes a disruption of ionic homeostasis by massive Na\(^+\) influx through Na\(^+\) channels and NMDARs, thus injuring neural functions. Activation of delta-opioid receptors (DOR), which reduces Na\(^+\) currents/influx in normoxia, had no effect on H\(_2\)S-induced Na\(^+\) influx, suggesting that H\(_2\)S-induced disruption of Na\(^+\) homeostasis is resistant to DOR regulation and may play a major role in neuronal injury in pathophysiological conditions, e.g., hypoxia/ischemia.

Key Words: hydrogen sulfide; Na\(^+\) homeostasis; ionotropic glutamate receptor; Na\(^+\) channel; delta-opioid receptor; cortex.

Hydrogen sulfide (H\(_2\)S) has been long known as a toxic environmental gaseous pollutant with the characteristic pungent odor of rotten eggs. Most of the environmental H\(_2\)S comes from the industrial by products released into the atmosphere and from natural sources such as petroleum, sewage, volcanic gases, and sulfur springs. H\(_2\)S is highly toxic and often fatal at higher concentrations, and a chronic exposure at lower concentrations or sublethal dose of H\(_2\)S could be harmful as well (Integrated Risk Information System, 2003; Szabó, 2007). H\(_2\)S toxicity in the central nervous system (CNS) can compromise neural functions (Abe and Kimura, 1996; Kilburn, 2003; Kombian et al., 1993; Partlo et al., 2001), alter metabolism (Elovaara et al., 1978; Hannah et al., 1989), exhaust mitochondrial cytochrome c oxidase and cause its deficiency (Meo et al., 2011), damage brain structures (Gaitonde and Sellar, 1987; Hooser et al., 2000; Solnyshkova, 2003; Solnyshkova et al., 2004), and lead to developmental abnormalities (Hannah and Roth, 1991) and neuronal death/loss (Brenneman et al., 2000; Cheung et al., 2007; Hooser et al., 2000). Therefore, it may cause an encephalopathic- and neuroasthenic-like syndrome and some neurological and/or neuropsychological abnormalities (Gaitonde and Sellar, 1987; Hirsch, 2002; Kilburn, 2003; Meo et al., 2011).

Hydrogen sulfide’s role as a neuromodulator in physiological functioning of the CNS was first recognized in 1996 (Abe and Kimura, 1996). H\(_2\)S is now acknowledged as the third endogenous gaseous transmitter succeeding its two prior cousins—nitric oxide and carbon monoxide (Wang, 2002). H\(_2\)S is produced endogenously in the body by the transsulfuration enzymes like cystathionine-γ-lyase, cystathionine β-synthase (CBS), and the recently discovered 3-mercaptopropylene sulfurtransferase (Kamoun, 2004; Wang, 2002). In the brain, CBS is the main enzyme responsible for the endogenous production of H\(_2\)S. The reported physiological levels in the human, rat, and bovine brain are relatively high, ranging from 50 to 160μM. As a gasotransmitter, H\(_2\)S exerts multiple effects at physiological concentrations in the CNS, for example, it potentiates the activity of N-methyl-D-aspartate receptors (NMDARs) and enhances long-term potentiation (LTP) in the hippocampus associated with learning and memory (Abe and Kimura, 1996). H\(_2\)S induces Ca\(^{2+}\) wave in neurons and glial cells, stimulates intracellular cyclic adenosine monophosphate (cAMP) production, regulates intracellular signaling activity (e.g., protein kinase A [PKA], mitogen-activated protein kinases), scavenges free radical species (such as hydrogen peroxide, nitric oxide, peroxynitrite, and hypochlorous acid), and increases intracellular glutathione (a major and effective antioxidant) levels (Tan et al., 2010).
Abnormality of endogenous H$_2$S production has been observed in several neurological diseases such as stroke (Qu et al., 2006; Ren et al., 2010), and Alzheimer’s disease (AD) (Eto et al., 2002; Gong et al., 2011).

The role of H$_2$S in the pathophysiological conditions in the brain remains incongruent. On the one hand, because of its ability to scavenge free radical species and increase the intracellular glutathione levels, H$_2$S offers neuroprotection against oxidative stress (Tan et al., 2010). In sharp contrast, some studies have showed that H$_2$S, even in physiological ranges, increases neuronal death (apoptosis and necrosis) and recruitment of death-inducing signal complexes associated with NMDAR-dependent pathways (Brenneman et al., 2000; Chen et al., 2011; Cheung et al., 2007; Hooser et al., 2000). As a matter of fact, NMDAR-dependent neuronal injury and functional changes are a well-known phenomenon associated with certain pathological conditions such as ischemia (Aarts et al., 2002; Lee et al., 1999) and AD (Bordji et al., 2011; Chohan and Iqbal, 2006; Doraiswamy, 2003; Farlow, 2004; Hu et al., 2012; Malinow, forthcoming; Parameshwaran et al., 2008). For example, NMDAR-mediated Ca$^{2+}$ overload in ischemic stress can lead to severe neuronal injury/death (Lee et al., 1999), and amyloid beta- and tau–induced neurotoxicity and deleterious effects in synaptic transmission and plasticity that contribute to memory and cognitive deficits in AD have been shown to be mediated by NMDAR (Bordji et al., 2011; Chohan and Iqbal, 2006; Doraiswamy, 2003; Farlow, 2004; Hu et al., 2012; Malinow, forthcoming; Parameshwaran et al., 2008).

Indeed, memantine, a noncompetitive NMDAR antagonist, has been approved in Europe for the treatment of moderately severe to severe AD and is an investigational drug in the United States (Doraiswamy, 2003; Farlow, 2004; Hu et al., 2012; Reisberg et al., 2003). Therefore, it is essential to clearly understand the role of H$_2$S in brain pathophysiology, at various concentrations and under hypoxic or ischemic conditions.

Neuronal function is critically dependent on the maintenance of homeostasis. A disruption of ionic homeostasis is considered to be a key initial step in neuronal injury/death that occurs in many pathological conditions (Chao and Xia, 2010). Na$^+$ is the predominant ion in the extracellular space. Changes in the extracellular sodium concentration ([Na$^+$]$_o$) have profound effects on the cellular functions, e.g., neuronal excitability, intracellular Ca$^{2+}$ homeostasis, pH stability, and glutamate uptake by altering the operating mode of Na$^+$/Ca$^{2+}$ exchange, Na$^+$/H$^+$ exchange, and Na$^+$-glutamate cotransport (Calabresi et al., 1999; Kiedrowski, 2007; Rojas et al., 2007; Sheldon and Church, 2004). A large amount of Na$^+$ influx and the subsequent Na$^+$ overload can induce neuronal injury and death (Chao and Xia, 2010). It is unknown whether H$_2$S has any effects on Na$^+$ homeostasis in the brain. Studies have shown that H$_2$S can modulate the neuronal activity through regulation of ionotropic glutamate receptors (Abe and Kimura, 1996; Cheung et al., 2007). A common feature of ionotropic glutamate receptor channels (including NMDAR channels and non-NMDAR channels) is Na$^+$ permeability (Mayer and Westbrook, 1987). In addition, voltage-gated Na$^+$ channels constitute the major route of Na$^+$ influx into neurons for normal neuronal activities as well as under pathological conditions (e.g., ischemia) (Chao and Xia, 2010; Jarecki et al., 2010). Both ionotropic glutamate receptor channels and voltage-gated Na$^+$ channels have been shown to mediate hypoxic/ischemic Na$^+$ influx in the cortex (Chao et al., 2009; Chao and Xia, 2010; Kang et al., 2009; Chao, He, Yang, Bazzy-Asaad, Lazarus, Balboni, Kim, and Xia, unpublished data). However, it still remains to be determined if these channels are also involved in H$_2$S regulation of Na$^+$ homeostasis.

In the present study, we have tried to determine the role of H$_2$S in Na$^+$ homeostasis at different concentrations by directly measuring Na$^+$ concentration in the cortical slices with Na$^+$ selective electrodes. In addition, we have explored the possible involvement of ionotropic glutamate receptors and Na$^+$ channels in the H$_2$S effect in normoxic conditions. After exploring the pathophysiological role of H$_2$S in the cortical tissues, we also checked the effect of activation of delta-opioid receptors (DOR) on H$_2$S regulation of Na$^+$ homeostasis because DOR has been well documented as a neuroprotector in the cortex (Chao and Xia, 2010). The results of this work will help us to better understand the mechanisms of hypoxic/ischemic injury in the cortex.

**MATERIALS AND METHODS**

**Slice preparation.** The Animal Care and Use Committee of Yale University School of Medicine, accredited by the American Association for Accreditation for Laboratory Animal Care, approved all our experiments. Slices of the frontoparietal cortex were prepared following the protocol described in our previous studies (Chao et al., 2007a,b). Transverse cortical slices (400 μm) were cut from 24- to 32-day-old male C57BL/6 mice (Charles River Laboratories, Wilmington, MA) with a vibratome containing carbogen (95% O$_2$, 5% CO$_2$) saturated ice-cold standard artificial cerebrospinal fluid (ACSF) and then transferred to an incubation holder placed in a beaker containing 150 ml ACSF vigorously aerated with carbogen at ~35°C. Standard ACSF consisted of (in mM) NaCl 125, KCl 3.1, NaHCO$_3$ 26, CaCl$_2$ 2.4, MgSO$_4$ 1.3, NaH$_2$PO$_4$ 1.25, and dextrose 10 at pH 7.4. Slices were used for recording after an equilibration period of at least 90 min in carbogen saturated ACSF at ~35°C. The recordings were made in the outer layer (corresponding to layer II and III) of the cortex.

A slice was transferred to the recording chamber (Model RC-22C; Warner Instrument Co., Hamden, CT) perfused with carbogen saturated ACSF (35.5 ± 0.5°C) at a flow rate of ~3 ml/min. Slices were completely submerged 0.5–1 mm below the ACSF surface in the tissue chamber and kept under normoxic conditions for at least 15 min at ~35.5°C before taking the experimental measurements.

**Measurements of extracellular [Na$^+$].** Extracellular Na$^+$ concentrations ([Na$^+$]$_o$) were measured using Na$^+$-sensitive microelectrodes. Na$^+$-sensitive microelectrodes were prepared as described previously (Kang et al., 2009). Glass pipette–pulled electrodes were silanized by exposure to hexamethyldisilazane for Na$^+$ electrode and subsequently baked at about 180°C for at least 2 h. The microelectrode tips were then broken back to ~0.5 μm. The internal filling solution (150mM NaCl + 10mM 4-(2-hydroxyethyl)-1-piperazinethanesulfonic acid) was injected into the electrode from the back. A cocktail mixture of internal filling fluid for Na$^+$ electrodes (10% Na$^+$ ionophore VI, 89.5% 2-nitrophenyl octyl ether, and 0.5% potassium tetraphenylborate), with height about 1 mm was sucked into the microelectrode tips. The reference
electrode was an Ag/AgCl bridge electrode embedded in 2% agar in 3M KCl solution. Calibrations were carried out by detecting the responses generated in NaCl solutions (5, 10, 20, 50, 80, 100, 120, 150mM NaCl) in triplicate. For each concentration, the average voltage change in three separate tests was used as the final measurement for voltage change. In this range, the electrode response was near ideal, showing a logarithmic relationship to [Na+].

Electrical signals were monitored on an oscilloscope, recorded by a DC amplifier (Model IE-210, LPF 200; Warner Instrument Co.) and digitized by an Axon mini-digitizer acquisition system (Model miniDigi 1A; Axon Instruments, Union City, CA) at a sampling rate of 100 Hz. The following parameters were used for assessment of Na+ activity: (1) the maximal drop in [Na+]o induced by NaHS; (2) the time of occurrence of the maximal drop in [Na+]o induced by NaHS (Tmax), which refers to the time interval from the beginning of NaHS perfusion to the point of maximal [Na+]o drop; and (3) the recovery period that refers to the total time taken for Na+ levels to return to baseline during reperfusion with normal ACSF.

The slices were subjected to further experiments after recording a stable baseline for at least 5 min. All electrophysiological recordings were performed continuously for 40–60 min.

**Drug administration.** Drugs were administered by switching from control superfuse to one containing drug(s) via a six-channel valve-controlled perfusion system (Model VC-6; Warner Instrument Co.). All drugs were perfused for 20 min before induction of [Na+]o drop and continued through the process. Under our experimental condition, H2S from NaHS could easily diffuse to the slice when NaHS reached the slice (theoretically no more than 20 s in maximum for total replacement of ACSF with NaHS-containing one in recording chamber). When NaHS arrives to the chamber, it quickly gets equilibrated with approximately one third of the H2S existing as the undissociated form (H2S), and the remaining two thirds existing as HS− at equilibrium with H2S (Abe and Kimura, 1996; Nagai et al., 2004). Because the perfusion was continuously made all the time, the H2S concentration was then maintained in a stable level in the whole duration of NaHS perfusion. Therefore, our experiment condition is well controlled and duplicable.

**Chemicals.** Na+ ionophore VI (Fluka 71739), 2-nitrophenyl octyl ether (Fluka 73732), sodium tetraphenylborate (Fluka 72018), hexamethyldisilazane (Fluka 52619), and sodium hydrosulfide (NaHS) were purchased from Sigma Chemicals Co. (St Louis, MO). Tetrodotoxin citrate (TTX), (-)-MK 801 maleate, and CNQX disodium salt were purchased from Tocris Cookson Inc. (Ellisville, MI). UFP 512 (H-Dmt-Tic-NH-CH(CH2-COOH)-Bid), a specific ionophore VI (Fluka 71739), 2-nitrophenyl octyl ether (Fluka 72018), hexamethyldisilazane (Fluka 52619), and sodium hydrosulfide (NaHS) were purchased from Sigma Chemicals Co. (St Louis, MO). Tetrodotoxin citrate (TTX), (-)-MK 801 maleate, and CNQX disodium salt were purchased from Tocris Cookson Inc. (Ellisville, MI). UFP 512 (H-Dmt-Tic-NH-CH(CH2-COOH)-Bid), a specific and potent DOR agonist (Balboni et al., 2002), was synthesized by our research team.

As a H2S donor, NaHS was first prepared as a stock solution with the concentration being 1000 times higher than that of the final concentration used in the work and stored in 4°C in a sealed glass bottle immediately before recording, and the appropriate volume was added to ASCF immediately before slice perfusion with NaHS to obtain the expected concentration. As previously documented (Abe and Kimura, 1996; Nagai et al., 2004), with NaHS as a source of H2S, “in physiological saline, approximately one third of the H2S exists as the undissociated form (H2S), and the remaining two thirds exists as HS− at equilibrium with H2S. The use of NaHS enables us to define the concentrations of H2S in solution more accurately and reproducibly than bubbling H2S.”

The extracellular [Na+] was around 152mM in our slices, although most of our experiments were done with 150 and 300mM of NaHS with the maximal concentration being used at 1.2mM. Under such a condition, the influence of sodium ion from NaHS on the electrophysiological experiments is negligible (Abe and Kimura, 1996; Nagai et al., 2004).

NaHS did not change the pH of buffered ACSF (Abe and Kimura, 1996) in perfused slices under our electrophysiological conditions. Indeed, we randomly checked the pH of NaHS-containing ACSF in our experiments and found that even with 1200mM of NaHS, no pH changes of perfused ACSF occurred under our experimental condition.

**FIG. 1.** Extracellular Na+ response to NaHS at different concentrations. The bars indicate the response rate of the examined cortical slices. Note that NaHS at concentration less than 100μM has very little effect on extracellular Na+ activity in all slices. With the increase in its concentration (≥150μM), NaHS evokes a large drop in [Na+]o (a direct index of Na+ influx) in a concentration-dependent fashion in almost all the slices that were investigated. Chi-square test showed a statistically significant difference in comparison with control when the concentration of NaHS increased to 150 (p = 0.0013), 300 (p < 0.0001), 600 (p = 0.0005), and 1200μM (p = 0.0001).

**Statistics.** All data are expressed as mean ± SEM, and the number of experiments (n) refers to the number of slices investigated. Except indicated in the text, one-way ANOVA followed by Newman Keuls test was used for multiple pairwise tests, and two-tailed unpaired Student’s t-test was used for comparison of the two experimental groups. Observations were identified as significant if p < 0.05.

**RESULTS**

**Concentration-Dependent Induction of Na+ Influx by H2S**

To explore whether H2S regulates [Na+]o, we perfused the cortical slices with NaHS. As shown in Figures 1 and 2, NaHS at a concentration less than 100μM has very little effect on [Na+]o. Of the six slices perfused with 100μM NaHS, five did not show any appreciable change in [Na+]o, and the remaining one showed a slow and slight decrease in [Na+]o, as shown in Figure 2B. All these slices showed a large sudden drop in [Na+]o in response to oxygen-glucose deprivation (OGD), indicating that they have a reliable viability. With an increase in concentration (≥150μM), NaHS evoked a large drop in [Na+]o in almost all the slices investigated in a concentration-dependent fashion. With an increase in NaHS concentrations from 150 to 1200μM, there was shortening of the interval to maximal drop of [Na+]o and prolongation of the recovery time (Fig. 2). These results suggest that low concentrations of H2S have little effect on ionic homeostasis; whereas higher levels disrupt the ionic homeostasis in the cortex.

**Effect of Blocking Na+ Channels on H2S-Evoked Na+ Influx**

Voltage-gated Na+ channels comprise the major route of Na+ influx into neurons under both normal and pathophysiological conditions (e.g., ischemia) (Chao and Xia, 2010).
These channels are well-known mediators of hypoxic/ischemic Na$^+$ influx in the cortex (Chao et al., 2009; Kang et al., 2009; Chao, He, Yang, Bazzy-Asaad, Lazarus, Balboni, Kim, and Xia, unpublished data). To investigate their possible involvement in NaHS-induced Na$^+$ influx, we applied TTX, a Na$^+$ channel blocker, to the cortical slices and examined its effect on NaHS-induced disruption of Na$^+$ homeostasis.

In eight of nine cortical slices, NaHS at 150 μM evoked a sudden drop of 92.1 ± 8.4 mM in [Na$^+$]o that appeared after 13.1 ± 1.7 min of NaHS perfusion and took 4.7 ± 0.6 min to recover from peak drop to baseline after reperfusion with normal ACSF. In the presence of TTX (1 μM), all the seven slices that were investigated (100%) did not have any response to NaHS (150 μM) (p = 0.0004, chi-square test), i.e., NaHS (150 μM) did not induce any appreciable change in [Na$^+$]o (Fig. 3A). OGD test showed that these slices had good viability with an obvious sudden drop in [Na$^+$]o in response to OGD in all these seven slices. With an increase in concentration to 300 μM, NaHS evoked a sudden drop of 100.5 ± 3.0 mM in [Na$^+$]o that appeared after 7.7 ± 1.1 min of perfusion with NaHS and took 7.2 ± 0.6 min for recovery from peak drop to baseline after reperfusion with normal ACSF in 12 cortical slices investigated. In the presence of TTX (1 μM), 21% (3 of 14) slices showed no response to NaHS (300 μM) (p = 0.088, chi-square test), and the OGD test in these slices had reliable viability with an obvious sudden drop in [Na$^+$]o in response to NaHS.

**FIG. 2.** NaHS-evoked Na$^+$ influx in the cortical slices. Trace recordings of (A) control, (B–F) 100, 150, 300, 600, 1200 μM of NaHS respectively. (G–I) Statistical results of each recording parameter. ***p < 0.001 as compared with the control; ###p < 0.001 as compared with 100 μM of NaHS; &p < 0.05, &&p < 0.01, &&&p < 0.001 as compared with 150 μM of NaHS; +p < 0.05, +++p < 0.001 as compared with 300 μM of NaHS. Note that NaHS, at the concentrations ≥ 150 μM, evoked a large concentration-dependent fall in [Na$^+$]o in almost all the slices investigated with a significantly shortened interval to maximal fall in [Na$^+$]o and prolonged the recovery time from Na$^+$ drop.
In the presence of MK 801 (10µM) and CNQX (10µM), respectively, 67% (8 of 12) and 73% (8 of 11) slices no longer showed responses to 150µM NaHS (p = 0.011 and 0.006, respectively, chi-square test) (Fig. 3A). In the remaining four slices, MK 801 (10µM) decreased the drop in [Na⁺]o from 92.1 ± 8.4mM to 59.5 ± 20.1mM. Given to the limited number of slices that showed a response to NaHS (150µM) with a huge variation, there is no statistically significant difference between MK 801 group and control with respect to the drop in [Na⁺]o, Tmax, and recovery. Similarly, the remaining three slices in CNQX group also showed no significant change in extracellular Na⁺ activity in response to NaHS (150µM) when compared with the control group.

In 300µM NaHS perfused slices with perfusion of MK 801 (10µM), 18% slices showed no response to NaHS (300µM) (p = 0.124, chi-square test), and the OGD test in these slices had reliable viability with an obvious sudden drop in [Na⁺]o in response to OGD. In contrast, in the presence of CNQX (10µM), there was no increase in the number of the slices that showed a lack of response to NaHS (300µM), all the nine slices investigated showed a greater response of [Na⁺]o to NaHS (300µM) (Fig. 3B). To further explore the effect of MK 801, CNQX on NaHS-induced changes in [Na⁺]o, we analyzed [Na⁺]o changes of slices that showed response to 300µM NaHS. As shown in Figure 4, MK 801 (10µM) significantly attenuated NaHS (300µM)-evoked drop in [Na⁺]o and accelerated the recovery from peak drop (n = 14) but had little effect on the occurrence of peak Na⁺ drop (Tmax). Unlike MK 801 (10µM) that totally blocked NaHS (300µM)-evoked changes in [Na⁺]o in some slices, CNQX (10µM) could not completely prevent NaHS (300µM)-evoked changes in [Na⁺]o in nine slices investigated (Fig. 3B). For all these slices, CNQX (10µM) had no effect on NaHS (300µM)-evoked drop in [Na⁺]o (Fig. 4). The presence of CNQX (10µM) delayed the recovery from peak Na⁺ drop, contrary to the acceleration noted with either TTX (1µM) or MK 801 (10µM) (Fig. 4).

Because MK 801 (10µM) significantly reduced NaHS (300µM)-evoked drop in [Na⁺]o but had little effect on the occurrence of peak Na⁺ drop (Tmax) (n = 14), whereas TTX (1µM) significantly delayed the occurrence of peak Na⁺ drop with little effect on the drop of [Na⁺]o (n = 11), we coapplied TTX (1µM) and MK 801 (10µM) to the cortical slices and examined their combined effect on NaHS (300µM)-evoked disruption of extracellular Na⁺ homeostasis. We observed a complete blockade of NaHS (300µM)-evoked drop in [Na⁺]o following a coperfusion of TTX (1µM) and MK 801 (10µM) in eight of nine slices investigated (89%) (p < 0.0001, chi-square test) (Fig. 3B). The only slice that showed a slight response to NaHS (300µM) is shown in Figure 4. In the presence of both TTX (1µM) and MK 801 (10µM), 20 min of NaHS (300µM) perfusion induced [Na⁺]o only a minor decrease from the baseline (around 152mM) to 126mM with a rapid recovery from Na⁺ drop when normal ACSF was reintroduced in this slice. This observation suggested that TTX (1µM) and MK 801
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FIG. 4. Different roles of Na⁺ channels, NMDAR, and non-NMDAR in NaHS-evoked Na⁺ influx. Trace recordings of (A) control, (B) TTX (1 μM), (C) MK 801 (10 μM), (D) CNQX (10 μM), and (E) TTX + MK 801. (F–H) Statistical results of each recording parameter. *p < 0.05, **p < 0.01, ***p < 0.001 as compared with the control. Note that 300 μM NaHS-evoked Na⁺ influx could be partially attenuated by Na⁺ channel blocker TTX or NMDAR antagonist MK 801 but could not be decreased by non-NMDAR antagonist CNQX.
additively blocked almost all the effects of NaHS on Na\(^+\) homeostasis.

**Effect of DOR Activation on H\(_2\)S-Evoked Na\(^+\) Influx**

Because our previous work has shown that DOR activation reduces Na\(^+\) currents and attenuates anoxia/ischemia-induced Na\(^+\) influx in the cortex (Kang et al., 2009; Chao, He, Yang, Bazzi-Asaad, Lazarus, Balboni, Kim, and Xia, unpublished data), we further investigated if DOR activation can attenuate H\(_2\)S-evoked Na\(^+\) influx. We perfused the cortical slices with UFP 512, a specific and potent DOR agonist (Balboni et al., 2002; Chao et al., 2007a). We chose 5\(\mu\)M UFP 512 for the perfusion because this concentration is more effective in suppressing anoxia/ischemia-induced Na\(^+\) influx in the cortex (Chao, He, Yang, Bazzi-Asaad, Lazarus, Balboni, Kim, and Xia, unpublished data). As shown in Figures 3 and 5, neither 150\(\mu\)M nor 300\(\mu\)M of NaHS-induced disruption of extracellular Na\(^+\) homeostasis could be attenuated by 5\(\mu\)M of UFP 512. The perfusion of UFP 512 (5\(\mu\)M) did not increase the number of slices showing a lack of response to NaHS (150 or 300\(\mu\)M) \((p = 0.881\) for 150\(\mu\)M NaHS, chi-square test). Furthermore, UFP 512 neither decreased the peak drop in [Na\(^+\)]\(_o\), delayed the occurrence of peak drop, nor accelerated the recovery from [Na\(^+\)]\(_o\) drop evoked by NaHS at the concentrations of 150–300\(\mu\)M.

**DISCUSSION**

The major findings in the present study are: (1) NaHS, a donor of H\(_2\)S, in lower concentrations (< 150\(\mu\)M) did not,
whereas in higher concentrations did disrupt the Na⁺ homeostasis under normoxic condition in a concentration-dependent manner; (2) NaHS (150 or 300μM)-evoked Na⁺ influx could be partially blocked by either Na⁺ channel blocker TTX or NMDAR blocker MK 801 but could not be blocked by non-NMDAR blocker CNQX (NaHS in 300μM); (3) TTX and MK 801 additively attenuated almost all of NaHS-induced Na⁺ influx; and (4) activation of the DOR with UFP 512, which significantly attenuated hypoxic/ischemic Na⁺ influx, has little effect on NaHS-evoked Na⁺ influx.

Under physiological conditions, Na⁺ concentrations, like other cations (K⁺ and Ca²⁺), are under a dynamic balance that often fluctuate transiently with neuronal activity within a small range but are brought back to their previous levels via multiple intrinsic mechanisms (Chao and Xia, 2010). A large alteration in extracellular/intracellular Na⁺ concentrations may trigger an event that leads to neuronal injury and death. For example, large Na⁺ influx induces cellular injury manifested by acute functional and morphological changes, e.g., loss of electrophysiological response to stimulus, cell swelling, bleb formation, and membrane injury (Calabresi et al., 1999; Friedman and Haddad, 1994; Hasbani et al., 1998; Itoh et al., 1998; Petrat et al., 2006; Shi et al., 2005). Removal of extracellular Na⁺ or blockade of Na⁺ entry, therefore, prevents hypoxic/ischemic neuronal damage and death (Banasik et al., 2004; Breder et al., 2000; Friedman and Haddad, 1994; Probert et al., 1997; Raley-Susman et al., 2001). In the present study, we found that NaHS in lower concentrations (<150μM) did not evoke an appreciable change in the extracellular Na⁺ homeostasis; however, at higher concentrations (150–1200μM), NaHS evoked a concentration-dependent large decrease in the [Na⁺]o under normoxic condition. This observation suggests that H₂S in relatively lower physiological levels has little effect on Na⁺ homeostasis, but it can disrupt Na⁺ homeostasis at levels beyond physiological. Because Na⁺ overload due to large Na⁺ influx induces neuronal injury and death, high-concentration H₂S-induced disruption of Na⁺ homeostasis may be harmful in the cortex. In fact, exposure to H₂S, even in lower concentrations, has been shown to impair the functions of the CNS (Brenneman et al., 2000; Hannah and Roth, 1991; Kombian et al., 1993). In higher concentrations, H₂S has been reported to suppress synaptic transmission (Abe and Kimura, 1996; Kombian et al., 1993) and cause structural damage (Solnyshkova et al., 2004) and neuronal death (Cheung et al., 2007). Cheung et al. (2007) reported that NaHS at <200μM induces apoptosis, whereas at concentrations >200μM, it induces necrosis in the primary cultured mouse cortical neurons. The necrotic neuronal death, cytoplasmic edema, and the vacuolated appearance in the cerebral cortex were also observed in H₂S-exposed animals (Hooser et al., 2000; Solnyshkova et al., 2004). These features were also observed in the cells with intracellular Na⁺ overload (Friedman and Haddad, 1994; Hasbani et al., 1998; Itoh et al., 1998; Petrat et al., 2006; Shi et al., 2005). Therefore, high-concentration H₂S induced large increase in Na⁺ influx that could be responsible for H₂S-induced necrotic death, cytoplasmic edema, and the vacuolated appearance in the cerebral cortex. In addition, H₂S-evoked disruption of Na⁺ homeostasis in the cortex may also be related to the phenomenon of H₂S inhalation-induced unconsciousness/unresponsiveness seen in both humans as well as the animals. H₂S inhalation has been reported to result in unconsciousness/unresponsiveness in some victim patients as well as livestock (Hirsch, 2002; Hooser et al., 2000; Integrated Risk Information System, 2003; Kilburn, 2003). As has been reported, a large Na⁺ influx causes widespread depolarization of the brain, disappearance of ongoing electrical activity, and interruption/blockade of synaptic transmission (Calabresi et al., 1999; Raley-Susman et al., 2001) and consequently makes the brain unresponsive, hence, unconscious in certain conditions such as stroke, epileptic seizures, and poisoning. The present observation of H₂S-evoked Na⁺ influx could also explain, at least partially, the effect of H₂S inhalation through this phenomenon.

Our data suggest that ionotropic glutamate receptor channels act as a direct mediator of H₂S-evoked Na⁺ influx because the ionotropic glutamate receptor blockers attenuate the H₂S-evoked Na⁺ influx in the cortex, and both NMDA and non-NMDAR channels are permeable to Na⁺ (Mayer and Westbrook, 1987). However, they also mediate H₂S-evoked Na⁺ influx via an indirect mechanism. Besides the permeability to Na⁺, these two receptor channels are also Ca²⁺ permeable (Mayer and Westbrook, 1987). It has been indicated that H₂S can induce the rise in intracellular Ca²⁺ through Ca²⁺ channels and thus induced Ca²⁺ releases from internal Ca²⁺ stores in neurons as well as glial cells (Tan et al., 2010). Increased [Ca²⁺]i can induce Na⁺ entry by activation of plasma Na⁺/Ca²⁺ exchangers to extrude excessive Ca²⁺ (Blaustein and Lederer, 1999). Therefore, ionotropic glutamate receptors also participate indirectly in the Na⁺ entry via Na⁺/Ca²⁺ exchangers in the cortex. We found NMDAR blocker MK 801 attenuates Na⁺ influx evoked by both 150 and 300μM NaHS. In contrast, non-NMDAR blocker CNQX could only attenuate the H₂S-induced Na⁺ influx when H₂S was in lower concentrations but had little effect on Na⁺ influx at higher concentrations of H₂S. This observation was similar to that of H₂S-induced cortical neuronal death made by Cheung et al. (2007) who showed that MK 801 and CNQX could selectively block neuronal death induced by H₂S in lower concentrations. In high concentrations of H₂S, however, only MK 801, but not CNQX, selectively blocked H₂S-induced neuronal death (Cheung et al., 2007). Our results indicate that MK 801 (10μM) significantly diminishes NaHS (300μM)-evoked drop in [Na⁺]o but does not affect the occurrence of peak Na⁺ drop (vs. TTX [1μM] that significantly delayed this interval but did not affect the level of peak drop in [Na⁺]o). Altogether, these observations suggest that NMDAR channels play a more dominant role than non-NMDAR channels in H₂S-induced Na⁺ entry.

The mechanisms underlying H₂S-evoked Na⁺ influx through ionotropic glutamate receptor channels need further elucidation.
One possibility is that H₂S interacts with disulfides bonds or free thiols (Mustafa et al., 2009) in the ionotropic glutamate receptors in a way of redox modulation to modify the gating properties of the receptor channels and therefore increases Na⁺ permeability and Na⁺ influx. This is supported by the notion that disulfide bonds play a role in the modulation of NMDAR function, and H₂S might be able to modify two cysteine residues (Cys744 and Cys798) of NR1 subunit of NMDAR to activate NMDAR (Aizenman et al., 1989; Sullivan et al., 1994). However, this may not be the case under conditions, for example, in NaHS potentiation of the induction of LTP in the hippocampus (Abe and Kimura, 1996). Alternatively, H₂S-induced activation of cAMP/PKA pathway (Kimura, 2000) may be responsible for NMDAR activation and the subsequently Na⁺ influx because it has been shown previously that an increased production of cAMP and PKA activation by H₂S in the neurons and glial cells phosphorylate NMDAR subunits at specific sites so as to enhance NMDA currents (Kimura, 2000; Leonard and Hell, 1997).

Voltage-gated Na⁺ channels constitute the major route for Na⁺ influx into the neurons in normal neuronal activities as well as certain pathophysiological conditions (e.g., ischemia) (Chao and Xia, 2010; Jarecki et al., 2010). H₂S is known to target various ion channels such as K_ATP channels, Ca²⁺ channels, BK channels, and Cl⁻ channels (Tang et al., 2010). It is not known whether H₂S targets voltage-gated Na⁺ channels to regulate Na⁺ influx in the cortex. We found that TTX (1μM) completely abolished Na⁺ influx induced by NaHS at lower concentrations in the cortex in all the slices we investigated and significantly delayed the occurrence of peak Na⁺ drop and accelerated the recovery from peak drop but had little effect on the drop of [Na⁺]o evoked by higher concentration of NaHS (300μM). There are at least two ways to interpret these results. The straightforward explanation is that TTX-sensitive voltage-gated Na⁺ channels are one of the pathways of H₂S-induced Na⁺ influx; therefore, H₂S-induced Na⁺ influx can be blocked by TTX, just like that in 150μM of NaHS-perfusing slices. Another possibility is that TTX-sensitive voltage-gated Na⁺ channels are indirectly involved in H₂S-evoked Na⁺ influx. Perfusion of TTX can lower the excitability and block the synaptic neurotransmission and therefore postpone the occurrence of peak Na⁺ drop, as it does in 300μM of NaHS-perfusing slices. Though a recently published study showed that NaHS increases peak sodium currents in myocytes obtained from the circular smooth muscle layer of human jejunum and rightly shifts the voltage dependence of Na⁺ current inactivation and activation in HEK293 cells heterologously expressing myocyte-type Nav1.5 (Strege et al., 2011), evidence has shown that in the brain, NaHS-induced inward currents of neurons and suppression of synaptic transmission are insensitive to TTX (Abe and Kimura, 1996; Kombian et al., 1993), making it unlikely that voltage-dependent Na⁺ channels may be involved in the brain. In our study, unlike NMDAR blocker that significantly attenuated NaHS (300μM)-evoked fall in [Na⁺]o without affecting the occurrence of peak Na⁺ drop, TTX greatly deferred the onset of NaHS (300μM)-evoked peak Na⁺ influx, while did not affect the peak drop in [Na⁺]o. Therefore, it is very likely that TTX-sensitive voltage-gated Na⁺ channels are indirectly involved in H₂S-evoked Na⁺ influx in the cortex. Coperfusion of TTX (1μM) and MK 801 (10μM) almost completely eliminated the NaHS (300μM)-evoked Na⁺ influx, suggesting Na⁺ channels and NMDAR channels as synergistically acting mediators of H₂S-evoked Na⁺ influx in the brain.

As to the resultant CNS lesions, H₂S shares some common features with anoxia/ischemia (Hooser et al., 2000). In fact, earlier reports indicated that H₂S can inhibit cytochrome oxidase in mitochondria through depleting cellular energy (Smith and Gosselin, 1966; Stene et al., 1976), and H₂S toxic effects deriving from cellular anoxia are comparable to those of cyanide anion (Brierley et al., 1977). As it has been known, anoxia/ischemia induces a massive Na⁺ influx and subsequently triggers neuronal injury and death in the brain (Chao and Xia, 2010), which is similar to the observations of the present study with high-concentration NaHS perfusion in the brain slices. Interestingly, recently published reports on a rat stroke model have shown that H₂S levels as well as the expression of H₂S synthesizing enzymes in the lesioned cortex and hippocampus are significantly high within 24 h of cerebral ischemia, and a pretreatment with high-concentration NaHS further induces an increase in infarct volume and neuronal injury (Qu et al., 2006; Ren et al., 2010), and a latest report also indicated a significant increase in H₂S production in the brain during hypoxic stress (Kwiatkoski et al., 2012). Therefore, H₂S disruption of Na⁺ homeostasis is a likely mechanism of hypoxic/ischemic neuronal injury and death.

The features of H₂S neurotoxicity (Abe and Kimura, 1996; Cheung et al., 2007; Hooser et al., 2000; Kombian et al., 1993; Solnyshkova et al., 2004) are similar, to a certain degree, to that in hypoxic/ischemic conditions (Chao and Xia, 2010; Kmjević, 2008). We previously found that DOR neuroprotection is related to its ability to attenuate anoxic/ischemic Na⁺ influx via regulation of Na⁺ channels in the cortex (Chao et al., 2009; Kang et al., 2009; Chao, He, Yang, Bazzy-Asaad, Lazarus, Balboni, Kim, and Xia, unpublished data). In the present study, we could not show an attenuation of H₂S-evoked Na⁺ influx after DOR activation under similar experimental conditions. DOR attenuation of anoxic/ischemic disruption of ionic homeostasis including Na⁺ influx and K⁺ leakage is largely dependent on the activation of protein kinase C (PKC) and in part on the inhibition of Ca²⁺ entry (Chao et al., 2007a,b; Pameunter and Buck, 2008). In contrast, the response of neurons to H₂S is predominantly cAMP-PKA–dependent (Abe and Kimura, 1996; Kimura, 2000). H₂S possesses the ability to increase intracellular Ca²⁺ levels in neurons as well as glial cells via Ca²⁺ entry and internal Ca²⁺ mobilization, which testifies the role of H₂S as a neurotransmitter to facilitate Ca²⁺-mediated signaling between neurons and glial cells (Tan...
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However, H₂S-induced neuronal injury and death seem independent of Ca²⁺ influx or internal Ca²⁺ mobilization (Cheung et al., 2007). Even though DOR activation can reduce NMDAR-mediated Ca²⁺ influx under anoxic condition (Pamenter and Buck, 2008), our data suggest that DOR activation cannot attenuate H₂S-evoked Na⁺ influx through NMDAR owing to the difference in target and signaling molecules of DOR involved in hypoxia from those of H₂S exposure. On the other hand, our present observations regarding DOR effects on H₂S-evoked Na⁺ influx reaffirm our previous reports that DOR activation specifically attenuates hypoxic/ischemic (vs. H₂S evoked) disruption of ionic homeostasis (such as Na⁺ influx and K⁺ leakage) via PKC-dependent and PKA-independent pathway as well as in part by inhibition of Ca²⁺ entry in the cortex (Chao et al., 2007a,b; Kang et al., 2009; Chao, He, Yang, Bazzy-Asaad, Lazarus, Balboni, Kim, and Xia, unpublished data).

In conclusion, our data suggest that H₂S in physiological concentrations exerts a minor effect on ionic homeostasis as a gasotransmitter, whereas at superphysiological levels it can be harmful and neurotoxic as it can disrupt the ionic homeostasis by markedly increasing Na⁺ influx through its action on ionotropic glutamate receptor channels, which cannot be attenuated by DOR activation that is neuroprotective against hypoxic/ischemic disruption of ionic homeostasis and insults.

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REFERENCES


