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Modulating effect of hydrogen sulfide on gamma-aminobutyric acid B receptor in recurrent febrile seizures in rats

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Abstract

Hydrogen sulfide (H_2S) is recognized as a new neuromodulator in regulating various brain functions. Some of our recent studies showed that H_2S alleviates the hippocampal damage induced by recurrent febrile seizures (FS). In the present study, we used a rat model of recurrent FS and found that sodium sulfhydrate (NaHS, a donor of H_2S) down-regulated the expression of c-fos and increased the expression of gamma-aminobutyric acid B receptor subunits 1 ($GABA_BR1$) and 2 ($GABA_BR2$). Hydroxylamine (an inhibitor of cystathionine b-synthase) up-regulated the expression of c-fos and down-regulated the expression of $GABA_BR2$, but did not change the expression of $GABA_BR1$. These results suggest that H_2S plays a regulatory role through modulating $GABA_BR$ function in the pathogenesis of recurrent FS.
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Keywords: Hydrogen sulfide; Cystathionine b-synthase; Gamma-aminobutyric acid; Receptor; Febrile seizure

Febrile seizure (FS) is a frequently encountered seizure type occurring in 2–4% of children between 6 months and 5 years of age. FS usually does not cause organic neurological abnormalities. However, many people still concern about the risk of neurological sequelae, such as temporal lobe epilepsy, after recurrent FS (Shinnar, 1998; Sloviter and Pedley, 1998; Lewis, 1999). Changes of brain function following FS therefore need to be further identified.

Hydrogen sulfide (H_2S) is recognized as an important intracellular and intercellular messenger, regulating a number of physiological and pathological processes (Eto et al., 2002). Endogenous H_2S is formed from cysteine by the pyridoxal-5′-phosphate-dependent enzymes, cystathionine beta-synthase (CBS) and cystathionine gamma-lyase (Stipanuk and Beck, 1982). In brains, CBS is highly expressed especially in hippocampus, but cystathionine gamma-lyase is undetectable (Abe and Kimura, 1996). H_2S is continuously generated, freely diffuses between biological membranes and shows extensive physiological activities in regulation of brain functions.

Gamma-aminobutyric acid (GABA) B receptor ($GABA_BR$) is a G protein-coupled receptor located at pre- and post-synaptic sites. Stimulation of the post-synaptic receptors generates long-lasting inhibitory post-synaptic potentials, which result in the increase of K^+ conductance and are important for the fine-tuning of inhibitory neurotransmission. At pre-synaptic sites, $GABA_BR$ mediates the release of neurotransmitters, such as GABA and glutamate, by inhibiting the voltage-sensitive Ca^{2+} channels (Kaupmann et al., 1997, 1998; Jones et al., 1998; White et al., 1998; Bowery and Enna, 2000). Consequently, $GABA_BR$ plays a crucial role in maintaining the excitation/inhibition balance in brain.

Our recent studies demonstrated that both $GABA_BR$ subunits and H_2S/CBS system were involved in FS and explored that H_2S functioned as a protective factor in the development of FS (Han et al., 2003, 2005). The mechanism by which H_2S reduces the hippocampal damage in FS is unclear. The present study is, therefore, to investigate whether H_2S play its role through regulating $GABA_B$ receptor subunits during recurrent FS.

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Animal experiment was approved by the Animal Research Committee of Peking University. Neonatal Sprague–Dawley male rats were housed with their mothers under standard laboratory condition until they were weaned at 21days (∼100 g). Our studies started at the age of 21 days. Rats were randomly divided into four groups: control group, FS group, FS with sodium sulfhydrate (NaHS) treatment group (FS + NaHS group) and FS with hydroxylamine (HA) treatment group (FS + HA group). The FS model has been described previously in detail (Jiang et al., 1999; Yang and Qin, 2004). Briefly, rats of control group were put into 37 °C water for 5 min, and rats of the three experimental groups were put into 45.2 °C water until a seizure was observed. Hyperthermia treatment initiates seizures in young rats, manifesting as facial clonus, head nodding, forelimb clonus, rearing (animal in a standing posture aided by tail and lateral spread of hindlimbs due to increased tone) and falling back (Jiang et al., 1999). Water immersion was carried out 10 times, once every 2 days. For rats in FS + NaHS group, NaHS, a donor of H_2S, 56 μmol/kg was administered intraperitoneally. For rats in FS + HA group, HA, an inhibitor of CBS, 12.5 mg/kg was intraperitoneally injected. An equal volume of normal saline was injected in the control and FS groups. Rats were killed within 2 h after the last time of water immersion.

For the measurement of plasma H_2S, 1% zinc acetate 0.5 ml, distillated water 2.5 ml, plasma 0.1 ml, 20 mM *N*,*N*-dimethyl-phenylenediamine dihydrochloride in 7.2 M HCl 0.5 ml and 30 mM $FeCl_3$ in 1.2 M HCl 0.4 ml were sequentially added into a test tube and incubated at room temperature for 20 min. Plasma protein was then removed by adding 10% trichloroacetic acid 1 ml to the reaction mixture and centrifugation. Optical absorbance of the resulting solution at 670 nm was measured using a spectrometer. H_2S concentration in the solution was calculated against a calibration curve obtained from standard H_2S solutions (Yan et al., 2004).

After anesthetized, rats were perfused through heart with normal saline followed by 4% paraformaldehyde in phosphate buffer solution for 30 min. Brain was removed, post-fixed for 20 h, sequentially equilibrated with sucrose up to 30% at 4 °C for 72 h, frozen in liquid nitrogen and stored at −70 °C for further analysis. Brain section of 10 μm thickness was incubated in 0.5% hydrogen peroxide for 10 min. Guinea pig anti-$GABA_BR1$ polyclonal antibody (Oncogene, Cambridge, MA, USA) diluted to 1:500, rabbit anti-$GABA_BR2$ polyclonal antibody (Santa Craz Biotech Inc., CA, USA) diluted to 1:100, rabbit anti-CBS polyclonal antibody (Santa Craz Biotech, CA, USA) diluted to 1:200 and rabbit anti-c-fos polyclonal antibody (Santa Craz Biotech Inc.) diluted to 1:200 were used as the primary antibodies and incubated with brain slides at 4 °C overnight. Goat anti-guinea pig IgG and goat anti-rabbit IgG conjugated with biotin were used as the secondary antibodies. Signals were visualized by streptavidin conjugated with horseradish peroxidase and diaminobenzidine. The yellow-brownish staining on membrane or in cytoplasm represents the signals of $GABA_BR1$, $GABA_BR2$, CBS and c-fos.

Fragments of rat $GABA_BR1$ cDNA (GenBank Y10369, nucleotide number 2521–2890) and rat $GABA_BR2$ cDNA (GenBank AF074482, nucleotide number 2116–2517) were cloned into plasmid pBluescript sk+ (Stratagene, La Jolla, CA). Digoxigenin labeled cRNA probes for in situ hybridization were in vitro transcribed from the two plasmids. Brain section was incubated in 0.3% hydrogen peroxide in methanol for 10 min. After treatment with proteinase K, pre-hybridization was performed at 37 °C for 1 h. Hybridization with labeled probes was carried out at 37 °C for 2 h. The section was then washed in 2 × SSC for three changes. Hybridized signal was detected by anti-digoxigenin antibody conjugated with horseradish peroxidase and diaminobenzidine.

The immunohistochemical signal intensity of $GABA_BR1$, $GABA_BR2$, CBS or c-fos and the mRNA signal intensity of $GABA_BR1$ and $GABA_BR2$ from in situ hybridization were roughly estimated by double blind method under microscope. The yellow-brown membrane or cytoplasm represented positive signals of $GABA_BR1$, $GABA_BR2$, CBS or c-fos. Six slides per rat and six microscopic sights in hippocampus per slide were randomly selected and examined under 40× microscope. The percentage of positive cells in each sight was calculated. The average percentage of positive cells of all the sights in each group represented the expression intensity of $GABA_BR1$, $GABA_BR2$, c-fos or CBS in each group. The intensity of signals in hippocampal neurons was roughly graded as following: (−) no positive signal, (+) the average percentage of positive cells is ∼15%, (++) the average percentage of positive cells is ∼30%, (+++) the average percentage of positive cells is ∼45% and (++++) the average percentage of positive cells is ∼60%.

All rats developed seizures within 5 min following 45.2 °C water immersion. The manifestations and severity of the seizures were similar among rats in FS, FS + NaHS and FS + HA groups. No seizures were found in control group. Immunohistochemistry showed that c-fos expression was up-regulated in FS group, further elevated in FS + HA group but declined in FS + NaHS group. The changes of c-fos were consistent with those from our previous studies (Han et al., 2005). Since c-fos expression is a marker for histological mapping of epileptic neuronal activity (Labiner et al., 1993), the down-regulation of c-fos expression in FS + NaHS group may indicate the lowered neuronal excitability induced by recurrent FS in the presence of elevated H_2S. H_2S functionally affects several neurotransmitter systems known to be involved in FS. For example, the relationship between H_2S and glutamatergic system has been well characterized. H_2S specifically potentiates the activity of NMDA receptors, and hippocampal long-term potentiation (LTP) is altered in CBS knockout mice (Abe and Kimura, 1996; Eto et al., 2002). The increased H_2S concentration in the present study

Table 1
Changes of $GABA_BR$ subunit proteins and mRNAs in hippocampus in rats of different groups

Group	Rats (*n*)	$GABA_BR1$		$GABA_BR2$	
		Protein	mRNA	Protein	mRNA
Control	6	++++	++++	++++	++++
FS	6	++	++	++	++
FS + NaHS	6	++++	++++	++++	++++
FS + HA	6	++	++	+	+

By double blind method, six slides per rat and six microscopic sights in hippocampus per slide were randomly selected and examined under 40× microscope. The percentage of positive cells in every sight was calculated. The average percentage of positive cells of all the sights in each group represented the expression intensity of $GABA_BR1$ or $GABA_BR2$ in each group. The intensity of signals in hippocampal neurons was roughly graded as following: (+) the average percentage of positive cells is ~15%, (++) the average percentage of positive cells is ~30%, (+++) the average percentage of positive cells is ~45% and (++++) the average percentage of positive cells is ~60%. FS: febrile seizures and HA: hydroxylamine.

may play a role in reducing the neuronal hyperexcitability in recurrent FS. However, as the role of excitatory amino acids in FS is yet poorly defined, the effect of H_2S on FS through factors other than excitatory amino acid receptors is also possible.

To investigate the effect of H_2S on $GABA_BR$ subunits, the rats with FS were treated with NaHS, a donor of H_2S. In FS + NaHS group, plasma H_2S elevated (Han et al., 2005) and the expression of $GABA_BR1$ and $GABA_BR2$ were significantly up-regulated to normal level (Table 1; Fig. 1). The above findings indicated that the changes of $GABA_BR$ subunits in recurrent FS were reversed by the enhanced level of H_2S. As a neuromodulator, H_2S activates K^+_{ATP} channels, and regulate the voltage-dependent Na^+ channels in neurons (Kimura, 2002). Ionic homeostasis in neurons may be rearranged in response to the changes of H_2S, which, in turn,

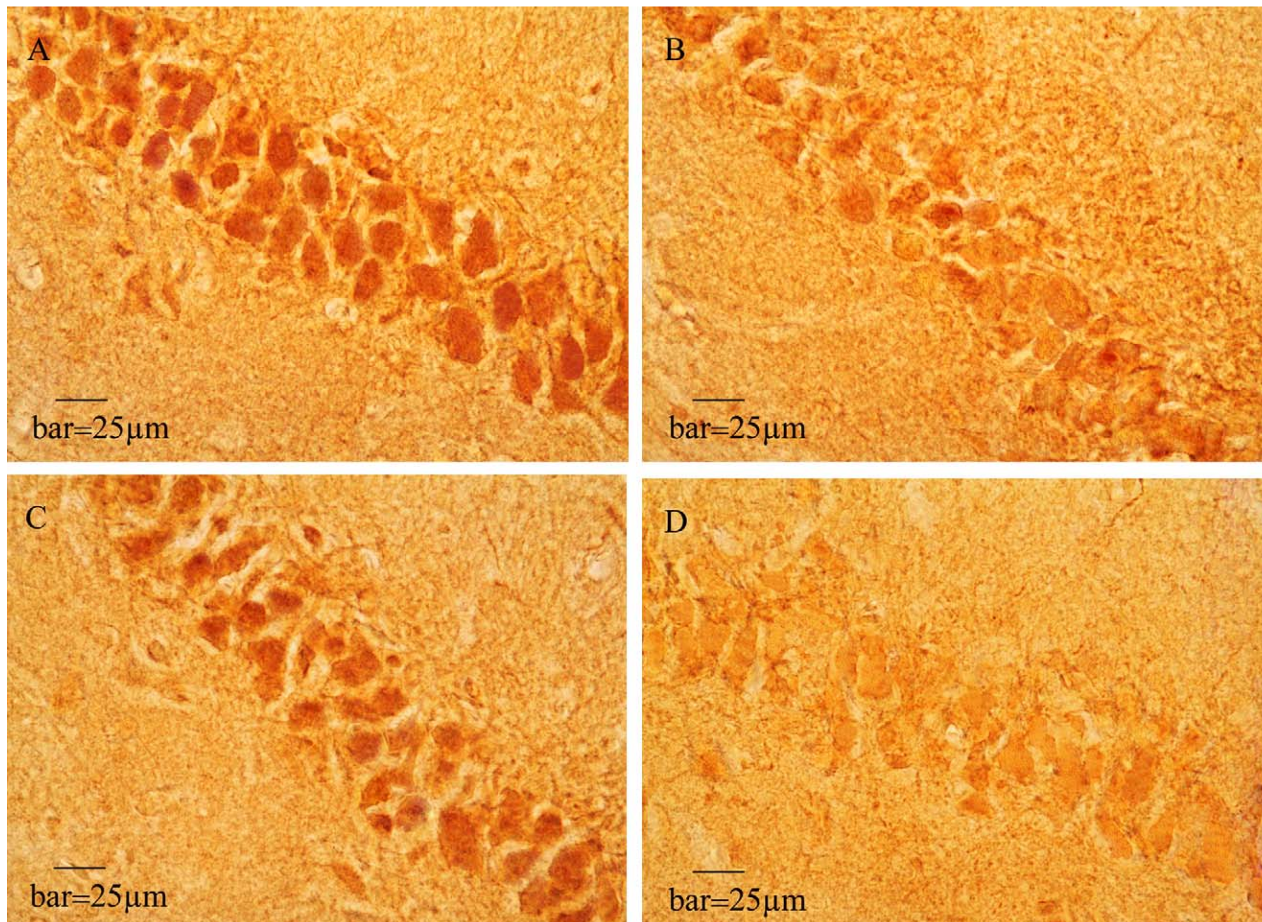

Fig. 1. In situ hybridization showing the changes of $GABA_BR2$ mRNA in hippocampus in rats. (A) Control group, (B) FS group, (C) FS + NaHS group and (D) FS + HA group. The expression of $GABA_BR2$ mRNA decreased notably in FS group (B) compared with that in control rats (A). The expression of $GABA_BR2$ mRNA increased conspicuously in FS + NaHS group (C) while obviously reduced in FS + HA group (D) compared with that in FS rats (B). FS: febrile seizures and HA: hydroxylamine.

may serve as a feedback signal for $GABA_BR$ function. In FS group, the down-regulation of $GABA_BR1$ and $GABA_BR2$ may indicate the imbalance of excitation/inhibition in brain. In FS + NaHS group, $GABA_BR1$ and $GABA_BR2$ returned to normal, therefore, the up-regulation of H_2S may be helpful for the maintenance of excitation/inhibition balance and protection of brain from seizure injury.

In FS + HA group, HA, which reduces the production of endogenous H_2S through inhibiting CBS (Abe and Kimura, 1996), was used. The $GABA_BR2$ expression decreased, while the $GABA_BR1$ expression remained unchanged, as compared with those of FS group (Table 1; Fig. 1). Since the heterodimer of $GABA_BR1$ and $GABA_BR2$ subunits is required for a functional $GABA_B$ receptor (Kaupmann et al., 1997, 1998; Jones et al., 1998; White et al., 1998; Bowery and Enna, 2000), $GABA_BR$ activity will be impaired if one of its subunits is insufficient. We speculate that reduced endogenous H_2S may inhibit the function of $GABA_BR$ through down-regulating $GABA_BR2$, which acts as the messenger in the whole $GABA_B$ receptor function.

H_2S, known as a toxic gas for a long time, is now recognized as a new neuromodulator in regulating various brain functions. Some studies suggest that H_2S affects the neurotoxicity caused by stimulation of excitatory amino acids to NMDA receptors (Abe and Kimura, 1996; Eto et al., 2002). The present study demonstrated that H_2S plays a regulatory role through modulating $GABA_BR$ function in recurrent FS.

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