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# Original Research Communication

# 3-Mercaptopyruvate Sulfurtransferase Produces Hydrogen Sulfide and Bound Sulfane Sulfur in the Brain

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## Abstract

Hydrogen sulfide ($H_2S$) is a synaptic modulator as well as a neuroprotectant. Currently, pyridoxal-5′-phosphate (PLP)-dependent cystathionine $\beta$-synthase (CBS) is thought to be the major $H_2S$-producing enzyme in the brain. We recently found that brain homogenates of CBS-knockout mice, even in the absence of PLP, produce $H_2S$ at levels similar to those of wild-type mice, suggesting the presence of another $H_2S$-producing enzyme. Here we show that 3-mercaptopyruvate sulfurtransferase (3MST) in combination with cysteine aminotransferase (CAT) produces $H_2S$ from cysteine. In addition, 3MST is localized to neurons, and the levels of bound sulfane sulfur, the precursor of $H_2S$, are greatly increased in the cells expressing 3MST and CAT but not increased in cells expressing functionally defective mutant enzymes. These data present a new perspective on $H_2S$ production and storage in the brain. *Antioxid. Redox Signal.* 11, 703–714.

## Introduction

Hydrogen sulfide ($H_2S$) is a multifunctional signaling molecule. It functions as a synaptic modulator in the brain (1, 19, 27). $H_2S$ enhances the activity of NMDA receptors and facilitates the induction of hippocampal long-term potentiation (LTP), a synaptic model of memory (1). $H_2S$ protects neurons from oxidative stress (20, 44, 49, 50). Two forms of glutamate toxicity exist: receptor-initiated excitotoxicity (8) and non–receptor-mediated oxidative glutamate toxicity (24). Oxidative glutamate toxicity is a programmed cell death pathway that is independent of NMDA receptors (24). $H_2S$ protects immature neurons, which do not express NMDA receptors, from the oxidative glutamate toxicity (20, 44), whereas the long-term exposure to $H_2S$ is toxic to neurons that express NMDA receptors (7). $H_2S$ has roles as a smooth muscle relaxant, cardiac protectant, and antiinflammatory as well as proinflammatory factor (4, 12, 14, 15, 21, 22, 38, 54, 56). Despite various roles in many tissues, the production and storage of $H_2S$ is not well understood.

$H_2S$ can be produced from cysteine by the pyridoxal-5′-phosphate (PLP)-dependent enzymes such as cystathionine $\beta$-synthase (CBS) and cystathionine $\gamma$-lyase (CSE) (6, 13, 35, 36, 37). PLP-independent 3-mercaptopyruvate sulfurtransferase (3MST) is another possible candidate for $H_2S$ production (36). 3-Mercaptopyruvate, a substrate for 3MST, is provided through the metabolism of cysteine and $\alpha$-ketoglutarate ($\alpha$-KG) by cysteine aminotransferase (CAT) that is identical with aspartate aminotransferase (2, 43). Because the maximal activity of 3MST is attained in nonphysiologic alkaline conditions (29, 36), its potential contribution to transsulfuration in general has been overlooked.

The sulfur of $H_2S$ can be incorporated into proteins as bound sulfane sulfur, divalent sulfur bound only to other sulfur, such as outer sulfur atoms of the persulfides and inner-chain atoms of polysulfides (48). Bound sulfane sulfur is rapidly labeled when [$^{35}$S]-cysteine is injected into an animal (11), suggesting that cysteine is metabolized to produce sulfide that is incorporated into a pool of bound sulfane sulfur (34). This pool of sulfur releases $H_2S$ under reducing conditions (30, 31).

$H_2S$ may be released immediately after its production by enzymes, as occurs with the release of nitric oxide (NO) from NO synthase (5). Alternatively, $H_2S$ can be transiently stored and then released when the cells are stimulated. For example, exogenously applied free $H_2S$ is immediately absorbed in a sulfur store as bound sulfane sulfur (16, 30, 46), and the

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endogenous bound sulfane sulfur in lysates of neurons and glia release $H_2S$ (16). However, the enzyme that produces endogenous bound sulfane sulfur has not been identified.

The present study shows that $H_2S$ is produced by 3MST from cysteine and α-KG through metabolism by CAT, and that the produced $H_2S$ may immediately be stored as bound sulfane sulfur in the brain. These observations provide a new insight into the production and storage of $H_2S$ in the brain.

## Materials and Methods

### *Plasmid construction*

Coding sequences for 3MST, cCAT, and mCAT were amplified by PCR from the cDNA clone IMAGE-5009481, MGC-3489628, and IMAGE-6832287 (Invitrogen, Carlsbad, CA), respectively. The three amplified fragments were ligated with pCI-neo Mammalian Expression Vector (Promega, Madison, WI) to generate 3MST/pCI, cCAT/pCI, and mCAT/pCI.

To construct expression vectors for 3MST mutants (R187G, R196G, and C247S), site- directed mutagenesis was performed with 3MST/pCI by using a PrimeSTAR mutagenesis basal kit (Takara Bio, Shiga, Japan), as described by the manufacturer. The following mutagenic primers were used: (a) R187G, (forward) 5′-agctggcggttccaaggcacccagc-3′ and (reverse) 5′-tggaaaccgccagctgcgcgggcgtc-3′; (b) R196G, (forward) 5′-agaacccggagatggcatcgaacctg-3′ and (reverse) 5′-ccatctccgggttctggctgggtgcc-3′; (c) C247S, (forward) 5′-agccacgagtggctccggtgtcacag-3′ and (reverse) 5′-gagccactcgtggctaccaagggctt-3′. The underlined portions of the forward and reverse primers represent mutagenized sequences. The coding sequences for mutagenized 3MST were confirmed by DNA sequencing (Bio Matrix Research, Chiba, Japan).

### *Transient transfection of HEK 293-F cells*

Transient transfection of human embryonic kidney HEK 293-F cells in suspension cultures was performed by using a FreeStyle 293 Expression System (Invitrogen). HEK 293-F cells efficiently express externally applied expression plasmids and grow in higher densities in suspension cultures than in regular plating. For transfection, 9.5 μg of expression plasmids and 0.5 μg of pCMV SPORT-βgal were mixed with 15 μl of transfection reagent 293fectin and then added to $1\times10^7$ cells in T-100 flasks with 10 ml of FreeStyle 293 Expression Medium. Cells were incubated with shaking at 125 rpm on a rotary shaker NR-3 (TAITEC, Saitama, Japan) at 37°C in a humid atmosphere with 10% $CO_2$. Transfection efficiency was >90%. The transfected cells were harvested at 48 h after transfection.

### *Determination of $H_2S$-producing activity*

All animal procedures were approved by the National Institute of Neuroscience Animal Care and Use Committee. CBS-knockout mice were obtained from Jackson Lab, which was initially established by Watanabe *et al*. (47), and their strain background was C57Black 6. Whole-brain homogenates were prepared from male C57BL/6N mice (Clea Japan, Tokyo, Japan) with nine volumes of ice-cold buffer A consisting of 100 m*M* potassium phosphate, pH 7.4, 1 m*M* dithiothreitol (DTT), and protease inhibitor cocktail "complete" (Roche Diagnostics, Mannheim, Germany) by using a Potter-type glass homogenizer with a Teflon pestle (700 rpm, 10 strokes). HEK 293-F cells ($1\times10^7$ cells) were precipitated by centrifugation at 1,000 *g* for 5 min. After washing with ice-cold PBS, the cell pellets were resuspended with ice-cold buffer A and sonicated for 10 sec by using a sonifier (Branson model 450; Branson Ultrasonics, Danbury, CT). For enzyme reactions, 11 μl of substrate was added to 0.1 ml of the homogenate or cell lysates in a 15-ml centrifugation tube to a final concentration, as indicated in the figure legends. The tube containing the reaction mixture was sealed with parafilm and was incubated at 37°C for 50 min. After adding 0.2 ml of 1 *M* sodium citrate buffer, pH 6.0, the mixtures were incubated at 37°C for 10 min with shaking at 125 rpm on a rotary shaker NR-3 (TAITEC) to facilitate release of $H_2S$ gas from the aqueous phase. Two milliliters of approximately 14.5 ml of head-space gas was applied to a gas chromatograph (GC-14B; Shimazu, Kyoto, Japan) equipped with a flame photometric detector and a data processor [C-R8A; Chromatopac (Shimazu)]. The concentrations of $H_2S$ were calculated by using a standard curve of 0–5 nmol of $Na_2S$, a source of $H_2S$.

### *Subcellular fractionation*

Mouse (Clea Japan) whole brains were homogenized with nine volumes of fractionation buffer consisting of 100 m*M* potassium phosphate, pH 7.4, 0.32 *M* sucrose, protease inhibitor cocktail by using a Potter-type glass homogenizer with a Teflon pestle (700 rpm, 10 strokes) and centrifuged at 1,500 *g* for 5 min to remove nuclei and intact cells. The postnuclear supernatant was centrifuged at 17,000 *g* for 15 min to collect synaptosomal and mitochondrial fractions, followed by centrifugation of the postsynaptosomal and mitochondrial fractions at 100,000 *g* for 60 min to obtain cytosolic and microsomal fractions. All pellets were resuspended in a volume of fractionation buffer equivalent to the supernatants.

Fractions of synaptosomes and mitochondria were prepared according to the method of Nagy and Delgado-Escueta (28) with a few modifications. Mouse brains were homogenized with nine volumes of isolation buffer consisting of 5 m*M* HEPES, pH 7.4, 0.32 *M* sucrose, protease inhibitor cocktail complete, and centrifuged at 1,000 *g* for 5 min. The supernatant was centrifuged at 12,000 *g* for 20 min, and the precipitate was resuspended in 3 ml of the isolation buffer per gram of original wet tissue. The resuspended fraction was diluted with eight volumes of 8.5% Percoll isoosmotic buffer (90% Percoll solution diluted 10.6-fold with dilution buffer consisting of 5 m*M* HEPES, pH 7.4, 0.25 *M* sucrose, protease inhibitor cocktail complete). This suspension was layered onto 10 and 20% discontinuous Percoll isoosmotic buffer gradient and centrifuged at 15,000 *g* for 20 min. The precipitates were recovered as a mitochondrial fraction, and the 10 and 20% Percoll interface was as a synaptosomal fraction.

### *Determination of bound sulfur*

HEK 293-F cells ($1\times10^7$ cells) were precipitated by centrifugation at 1,000 *g* for 5 min. After washing with ice-cold PBS, the cell pellets were lysed with 0.5 ml of ice-cold lysis buffer B consisting of 10 m*M* potassium phosphate, pH 7.4, 0.1% TritonX-100, 10 m*M* hydroxylamine, which was used to suppress the activity of PLP-dependent enzymes including CAT, involved in enzymatic $H_2S$ production, and the protease inhibitor cocktail. To lyse intracellular membranes in brain fractions, TritonX-100 was added to a final concentration of 0.5%. The lysates were centrifuged at 12,000 g for 10 min, and

the supernatants were applied onto a PD-10 desalting column (GE Healthcare, Little Chalfont, Buckinghamshire, England) preequilibrated with lysis buffer B containing 150 m*M* NaCl, and the high-molecular-weight fraction (>5,000 M*r*) was recovered.

For measurement of $H_2S$ released from bound sulfur, 0.1 ml of sample (2 mg protein/ml) and 0.1 ml of 15 m*M* DTT in 100 m*M* Tris/HCl, pH 9.0, were placed in a 15-ml centrifugation tube, sealed with parafilm, and then incubated at 37°C for 50 min. After adding 0.4 ml of 1 *M* sodium citrate buffer, pH 6.0, the mixtures were incubated at 37°C for 10 min with shaking at 125 rpm on a rotary shaker NR-3 (TAITEC) to facilitate release of bound sulfur as $H_2S$ gas from the aqueous phase. $H_2S$ concentrations were determined according to the method for determination of $H_2S$ produced by enzymes. A reaction mixture without samples was used as a control for a release of $H_2S$ from DTT.

### *Western blot analysis*

Ten micrograms of protein samples were fractionated by SDS-PAGE on a 12.5% polyacrylamide gel (DRC, Tokyo, Japan) and transferred to a polyvinylidene difluoride membrane (Millipore, Bedford, MA). The membrane was blocked by PBS-T (137 m*M* NaCl, 10 m*M* $Na_2HPO_4$, 2.7 m*M* KCl, 1.8 m*M* $KH_2PO_4$, 0.1% Tween 20) containing 2% skim milk (Wako, Osaka, Japan) overnight at 4°C and incubated with either anti-MPST (1:3,000; Atlas antibodies AB, Stockholm, Sweden), anti-aspartate aminotransferase (AST) [Pig Heart] [Sheep] (1:30,000; Rockland, Gilbertsville, PA), anti-GOT2 polyclonal antibody (1:3,000; Lifespan biosciences, Seattle, WA), rabbit anti-synaptotagmin I affinity purified, polyclonal antibody (1:1,000; Chemicon, Temecula, CA), anti-Mn SOD (1:3,000; Stressgen Bioreagents, Victoria, BC, Canada), G6PD antibody (BL341) affinity purified (1:3,000; Bethyl Laboratories, Montgomery, TX), or anti-cytochrome P450 reductase (1:3,000; Stressgen) for 4 h at 4°C. After additional 2-h incubation with secondary antibodies diluted at 1:10,000 of either horseradish peroxidase–conjugated anti-mouse (GE Healthcare), anti-rabbit (GE Healthcare), or anti-sheep (Rockland), the binding of antibodies was visualized with Millipore Immobilon Western Chemiluminescent HRP substrate (Millipore).

### *Immunohistochemical studies*

Brains were fixed with a paraformaldehyde-based fixative (Genostaff, Tokyo, Japan), and 6-$\mu$m-thick sections were prepared after being embedded in paraffin. Deparaffinized sections were subjected to antigen retrieval by microwave for 10 min and treated with 0.3% $H_2O_2$ in methanol for 30 min at room temperature. Sections were blocked with Dako Protein Block (Dako, Carpentaria, CA) for 10 min at room temperature and incubated with anti-MPST or normal rabbit IgG (2 $\mu$g/ml) overnight at 4°C. After endogenous biotin activity was blocked with Biotin blocking system (Dako), sections were incubated with anti-rabbit IgG Biotin (Dako) for 30 min at room temperature, followed by incubation with horseradish peroxidase–conjugated streptavidin (Nichirei, Tokyo, Japan). Sections were stained with diaminobenzidine and counterstained with hematoxylin. Stained samples were observed with an Axiophoto microscope (Carl Zeiss, Jena, Germany) equipped with a cooled CCD digital camera (Penguin 600CL; Pixera, Los Gatos, CA).

### *Statistical analysis*

All statistical analyses of the data were performed by using Excel 2003 (Microsoft, Redmond, WA) with the add-in software Statcel2 (OMS, Saitama, Japan). Differences between two groups were analyzed with Student's *t* test. Differences between three or more groups were analyzed with one-way analysis of variance (ANOVA). *Post hoc* multiple comparisons were made by using the Bonferroni test.

## Results

### *An enzyme distinct from CBS produces $H_2S$*

When we examined the production of $H_2S$ from brain homogenates of CBS-knockout mice by using cysteine as a substrate but in the absence of PLP at pH 7.4, we found that $H_2S$ was produced at a level similar to that of wild-type mice. No statistical difference was found in the production of $H_2S$ between the homozygous CBS-knockout mice ($0.87 \pm 0.10$ nmol $H_2S$/mg protein, $n = 3$) or heterozygous mice ($0.97 \pm 0.06$ nmol $H_2S$/mg protein, $n = 3$) and the wild-type ($0.99 \pm 0.15$ nmol $H_2S$/mg protein, $n = 3$). These observations suggested that another enzyme produces $H_2S$ in the brain.

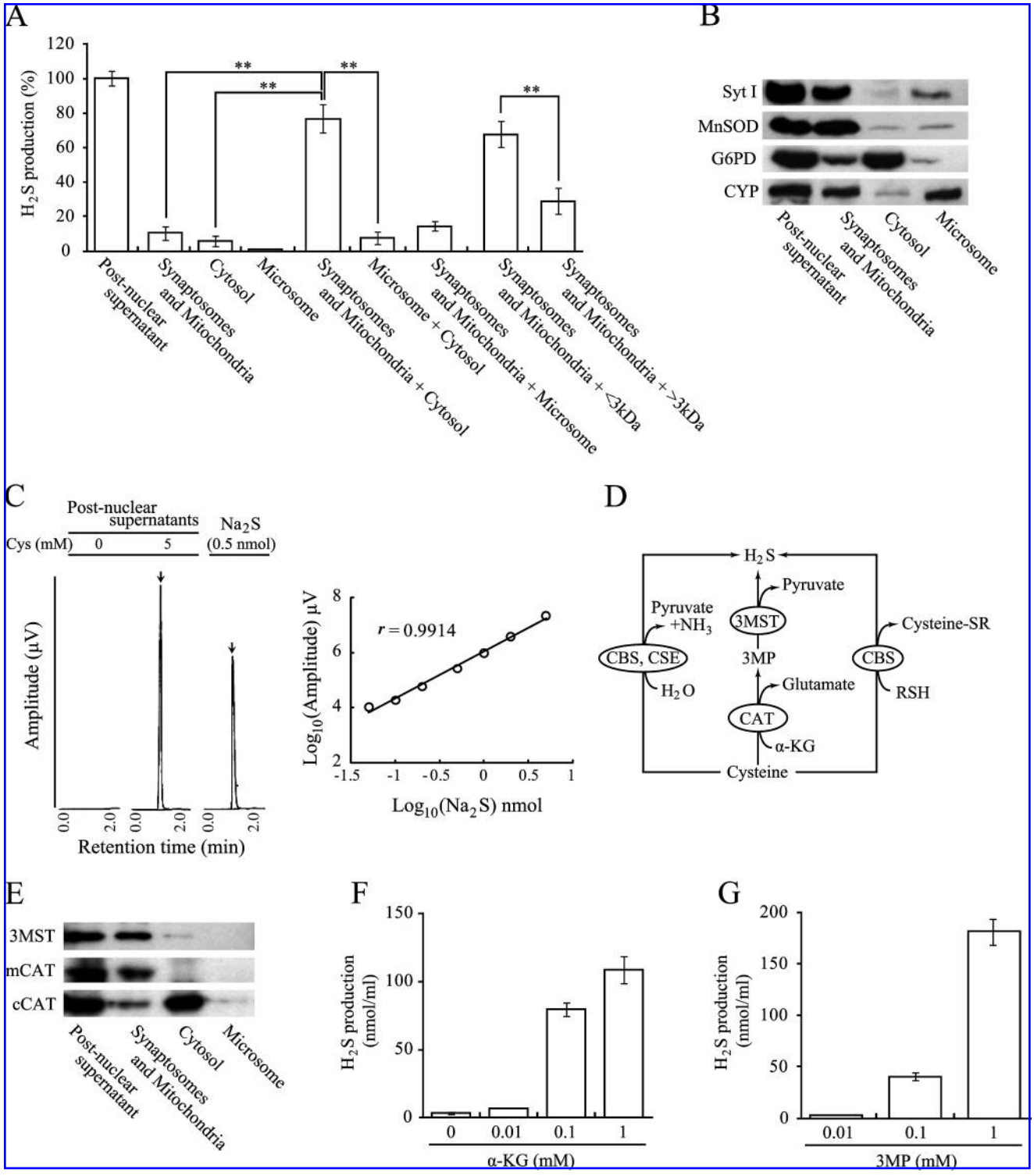
To determine the subcellular localization of the $H_2S$-producing enzyme, we prepared a postnuclear supernatant fraction from brain homogenates of the wild-type mice. The fraction was further fractionated into synaptosomes and mitochondria-, cytosol-, and microsome-enriched fractions. The $H_2S$-producing activity of synaptosomes and mitochondria, cytosol, or microsomes was much weaker than postnuclear supernatant activity, and the activity of even the sum of the three fractions was less than one-fifth of postnuclear supernatant activity (Fig. 1A and B). It is therefore possible that two components in different fractions were required for $H_2S$ production. To examine this possibility, the $H_2S$-producing activity in three combinations of two fractions was measured. The activity of synaptosomes and mitochondria plus cytosol was ~80% of activity of postnuclear supernatant, whereas that of microsome plus cytosol or synaptosomes and mitochondria plus microsome was <15% of activity of postnuclear supernatant (Fig. 1A). These observations suggest that separate components contained in synaptosomes, mitochondria, and cytosol are required for $H_2S$ production.

Cytosol was further fractionated by passing it through a filter that removes substances >3 kDa. The $H_2S$-producing activity of synaptosomes and mitochondria with a fraction of <3 kDa of cytosol showed ~70% of the activity of postnuclear supernatant, whereas synaptosomes and mitochondria with >3 kDa of cytosol showed <30% of the activity of postnuclear supernatant (Fig. 1A). It follows that for $H_2S$ production, a component of cytosol is required along with another component in synaptosomes and mitochondria.

### *3MST with CAT produces $H_2S$*

The existence of 3MST activity has been reported in the brain (41, 52, 53). Because PLP-independent 3MST is a possible alternative to CBS for $H_2S$ production (Fig. 1D) (36) and because its substrate 3-mercaptopyruvate (3MP) can be provided by the metabolism of cysteine and $\alpha$-KG by cysteine aminotransferase (CAT) that is identical with aspartate aminotransferase (Fig. 1D) (2, 43), we asked whether the active synaptosome and mitochondria fraction contains 3MST

and CAT by using Western blot analysis. The synaptosomes and mitochondria fraction contains 3MST, mitochondrial CAT (mCAT), and cytosolic CAT (cCAT) (Fig. 1E). It is therefore possible that the less than 3-kDa factor in cytosol is α-KG. To examine this possibility, α-KG was added to synaptosomes and mitochondria, and the production of $H_2S$ in the presence of cysteine was examined. α-KG enhanced the $H_2S$-producing activity of synaptosomes and mitochondria in a dose-dependent manner (Fig. 1F), suggesting that the less than 3-kDa component is α-KG. Because the synaptosomes and mitochondria fraction contains 3MST, it should produce $H_2S$ in the presence of its substrate 3MP. Figure 1G shows that the synaptosomes and mitochondria fraction produced $H_2S$ from 3MP in a dose-dependent manner. These data indicate that 3MST produces $H_2S$ from 3MP that is generated from cysteine and α-KG by CATs.



To confirm the $H_2S$-producing activity of 3MST in combination with CAT, we prepared lysates of HEK 293-F cells expressing 3MST, cCAT, mCAT, or combinations thereof (Fig. 2A) and measured the amounts of $H_2S$ produced in the presence or absence of their substrates at pH 7.4 (Fig. 2B–D). Lysates of the cells expressing 3MST produced $H_2S$ in the presence of 3MP (Fig. 2B). We next determined whether 3MP is supplied by the metabolism of cysteine and α-KG by CAT. In the presence of cysteine and α-KG, the lysates of cells expressing 3MST with either cCAT or mCAT produced $H_2S$ (Fig. 2C and D). No $H_2S$ was detected in the lysates of cells transfected with an empty vector (data not shown). In the absence of either α-KG or cysteine, even the lysates of the cells expressing both 3MST and CAT produced little $H_2S$ (Fig. 2C and D). Aspartate, a competitive inhibitor of CAT (43), suppressed the $H_2S$ production from cysteine and α-KG in a dose-dependent manner, whereas $H_2S$ production from 3MP was intact even in the presence of aspartate (Fig. 2F and G). These observations indicate that $H_2S$ is produced from cysteine and α-KG by 3MST with the help of CAT. Similar results were obtained with brain homogenates and their fractions (Fig. 2E, H, and I).

### *3MST with CAT increases bound sulfane sulfur in cells*

Because exogenously applied $H_2S$ is rapidly stored as bound sulfane sulfur (16, 30, 46), it is possible that $H_2S$ produced by 3MST may immediately be stored as bound sulfane sulfur in the cells. To examine this possibility, we measured the amounts of bound sulfane sulfur of HEK 293-F cells expressing 3MST, cCAT, mCAT, or their combination. Expression of 3MST with either cCAT or mCAT increased the levels of bound sulfane sulfur ~220 and 200%, respectively (Fig. 3A). Cells expressing 3MST alone increased their endogenous bound sulfane sulfur by 180%, suggesting that endogenous CATs may be sufficient to supply the substrate for 3MST (Figs. 2A and 3A). In contrast, the expression of cCAT alone or mCAT alone only weakly increased the levels of bound sulfane sulfur but failed to reach statistical significance, indicating that the endogenous levels of 3MST are limiting for the production of bound sulfane sulfur (Figs. 2A and 3A). These observations indicate that the levels of bound sulfane sulfur in the cells correlate well with the $H_2S$-producing activity of 3MST.

Because CBS is expressed in the brain (1), we also examined whether the expression of CBS increases the levels of bound sulfane sulfur in HEK 293-F cells. Although expression of CBS weakly increased the levels of bound sulfane sulfur, it was not statistically significant (Fig. 3A).

To confirm that the levels of bound sulfane sulfur were increased by the $H_2S$-producing activity of 3MST, we constructed expression vectors with mutants of 3MST and examined the production of $H_2S$ and the levels of bound sulfane sulfur. Because the cysteine residue at position 247 of 3MST is a catalytic site for 3MP, and the arginine residues at positions 187 and 196 determine substrate specificity (25), we introduced the following mutations: cysteine 247 to serine (C247S), arginine 187 to glycine (R187G), and arginine 196 to glycine (R196G). C247S and R187G diminished the $H_2S$-producing activity of 3MST (Fig. 3B). The levels of bound sulfane sulfur in the cells expressing the C247S mutant remained at the basal level, and those with the R187G mutant were only slightly increased from the basal level (Fig. 3C). The R196G mutant maintained $H_2S$-producing activity at 50% of the wild type and contained a level of bound sulfane sulfur similar to that of the wild type (Fig. 3B and C). These results indicate that the levels of bound sulfane sulfur depend on the $H_2S$-producing activity of 3MST and suggest that $H_2S$ produced by 3MST is stored as bound sulfane sulfur in the cells.

To examine the localization of bound sulfane sulfur within the brain, the amounts of bound sulfane sulfur in fractions of postnuclear supernatant, synaptosomes and mitochondria, cytosol, and microsome were measured. The synaptosomes and mitochondria fraction contained approximately 60% of bound sulfane sulfur of postnuclear supernatant (Fig. 3D). The levels of bound sulfane sulfur and the $H_2S$-producing activity were further examined by fractionating synaptosomes and mitochondria into synaptosomal and mitochondrial subfractions. Both subfractions have similar $H_2S$-producing activity and levels of bound sulfane sulfur (Figs. 3D–F ).

### *The expression and localization of 3MST in the brain*

To examine the developmental changes in the expression of 3MST in the brain, we investigated the relative amount of

**FIG. 1. Subcellular localization of the $H_2S$-producing activity in the brain.** (**A**) The $H_2S$-producing activities of the brain fractions. A postnuclear supernatant was fractionated into synaptosomes and mitochondria-, cytosol-, and microsome-enriched fractions. Each fraction was incubated with 10 m*M* cysteine, and the production of $H_2S$ was measured. A concentration of cysteine was used as in the previous studies (1, 36). Cytosol was further fractionated into two fractions with a molecular weight larger (>3 kDa) than or smaller (<3 kDa) than 3 kDa. $^{**}p < 0.01$. (**B**) Western blot analysis of subcellular fractions of brain homogenates. Antibodies were used against synaptotagmine I (Syt I), manganese superoxide dismutase (MnSOD), glucose-6-phosphate dehydrogenase (G6PD), and cytochrome P450 reductase (CYP) as markers for synaptosomes, mitochondria, cytosol, and microsomes, respectively. (**C**) *Left panel*: Representative chromatographic trace of $H_2S$ released from brain homogenates and $Na_2S$ solution. *Arrows*, $H_2S$ (retention time: 1.2 min). *Right panel*: Calibration curve for determination of $H_2S$ concentrations with gas chromatography. $Na_2S$ was diluted with 0.01 *M* NaOH. The logarithm of the amplitude was plotted against the logarithm of the amounts of $Na_2S$ in a tube. (**D**) Schematic representation of possible pathways of $H_2S$ production from cysteine in mammalian cells. Possible pathways previously demonstrated (36) were summarized. (**E**) Western blot analysis of subcellular localization of 3MST and CATs. Antibodies were used against 3MST, mitochondrial CAT (mCAT), and cytosolic CAT (cCAT). (**F**) The dose-dependent effect of α-KG on the $H_2S$-producing activity of synaptosomes and mitochondria. Synaptosomes and mitochondria were incubated with 10 m*M* cysteine in the presence of increasing amounts of α-KG. (**G**) The $H_2S$-producing activity of synaptosomes and mitochondria with 3MP as a substrate. Synaptosomes and mitochondria were incubated with different amounts of 3MP. All data are expressed as the mean ± SEM of three experiments.

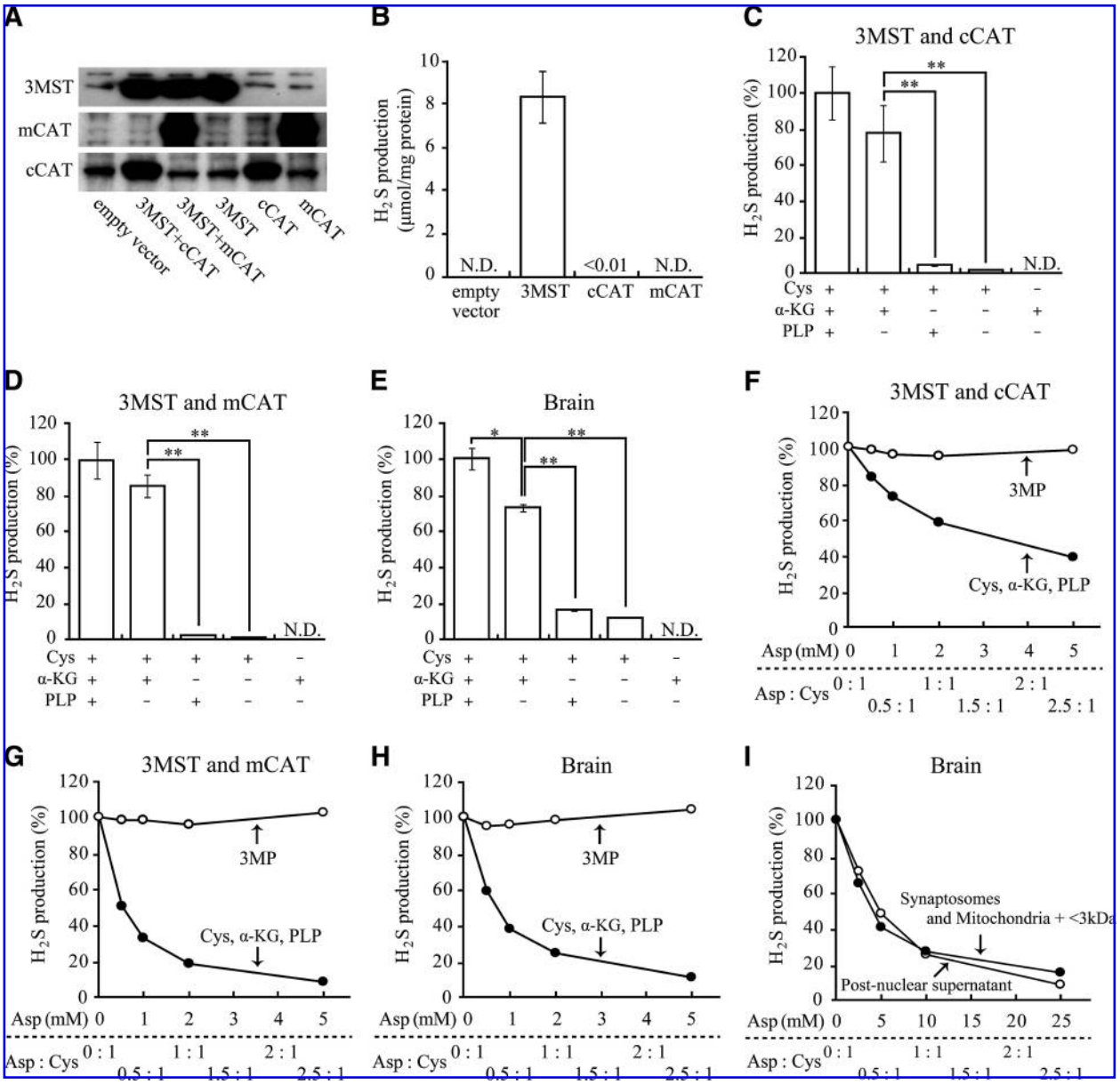



**FIG. 2. 3MST with CAT produces $H_2S$.** (**A**) Western blot analysis of expression of 3MST and CATs in HEK 293-F cells. HEK 293-F cells were transfected with the expression plasmids containing cDNAs of 3MST and CATs. Antibodies were used against 3MST, mitochondrial CAT (mCAT), and cytosolic CAT (cCAT). (**B**) $H_2S$-producing activity of 3MST. The lysates of HEK 293-F cells expressing 3MST, cCAT, or mCAT were mixed with 0.2 m*M* 3MP, and released $H_2S$ was measured. (**C–E**) Requirement of cysteine, α-KG, and PLP for $H_2S$ production. The lysates of cells coexpressing 3MST and cCAT (**C**), 3MST with mCAT (**D**), or brain homogenates (**E**) were mixed with 2 m*M* cysteine, 0.5 m*M* α-KG, or 0.05 m*M* PLP. $H_2S$ production was measured. $^*p < 0.05$; $^{**}p < 0.01$. (**F–H**) The inhibitory effect of aspartate on $H_2S$ production. The lysates of cells expressing 3MST and cCAT (**F**) or 3MST and mCAT (**G**), or brain homogenates (**H**) were incubated with 2 m*M* cysteine, 0.5 m*M* α-KG, and 0.05 m*M* PLP (●), or with 0.2 m*M* 3MP (○) in the presence of aspartate. (**I**) The inhibitory effect of aspartate on $H_2S$ production of brain fractions. Postnuclear supernatant (○) or synaptosomes and mitochondria plus <3 kDa (●) were incubated with 10 m*M* cysteine in the presence of aspartate. All data are expressed as the mean ± SEM of three experiments.

3MST in mouse brains by Western blot analysis. The levels of 3MST are maintained from embryonic day 16 (E16) to postnatal day 14 (P14) and decreased during P28 and P52 (Fig. 4A). It then slightly increases with further aging up to 156 days old. These observations are consistent with a proteomics analysis, finding that the expression of 3MST is downregulated between E16 and P56 (45).

CATs localize to neurons in several areas of the brain, including the olfactory bulb, cerebral cortex, and cerebellum (3, 18), but the localization of 3MST has not been determined. Immunohistochemical analysis of the brain showed that 3MST was localized to neurons of mitral cell layers, glomerular, and external plexiform layers in the olfactory bulb (Fig. 4B–E). 3MST was also found in Purkinje

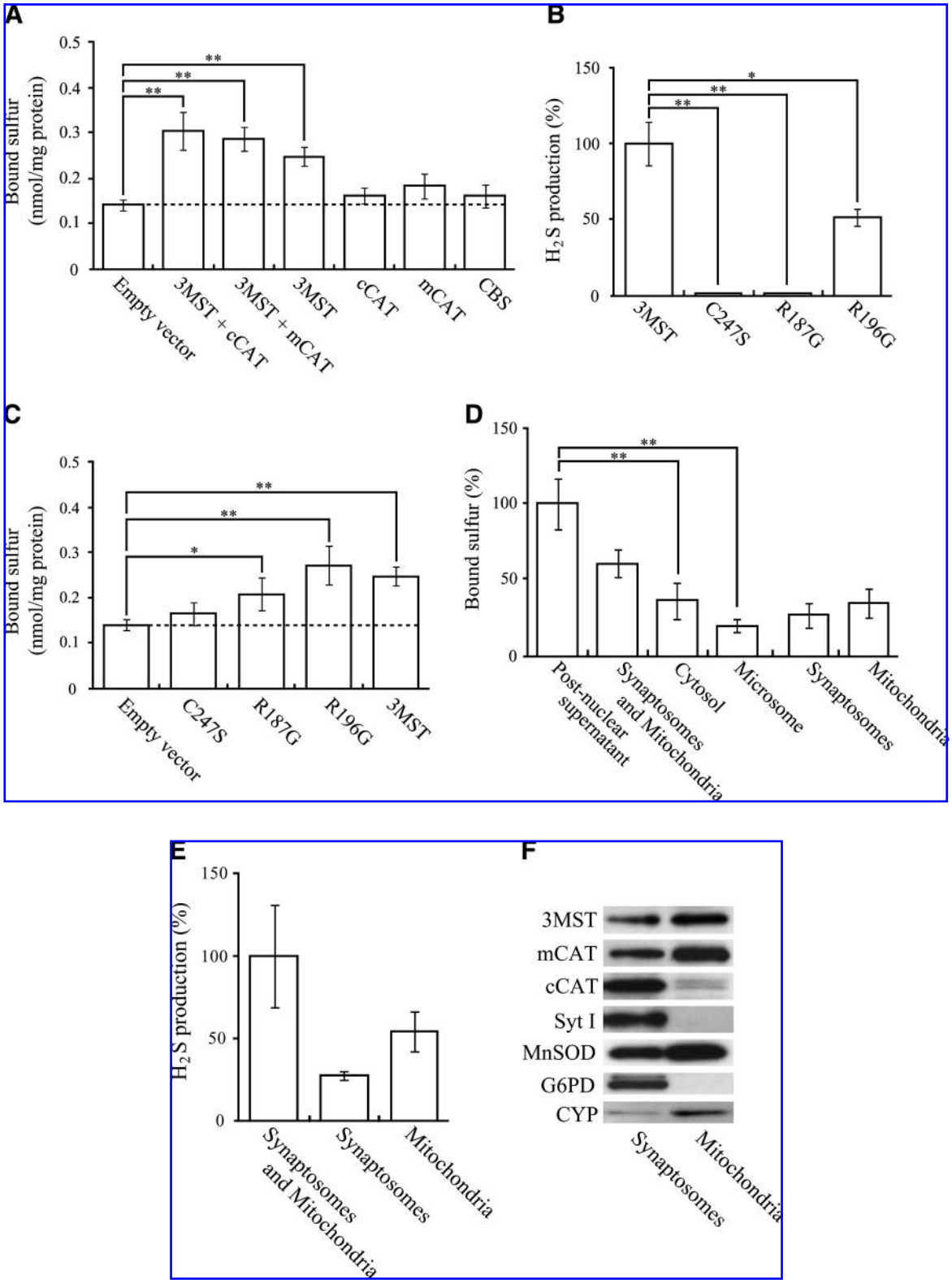
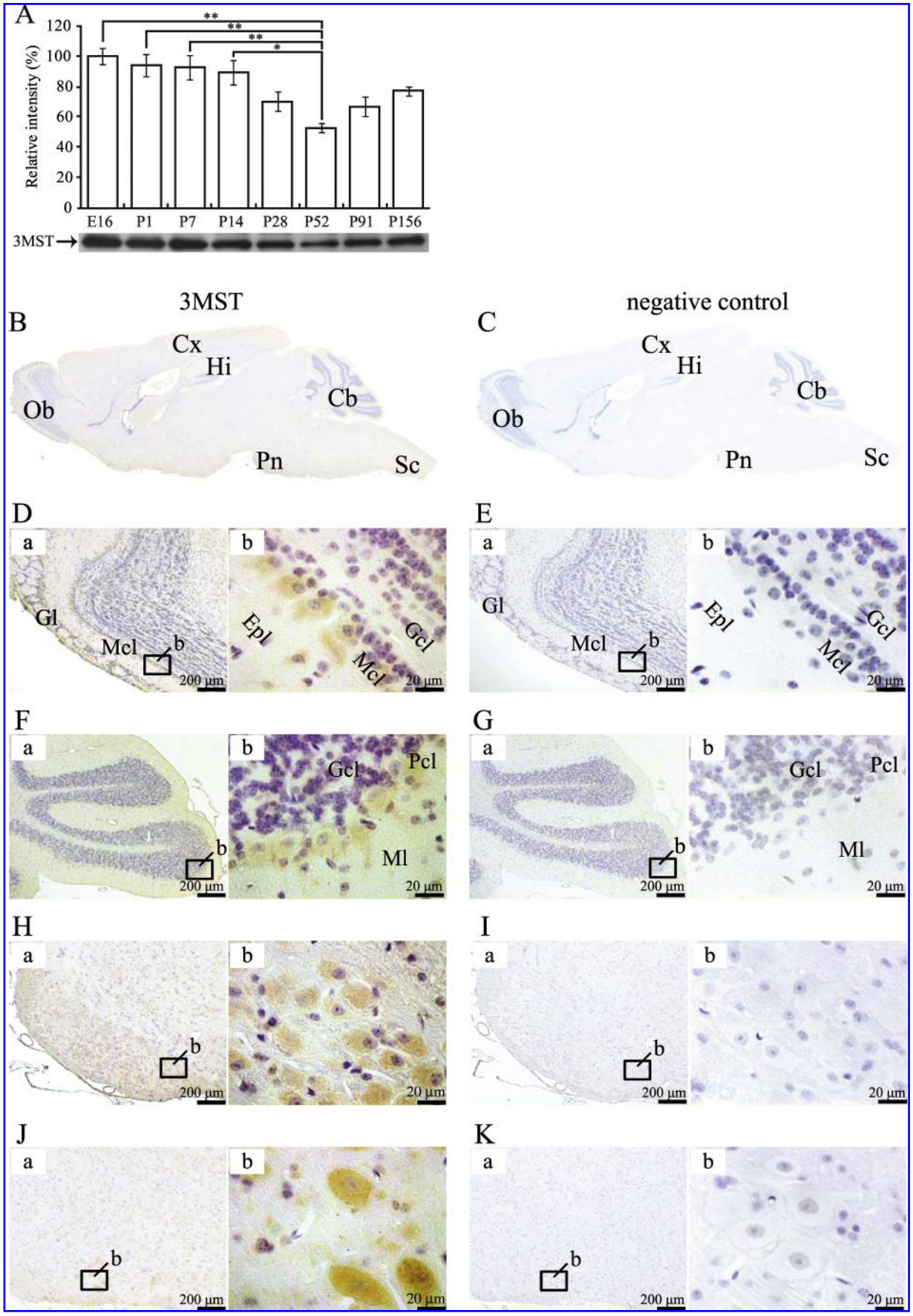



**FIG. 3. 3MST with CAT increases bound sulfane sulfur in cells.** (**A**) The levels of bound sulfane sulfur in cells expressing 3MST, CAT, and CBS. The lysates of HEK 293-F cells expressing with 3MST, cytosolic CAT (cCAT), mitochondrial CAT (mCAT), or CBS were incubated with 7.5 m*M* dithiothreitol at pH 9.0, and released $H_2S$ was measured. (**B**) The $H_2S$-producing activities of the 3MST mutants. The lysates of the cells expressed with the 3MST mutants were mixed with 0.2 m*M* 3MP, and released $H_2S$ was measured. (**C**) The levels of bound sulfane sulfur in cells expressing 3MST mutants. $^*p < 0.05$; $^{**}p < 0.01$. (**D**) Subcellular localization of bound sulfane sulfur. (**E**) $H_2S$-producing activity in synaptosomes and mitochondria. (**F**) Western blot analysis of the localization of enzymes in synaptosomes and mitochondria. All data are expressed as the mean ± SEM of at least three experiments. $^*p < 0.01$; $^{**}p < 0.001$.


A
Relative intensity (%)
120
100
80
60
40
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0
**
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*
E16
P1
P7
P14
P28
P52
P91
P156
3MST
B
3MST
Cx
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Sc
C
negative control
Cx
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200 μm
20 μm

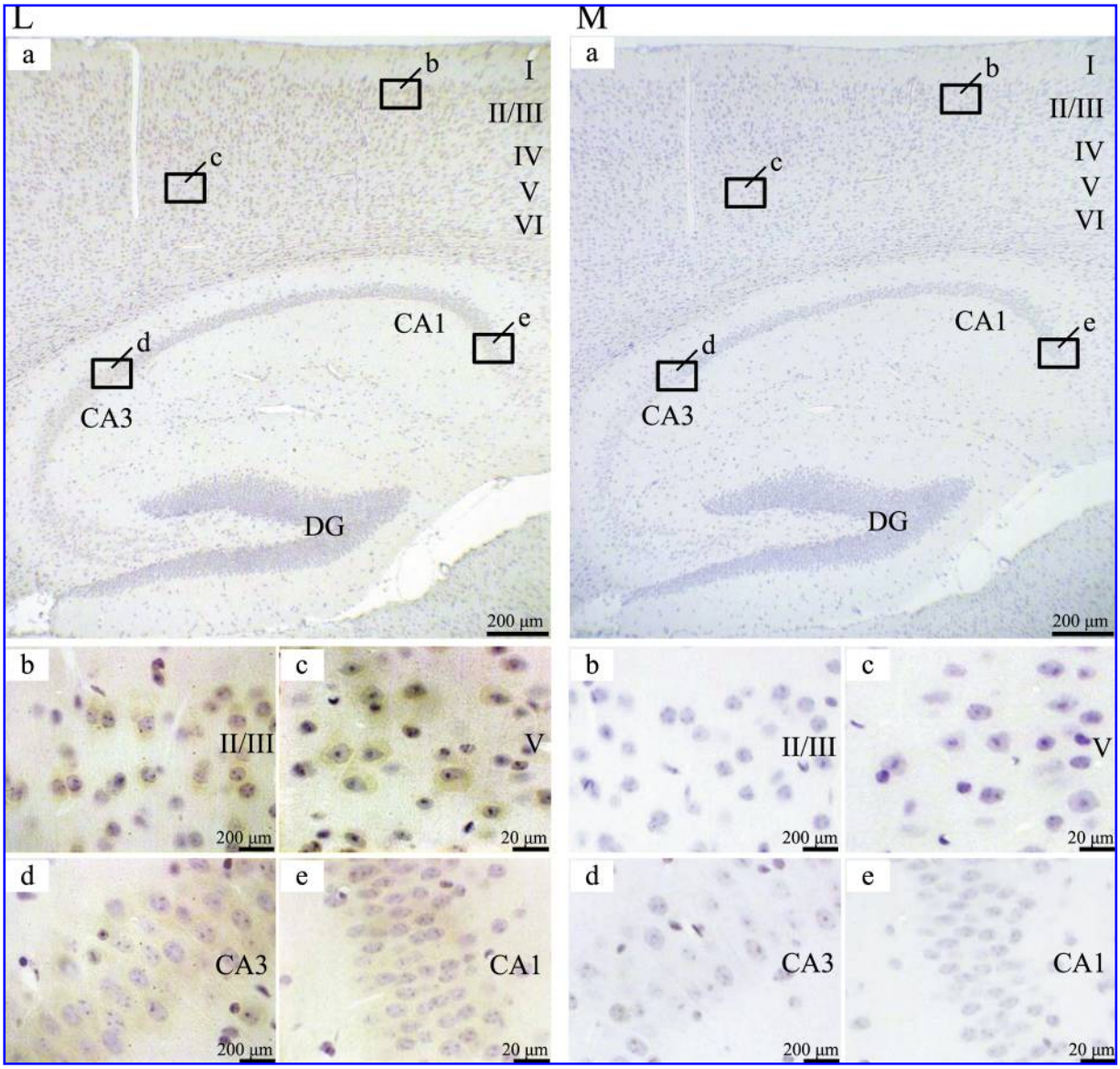


**FIG. 4. (*Continued*)**

cell somata and proximal dendrites (Fig. 4F and G), and in the pontine nuclei in the pons (Fig. 4H and J). In the spinal cord, it was localized to large neurons (Fig. 4J and K). In the cerebral cortex, 3MST was localized to pyramidal neurons in layers II/III and V, and in layers I–VI of the neocortical areas (Fig. 4L and M, a, b, and c). In the hippocampus, 3MST was found in the CA1 and CA3 pyramidal cells (Fig. 4L and M, d and e). These observations indicate that 3MST is primarily associated with neurons in the brain and spinal cord.

## Discussion

The present study shows that a major source of $H_2S$ production in the brain is from the enzyme 3-mercaptopyruvate

**FIG. 4. Immunohistochemical staining of 3MST in the brain.** (**A**) Developmental changes in the expression of 3MST in mouse brains. Western blot analysis with an antibody against 3MST. Relative amounts of 3MST quantitatively analyzed by NIH Image are shown in the upper column ($n = 3$). (**B, C**) Sagittal sections of 12-week-old mice were stained with an antibody against 3MST (**B**) or with normal rabbit IgG (**C**). The sections were counterstained in hematoxylin. (**D–K**) Images of the olfactory bulb (**D** and **E**), cerebellum (**F** and **G**), pons (**H** and **I**), and spinal cord (**J** and **K**) with an antibody against 3MST (**D, F, H**, and **J**) or normal rabbit IgG (**E, G, I**, and **K**). The higher-magnification images of the boxes in (**a**) were shown in (**b**). (**L, M**) Images of the cerebral cortex and hippocampus with an antibody against 3MST (**L**) or with normal rabbit IgG (**M**). The higher-magnification images of the boxes in (**a**) are shown in (**b–e**). Cb, cerebellum; Cx, cerebral cortex; DG, dentate gyrus; Epl, external plexiform layer; Gcl, granule cell layer; Gl, glomerular layer; Mcl, molecular cell layer; Ml, molecular layer; Ob, olfactory bulb; Pcl, Purkinje cell layer; Pn, pons; Sc, spinal cord. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article at www.liebertonline.com/ars).

sulfurtransferase (3MST). 3MST produces $H_2S$ more efficiently than does CBS, which was previously believed to be the sole $H_2S$-producing enzyme. The striking difference between 3MST and CBS is that 3MST produces bound sulfane sulfur more efficiently than CBS in the cells (Fig. 3). This is also the first experimental evidence for the hypothesis that enzymes that produce $H_2S$ can also generate bound sulfane sulfur in the cells (16, 30). Because free $H_2S$ is immediately absorbed and stored as bound sulfane sulfur, $H_2S$ produced by 3MST may also immediately be incorporated as bound sulfane sulfur and stably stored until released in response to physiologic signals (16). A difference between 3MST and CBS in the efficiency of producing bound sulfane sulfur may be the result of the following. Considering the fact that 3MST has ~66% homology with rhodanese (26), which was shown to have sulfur-carrier activity from bound sulfane sulfur to acid-labile sulfur (23, 32, 33), it is possible that 3MST has the sulfur-carrier activity from $H_2S$ to bound sulfur, whereas CBS has only weak sulfur-carrier activity.

Although a high concentration of substrates was require for the reaction, 3MST in protozoa produces $H_2S$ from the reaction between 5 m*M* each of 2-mercaptoethanol and 3MP or thiosulfate (51). Lysates of erythrocytes produce $H_2S$ in the presence of 2 m*M* $\beta$-mercaptopyruvate, suggesting a possible involvement of 3MST in $H_2S$ production, although $H_2S$ is not produced from cysteine (55). In the present study, only 0.2 m*M* 3MP was required to produce $H_2S$ by 3MST (Fig. 2B). The important finding in the present study is that $H_2S$ is produced by 3MST with CAT in the presence of cysteine and $\alpha$-KG, supporting the existence of 3MP, which has not been identified. The existence of 3MP has been suggested based on the observation that mercaptolactate-cysteine disulfide, a metabolite of 3MP, was found in urine (9, 17, 42). The present observation that 3MST with CAT in the presence of cysteine and $\alpha$-KG produces $H_2S$, even without the addition of 3MP, indicates the existence of 3MP.

Correlation between the activity of $H_2S$ production and the levels of bound sulfur was confirmed by using mutants of 3MST (Fig. 3). The C247S mutant completely lost the activity to metabolize 3MP that was assessed by measuring its products, either pyruvate or $H_2S$ (Fig. 3B) (25). In this mutant, the levels of bound sulfane sulfur were not increased (Fig. 3C). In contrast, the R196G mutant, which partially lost the activity, did not decrease the levels of bound sulfane sulfur (Fig. 3B and C). The R187G mutant, which greatly lost the activity, decreased the levels of bound sulfane sulfur, but not statistically significantly. Both C247S and R187G abolished $H_2S$ production, but only C247S abolished bound sulfur production. It is possible that cysteine 247 is involved in both $H_2S$ production and the sulfur-carrier activity from $H_2S$ to bound sulfane sulfur, whereas arginine 187 may be involved only in the sulfur-carrier activity.

An additional experiment to support the role of 3MST to produce bound sulfane sulfur is to test whether suppression of 3MST in cells decreases the levels of bound sulfane sulfur. Although the protein levels of 3MST in Neuro 2A cells were decreased by the RNA interference to ~60% of a control, no statistically significant suppression in the levels of bound sulfane sulfur was observed (data not shown). Because low levels of endogenous 3MST were enough to produce endogenous levels of bound sulfane sulfur (Fig. 3), complete suppression of the 3MST activity may be required.

Bound sulfane sulfur is the major physiologically relevant pool of $H_2S$, for it regulates the activity of enzymes and growth of cells (39). In addition, our recent study showed that bound sulfane sulfur of the lysates of neurons and astrocytes releases $H_2S$ in the presence of physiologic concentrations of the endogenous reducing substances, glutathione and cysteine (16).

3MST is localized to neurons such as hippocampal pyramidal cells (Fig. 4). $H_2S$ facilitates the induction of hippocampal long-term potentiation, a synaptic model of learning and memory, and induces $Ca^{2+}$ waves in astrocytes (1, 27, 40). $H_2S$ may mediate the reciprocal interaction between neurons and astrocytes (10), resulting in the regulation of synaptic activity. Because 3MST is localized to mitochondria and synaptosomes (Fig. 3D–F), and because mitochondria produce reactive oxygen species, $H_2S$ may efficiently protect cells from oxidative stress, as has been demonstrated by the neuroprotective effect of $H_2S$ (19, 20).

In conclusion, $H_2S$ is produced by 3MST from cysteine and $\alpha$-KG through the metabolism with CAT, and 3MST produced bound sulfane sulfur in the brain. 3MST is specifically localized to neurons. These observations provide a new insight into the production and storage of $H_2S$ in the brain.

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## Abbreviations

ANOVA, analysis of variance; CAT, cysteine aminotransferase; CBS, cystathionine $\beta$-synthase; CSE, cystathionine $\gamma$-lyase; DTT, dithiothreitol; G6PD, glucose-6-phosphate dehydrogenase; $H_2S$, hydrogen sulfide; $\alpha$-KG, $\alpha$-ketoglutarate; MnSOD, manganese superoxide dismutase; 3MP, 3-mercaptopyruvate; 3MST, 3-mercaptopyruvate sulfurtransferase; $Na_2S$, sodium sulfide; PLP, pyridoxal-5′-phosphate; SDS, sodium dodecyl sulfate.

## Disclosure Statement

No competing financial interests exist.

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