H_2S, THE POTENTIALLY NOVEL GASOTRANSMITTER DURING EXPERIMENTAL CEREBRAL ISCHEMIA

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H_2S, the potentially novel gasotransmitter during experimental cerebral ischemia

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SUMMARY

Background and Purpose—We observed recently that elevated plasma cysteine (Cys) levels are associated with poor clinical outcome in acute stroke patients. In a rat stroke model, Cys administration increased the infarct volume apparently via its conversion to hydrogen sulfide (H_2S). We therefore investigated the effects of H_2S and the inhibition of its formation on stroke.

Methods—Cerebral ischemia was studied in a rat stroke model created by permanent occlusion of the middle cerebral artery (MCAO). The resultant infarct volume was measured 24 hours after occlusion.

Results—Administration of sodium hydrosulfide (NaHS, a H_2S donor) significantly increased the infarct volume after MCAO. The NaHS-induced increase in infarct volume was abolished by the administration of MK-801 (an *N*-methyl-D-aspartate receptor channel blocker). MCAO caused an increase in H_2S level in the damaged cortex as well as an increase in the H_2S synthesizing activity. Administration of 4 different inhibitors of H_2S synthesis reduced MCAO-induced infarct volume dose dependently. The potency of these inhibitors in effecting neuroprotection in vivo appeared to parallel their potency as inhibitors of H_2S synthesis in vitro. It also appeared that most of the H_2S synthesizing activity in the cortex results from the action of cystathionine-β-synthase (CBS).

Conclusions—The present results clearly demonstrate that H_2S, produced from Cys in the cerebral cortex most probably by CBS, is an important mediator of ischemic damage. H_2S acts via the NMDA receptor, which has become a prime target for stroke research over the past decade. Indeed, some NMDA antagonist and glycine antagonists have shown promise in clinical trials. Current evidence suggests that H_2S promotes ischemic damage by a

direct degenerative effect on cerebral neurons, although effect on cerebral blood flow may not be, as yet, excluded. Whatever the mechanism of action, these results suggest, for the first time, that inhibition of H_2S production using a CBS inhibitor may represent a novel therapeutic approach to the treatment of stroke.

PUBLICATIONS

- **K. Qu**, S.W. Lee, J.S. Bian, C.M Low and P.T.-H. Wong (2007) "Hydrogen sulfide: neurochemistry and neurobiology". ***Neurochemistry International*** [accepted]
- **K. Qu**, C.P.L.H. Chen, B Halliwell, P.K. Moore, and P.T.-H. Wong (2006) "Hydrogen sulfide is the mediator of cysteine neurotoxicity in cerebral ischemia". ***Stroke.2006; 37: 889-893*** [Print ISSN: 0039-2499; Online ISSN: 1524-4628]
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CONFERENCE PAPERS

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- The 58^{th} Annual Meeting of American Academy of Neurology (San Diego, USA 2006): "Hydrogen sulfide is a mediator of cysteine neurotoxicity in cerebral ischemic damage". (Poster presentation)

- 7th Biennial Meeting of the Asian Pacific Society for Neurochemistry (APSN, Singapore 2006): “Hydrogen sulfide is a mediator of cerebral ischemic damage”. (Poster presentation)

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5-methyl THF	5-methyltetrahydrofolate
ABI	atherothrombotic brain infarctions
ACA	anterior cerebral artery
ACPD	1-aminocyclopentane-1, 3-dicarboxylic acid
AD	Alzheimer's disease
AHA	American Heart & Stroke Association
AIFs	apoptosis initiating factors
AMPA	α-amino-3-hydroxy-5-methyl-isoxazolepropionate
AOAA	aminooxyacetic acid
AP-1	activating protein-1
BBB	blood brain barrier
CAD	caspase activated deoxiribonuclease
CAPON	carboxy-terminal PDZ ligand of nNOS
CAT	cysteine aminotransferase
CBF	cerebral blood flow
CCA	common carotid artery
cGMP	cyclic guanosine monophosphate
CNS	central nervous system
CO	Carbon oxide
COX-2	cyclooxygenase-2
DAG	diacylglycerol
dNTPs	deoxynucleotide 5'-triphosphate
dTT	dithiothreitol
ECA	external carotid artery
EEG	electroencephalogram
eNOS	endothelial NOS
FAD	flavin adenine dinucleotide
FMN	flavin adenine mononucleotide
H_2S	hydrogen sulfide

HA	hydroxylamine
Hcy	homocysteine
HO	heme oxygenase
i.p.	intraperitoneal injection
IACUC	Institutional Animal Care and Use Committee
ICE	interleukin-1 converting enzyme
iNOS	inducible NOS
IP3	inositol-1, 4, 5-trisphosphate
KA	kainite
L-Cys	L-cysteine
LTP	long-term potentiation
MCA	middle cerebral artery
MCAO	middle cerebral artery occlusion
mGluRs	Metabotropic glutamate receptor
MPST	3-mercaptopyruvate sulfurtransferase
MS	methionine synthase
MTHFR	methylenetetrahydrofolate reductase
NaHS	sodium hydrosulfide
NANC	nonadrenergic/noncholinergic
NAPDH	nicotinamide adenine dinucleotide phosphate
NF-κB	nuclear factor kappa-B
NH_3	ammonia
NMDA	N-methyl-D-aspartate
nNOS	neuronal NOS
NO	Nitrogen oxide
NOS	NO synthase
P5P	pyridoxal-5'-phosphate
PAG	propargylglycine
PARP	poly (ADP-ribose) polymerase
PCA	posterior cerebral artery
PKC	protein kinase C
PLC	phospholipase C

pro-IL-1β	pro-interleukin-1 β
PSD	postsynaptic density protein
ROS	reactive oxygen species
rtPA	recombinant tissue plasminogen activator
SAM	S-adenosylmethionine
sGC	soluble guanylyl cyclase
SIN-1	3-morpholinosydnonimine
SNAP	S-nitroso-N-acetyl-penicillamine
SO_2	sulfur dioxide
TMCAO	Transient middle cerebral artery occlusion
TTC	2, 3, 5-triphenyltetrazolium chloride
VACCs	voltage activated calcium channels
WHO	World health organization
β-CNA	β-cyanoalanine

1 INTRODUCTION

1.1 Neurotransmitter

Neurotransmitters are the most common class of chemical messengers in the nervous system. They have attracted extensive attention because of their multiple roles in physiologic and pathophysiologic conditions since the first neurotransmitter, acetylcholine was discovered in 1921[1]. The central nervous system (CNS) is a complex system with many neurotransmitters working in concert to maintain proper functioning.

Before a neuroactive substance can be classified as a neurotransmitter, it has to fulfill certain criteria as below [2]:

- It must be of neuronal origin and accumulated in presynaptic terminals, from where it is released upon depolarization.
- The released neurotransmitter must induce postsynaptic effects mediated by neurotransmitter-specific receptors.
- The substance must be metabolically inactivated or cleared from the synaptic cleft by reuptake mechanisms.
- Experimental application of the substance to nervous tissue must produce effects comparable to those induced by the naturally occurring neurotransmitter.

Various neurotransmitters are probably involved in synthesizing, transporting and recycling in the chemical milieus of CNS which consist of its whole living environment. They are used to relay, amplify and modulate electrical signals between a neuron and another cell. Within the cells, small-molecule neurotransmitter molecules are usually packaged in vesicles and are released from the axon terminal of a presynaptic neuron

either by exocytosis. They could travel across the synaptic cleft and bind to specific receptors to either excite or inhibit the postsynaptic cell membrane. Neurotransmitters also act on “autoreceptors” located on presynaptic membranes to regulate the progress of synaptic transmission.

The three major categories of neurotransmitters are (a) amino acids (primarily glutamic acid, GABA, aspartic acid & glycine), (b) monoamines (norepinephrine, dopamine & serotonin) plus acetylcholine and (c) neuropeptides (vasopressin, somatostatin, neurotensin, etc.). Glutamate[3] and GABA[4] are the major "workhorse" neurotransmitters in the brain. The monoamines and acetylcholine perform specialized modulating functions, often confined to specific structures. The neuropeptides perform specialized functions in the hypothalamus or act as co-factors elsewhere in the brain[5]. It is anticipated that future studies will find more potential neurotransmitters and better reveal the underlying molecular mechanisms.

1.2 Gasotransmitters

Among the potential neurotransmitters, nitric oxide (NO), carbon monoxide (CO) and hydrogen sulfide (H_2S) are distinctive from classical neurotransmitters and hormonal factors but sharing common characteristics among themselves. They are small signaling molecules with physiological importance, which have been termed as “gasotransmitters” as they are endogenous gases.

In 2002, the criteria for classifying gasotransmitters were first suggested by Wang Rui [6].

- They are small gaseous molecules.

- They are freely permeable to membranes. As such, their effects do not rely on the cognate membrane receptors, and they can have endocrine, paracrine, and autocrine effects.

- They are endogenously and enzymatically generated and regulated.

- They have well defined and specific functions at physiologically relevant concentrations.

- Their cellular effects may or may not be mediated by second messengers, but should have specific cellular and molecular targets.

Following the identification of NO and CO as gasotransmitter based on these criteria, H_2S may be qualified as the third one.

1.3 H_2S, the 3rd putative gasotransmitter

H_2S is a well-known toxic gas so that it had been assumed to exist in animal tissues only at very low concentrations even though it could be produced endogenously by enzymes and non-enzymatic pathways. However, recent studies of have shown that H_2S level in mammalian tissues is more considerable than first expected. Measured in rat, human and bovine brain tissues, the concentrations of H_2S were up to 50-160 µmol/l[7;8]. Abe and Kimura [9] have shown that at concentrations similar to the physiological concentrations of H_2S, sodium hydrosulfide (10 - 130 µM) selectively enhances N-methyl D-aspartate (NMDA) receptor-mediated responses and facilitates the induction of hippocampal long-term potentiation (LTP). These findings suggest that endogenous H_2S functions as a neuromodulator.

1.3.1 Physical properties of H_2S

H_2S is a colorless gas with an odour described as the smell of rotten eggs. It is the sulfur analog of water (H_2O) with a molecular weight of 34.08 (**Fig. 1-1**). Relative to water, H_2S has intermolecular forces and then exist in gaseous form at room temperature and pressure. It can be oxidized by a variety of agents to form sulfur dioxide (SO_2), sulfates such as sulfuric acid and elemental sulfur[10]. In the mammalian body, at a physiological pH of 7.4, approximately one-third of H_2S exists as the un-dissociated form and two-thirds as the hydrosulfide anion (HS^-)[10] (**Formula 1**). It can easily penetrate the plasma membranes of cells in the undissociated form because of its lipid solubility.

$$\mathbf{H_2S \leftrightarrow H^+ + HS^- \leftrightarrow 2H^+ + S^{2-}} \quad \textbf{……Formula 1}$$

1.3.2 Toxicity of H_2S

The toxicity of H_2S was first described almost 300 years ago[10]. Lot of works had been done to investigate H_2S toxicity by H_2S exposure because it is still a pollutant and a working hazard in modern society. This toxic gas has often been regarded as a broad-spectrum toxicant since most organ systems are susceptible to the effects of H_2S. The early symptoms of H_2S exposure include sore throat, dizziness, nausea, and respiratory effects attributed to airway irritation. Acute exposure to H_2S exhibits a very steep dose-response relationship with an LD_{50} of 15 mg/kg (rats), especially for CNS and respiratory depression, which is the major cause of death in acute H_2S poisoning[8]. The primary cause of death in H_2S poisoning has been attributed to respiratory paralysis as a result of the toxic effect of sulfides on the respiratory centers of the brain[11]. In addition, pulmonary edema has consistently been reported as the single most notable lesion in

Fig. 1-1 Structures of H_2O and H_2S

H_2S has a structure similar to that of water but comparatively weak intermolecular forces exist for H_2S.

autopsies of individuals killed by this gas[12]. Mechanistically, it is believed that the H_2S poisons the mitochondria at low micromolar concentrations via reversible inhibition of cytochrome *c* oxidase [10].

1.3.3 Endogenous biosynthesis of H_2S

In mammalian tissues, H_2S is mainly produced from L-cysteine (L-Cys) through various pathways. Two pyridoxal-5'-phosphate (P5P)-dependent enzymes—cystathionine-β-synthase (CBS, EC 4.2.1.22) and cystathionine-γ-lyase (CSE, EC 4.4.1.1)—were reported to be responsible for the majority of the endogenous production of H2S which could function as an intracellular messenger[13;14]. These two key enzymes are also involved in the transsulfuration pathway of homocysteine (Hcy) metabolism. The pathways of endogenous biosynthesis of H_2S are simplified in the following figure (**Fig. 1-2**).

As described in **Fig. 1-2**, catabolism of L-Cys will release H_2S as a final product via several desulfuration pathways [13] . Firstly, L-cysteine is hydrolyzed by CBS, producing equimolar amounts of H_2S and L-serine. In the second pathway, two L-cysteine molecules dimerize to form cystine, which is transformed into thiocysteine, pyruvate and ammonia (NH_3) by CSE. Then the thiocysteine can form H_2S via two reactions: the CSE-catalyzed reaction of thiocysteine with another thiol compounds (e.g. glutathione or cysteine) to form H_2S and CysSR, or thiocysteine can form cysteine and H_2S enzymatically (by CSE activation) or possibly non-enzymatically[15]. This figure also shows the role of CBS and CSE in Hcy and L-Cys metabolism pathways. CBS catalyzes the condensation of Hcy and L-serine to form cystathionine in an irreversible reaction, the cystathionine is then hydrolyzed by CSE to form L-Cys, together with α-ketobutyrate plus ammonia (not

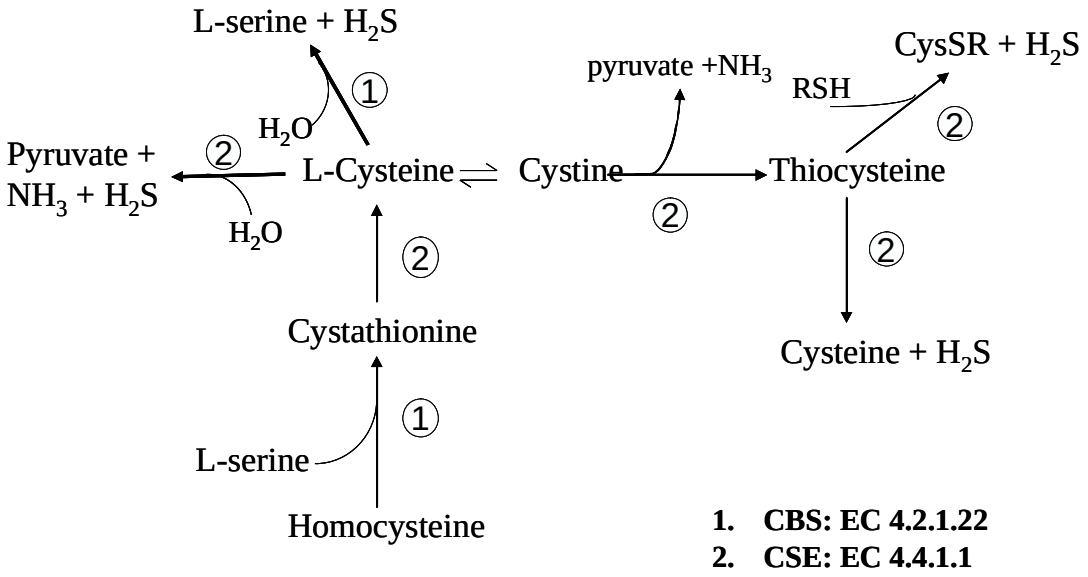


Fig.1-2 Endogenous biosynthesis of H_2S in mammalian. Catabolism of L-Cys will release H_2S as final production via few various desulfuration pathways. Two pyridoxal-5'-phosphate (P5P)-dependent enzymes, cystathionine-β-synthase (CBS, EC 4.2.1.22) and cystathionine-γ-lyase (CSE, EC 4.4.1.1), are key enzymes in H_2S biosynthesis.

shown in figure).

There are a few other pathways (not shown in **Fig. 1-2**) that may release H_2S from L-cysteine although these pathway may be subservient to the pathways described above. As reviewed by Julian D. et al[16], L-Cys may react with a ketoacid (e.g. α-ketoglutarate) to form 3-mercaptopyruvate and an amino acid (e.g. L-glutamate) by the catalysis of cysteine aminotransferase (CAT, EC 2.6.1.3). The 3-mercaptopyruvate can then be desulfurated by 3-mercaptopyruvate sulfurtransferase (MPST, EC 2.8.1.2) to form H_2S and pyruvate. In addition, CSE also can convert L-cysteine and sulfite to L-cysteate and H_2S. More recently, Chen X.L. et al[17] suggested a novel mechanism that CBS could catalyze the condensation of L-Cys with Hcy to form cystathionine and H_2S. Kinetic studies demonstrated that the production of H_2S by this reaction is more efficient than the traditional hydrolysis of L-cys by CBS. Although this finding confirms the ability of CBS to produce H_2S, further experimental evidence in needed to verify the extent to which it occurs in vivo.

1.3.3.1 Precursors: homocysteine and L-cysteine

Met is an essential amino acid in mammals and thus generally considered as the source of all sulfur-containing amino acids. Cys, on the other hand, is non-essential and can be synthesized from Met via Hcy (the transsulfuration pathway). The mammalian liver regulates its free Cys pool tightly even when dietary source of sulfur-containing amino acid varies from sub- to over-requirement[18]. This is achieved by regulating the synthesis of glutathione, which acts as a reservoir of Cys, and the catabolism of Cys via Cys dioxygenase, which converts Cys to Cys sulfinate[19]. The plasma concentration of cystine (100 – 200 μM) is about 10 times higher than those of Cys (10 – 20 μM)[20] and

Hcy (3 – 15 μM)[21]. Cystine undergoes influx transport across the blood-brain barrier via a cystine-Glu exchange transporter (system x_c^-)[22]. Just as Met and Hcy, Cys may also enter the brain via a neutral amino acid transporter such as system L, but the significance of this transport in vivo is not certain[23]. In the brain, astrocytes constantly release glutathione which then reacts extracellularly with cystine transported from blood to form Cys and cysteine-glutathione disulfide[24]. It appears that neurons rely on this extracellular thiol/disulfide exchange reaction for Cys as it is not able to provide thiols by themselves.

Both Hcy and Cys have recently received greater attention as important risk factors for vascular diseases and CNS diseases. Their levels were found to be correlated with age in the whole study population[25;26]. The elevated plasma total homocysteine (tHcy) and cysteine concentration were linked with coronary atherosclerosis[27], stroke[28;29] and several neurodegenerative diseases[30;31].

1.3.3.1.1 Homocysteine

Homocysteine is an intermediate product of methionine metabolism and is metabolized by the re-methylation and transsulfuration pathways (see **Fig. 1-3**). The re-methylation pathway, which regenerates methionine, is controlled by the vitamin B_{12}-dependent methionine synthase (MS)[32] and methylenetetrahydrofolate reductase (MTHFR). After re-methylation, methionine can be re-utilized to produce S-adenosylmethionine (SAM), a methyl donor, which participates in several key metabolic pathways, including methylation of DNA and myelin, and synthesis of important substances, such as carnitine, coenzyme Q10, creatine, epinephrine and melatonin[33].

The transsulfuration pathway is important in the synthesis of glutathione which is regulated at the substrate level by Cys, which is an important nutrient for cardiac health,

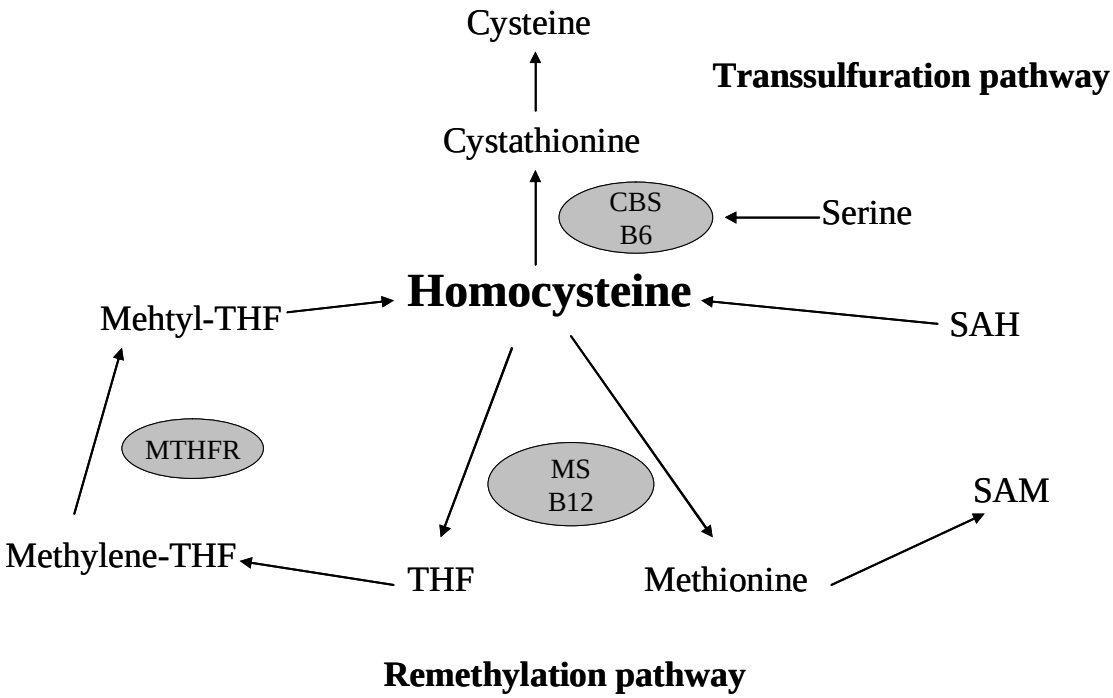


Fig. 1-3 Simplified homocysteine metabolic pathways. Homocysteine is an intermediate product of methionine metabolism and is metabolized by remethylation and transsulfration pathways. The re-methylation pathway, which regenerates methionine, is controlled by vitamin B_{12}-dependent methionine synthase (MS)[32] and methylenetetrahydrofolate reductase (MTHFR). The trans-sulfuration pathway of homocysteine catabolyzed by CBS produces cystathionine which is the precursor of cysteine. After re-methylation, methionine can be re-utilized to produce a methyl donor, S-adenosylmethionine (SAM), which can be converted back to homocysteine.

hepatic detoxification, cholesterol excretion, bile salt formation. The glutathione level in the brain of transgenic mice with homozygous disruption of the CBS gene is reported to be decreased by 30%[34]. More recently, Vitvitsky et al. (2006)[35] confirmed that glutathione depletes quickly in brain slices following inhibition of CSE. Elevated Hcy and decreased glutathione are features seen in Alzheimer's and Parkinson's disease patients indicating impairment of the transsulfuration pathway in these neurodegenerative diseases. This pathway depends on an adequate dietary intake and conversion of vitamin B6 into its active form, P5P in the liver. In essence, the intermediate metabolite homocysteine is located at a critical metabolic crossroad, and therefore impacts on methyl and sulfur metabolism in the body directly and indirectly. Reduced activities of the key catabolizing enzymes, would result in elevated plasma level of homocysteine, which is called hyperhomocysteinemia[36;37]. It has been widely accepted that hyperhomocysteinemia is an independent risk factor for vascular diseases. Elevated homocysteine in serum induced arteriosclerosis-like alterations of the aorta in both normotensive and hypertensive rats after loading with high doses of the Hcy precursor, methionine[38]. Homocysteine appeared to alter the anticoagulant properties of endothelial cells to a procoagulant phenotype and mildly increased homocysteine caused dysfunction of the vascular endothelium[39].

In the nervous systems, homocysteine was reported to induce apoptosis and increase neuronal vulnerability to excitotoxicity by several mechanisms, including DNA damage, associated activation of poly-ADP-ribose polymerase (PARP) and nicotinamide adenine dinucleotide (NAD) depletion[40]. Chronic experimental hyperhomocysteinemia caused cognitive dysfunction in rats and in clinical cases, it may present as mental retardation and

other neurological symptoms[41]. Clinical evidence and animal experiments also showed an association between brain atrophy and increased plasma total Hcy level in chronic alcoholism[42]. Furthermore, hyperhomocysteinemia is also one of the known risk factors for developing Alzheimer's disease (AD) since it not only sensitizes hippocampal neurons to β-amyloid-induced damage in cell cultures but also enhances β-amyloid generation by inducing the stress protein Herp through interaction with both presenilin 1 and 2[43]. In stroke patients, epidemiological studies also linked elevated tHcy with an increased risk of ischemic stroke because of its damage to arteries. Large randomized trials showed that multivitamin therapy reduced the rate of recurrent stroke and other serious vascular events in patients with prior stroke or transient ischemic attack[28;29].

1.3.3.1.2 L-cysteine

The availability of Cys from dietary sources becomes critical when there is a deficiency in the transsulfuration pathway resulting from conditions such as prematurity[44;45] or liver disease[46]. It is a very important amino acid for the synthesis of proteins, coenzyme A, taurine, and GSH which have important physiological functions in the body. For example, GSH is the predominant low-molecular-weight thiol in mammalian cells and a major cellular antioxidant[47]. Although less reactive than Hcy, Cys is the most abundant plasma thiol and may function as an extracellular regulating factor of thiol/disulfide exchange in order to maintain an adequate redox status. Administration of Cys to rats protects against some neurotoxic compounds. For example, Cys can prevent the depletion of dopamine and related compounds caused by amphetamine and p-chloroamphetamine in mouse and rat brains[48]. On the other hand, Cys has been found to be cytotoxic both in vitro[49] and in vivo[50-53]. It is toxic to cultured hepatocytes (4

mM)[54], kidney cell lines (4 mM)[55], and primary neurons (1 mM)[56]. In *in vivo* studies, water-soluble, chemically defined diets containing Cys (0.92 g/ L) were disastrous to rats; the animals died within 3 days[57]. Administration of Cys was reported to lead to necrosis of the retina and hypothalamus in infant mice[53], brain atrophy in infant rats[51;58], and lethargy or convulsions at a dose of 10 mmol/kg in mice[50]. Current evidence shows that Cys toxicity depends highly on its auto-oxidation rate and on the total amount of Cys being oxidized, suggesting that the toxicity can be attributed to free radicals produced from auto-oxidation, but not to Cys itself[55]. Catalase and pyruvate were found to inhibit the production of hydroxyl radicals generated by Cys autoxidation so that they both protected primary neurons against Cys toxicity in tissue cultures. This protection is attributed to their ability to react with hydrogen peroxide (H_2O_2), preventing the formation of hydroxyl radicals[59].

Although Cys lacks the omega carboxyl group required for excitotoxic actions through direct activation of the excitatory amino acid receptors, it nevertheless evokes NMDA-like excitotoxic neuronal death and potentiates the Ca^{2+} influx evoked by NMDA. There are a number of possible mechanisms as reviewed by Janaky et al. (2000)[60]. Briefly, NMDA receptor activity may be up-regulated by increases in extracellular glutamate through increased release or inhibition of reuptake, removal of Zn^{2+}-induced inhibition on the NMDA receptor and/or direct interaction at the redox site. The generation of toxic Cys derivatives, including cysteine α-carbamate, cysteine sulfinate, S-nitrosocysteine and 5-S-cysteinyl-3, 4-dihydroxyphenylacetate, and free radicals as described above may also be contributing mechanisms. The formation of H_2S from Cys is yet another possibility.

1.3.3.2 Key enzymes of H_2S biosynthesis

Significant amount of H_2S is produced by mammalian cells, and this substance was measured in blood, isolated tissues and cells[9;61;62]. As previously described, two pyridoxal-5'-phosphate-dependent enzymes, cystathionine β-synthase (CBS, EC 4.2.1.22) and cystathionine γ-lyase (CSE EC 4.4.1.1), are responsible for the majority of the endogenous production of H_2S in mammalian tissues, where L-cysteine in used as the main substrate. CBS and CSE are also the first enzymes in the transsulfuration and reverse transsulfuration pathways, respectively.

1.3.3.2.1 CBS

In eukaryotes, CBS is directly involved in the homocysteine removal and biosynthesis of cysteine and H_2S. In these complex pathways, CBS was reported to have a much higher Km for L-cysteine (36mM) than for its natural substrates, L-serine (2–8 mM) and L-homocysteine (0.1–9 mM)[63]. It is activated approximately two-fold by the allosteric regulator, S-adenosylmethionine (SAM).

CBS is a cytosolic enzyme firstly purified from vertebrate liver[64]. The primary translational product of both the human and the rat CBS gene is a precursor protein with a molecular weight of 63 kDa[65] that forms tetramers or higher oligomers. Proteolysis of the precursor protein yields the active enzyme of CBS (amino acid residues 40-413)[66;67]. The reduction in size is accompanied by a significant increase in the specific activity of the enzyme and change from a tetramer to a dimer[68;69]. The purified CBS firmly bound with pyridoxal 5'-phosphate (P5P), which is necessary for its activity[70-72]. CBS is also continuously produced at an especially high level in the neural and cardiac systems[73]. CBS activity has been measured in various regions of the developing rat brain. CBS

activity gradually increases during development at almost the same rate in each region, until the adult level is reached at week 4 (about 4-fold increase)[74]. The level of CBS gene expression was studied during early human embryogenesis by in situ hybridization and in fetal and adult tissues by northern-blot analysis. Studies on the mutagenesis of CBS[75] showed that CBS is involved in the production of H_2S in the brain. Endogenous H_2S could not be detected in CBS knock-out mice and intermediate levels were detected in heterozygous mice[76]. These observations are in agreement with the inhibition of in vitro H_2S production from L-cysteine by brain homogenate in the presence of CBS inhibitors, such as hydroxylamine (HA) and aminooxyacetate (AOAA)[9].

Activity of CBS is believed to be highly regulated[77], tissue-specific[78] which present in brain and adipose tissue, absent in heart lung, testes, adrenal and spleen and compartmentalized in the endoplasmic reticulum[79]. These characteristics strongly suggest that any alterations in production, dissemination or consumption of the enzyme, products or substrates could have potentially damaging outcomes. CBS deficiency is an inherited metabolic disease characterized by lens dislocation, skeletal problems, vascular disease and mental retardation, etc. based on a clinical description of CBS deficiency in 629 patients in 1985[80], some of the most important clinical aspects of CBS deficiency are discussed below. Lens dislocation is one of the typical features of CBS deficiency, and the most common sign leading to diagnosis[81;82]. Numerous skeletal abnormalities may be observed in patients with CBS deficiency both by clinical and X-ray examinations. The most remarkable abnormalities included scoliosis/kyphosis, dolichostenomelia (long and thin extremities), decreased upper/lower segment ratio and arachnodactyly. Vascular disorders are another peculiar feature of this disease. Generally, they can be

characterized as a thrombotic diathesis that may manifest in the venous or arterial system and/or as accelerated atherosclerosis. In CNS, mental retardation is a frequent finding in CBS deficient patients. In an international survey, quantitative data from 284 patients showed a median IQ of 78 and 56 for the pyridoxine responders and non-responders, respectively[83].

1.3.3.2.2 CSE

CSE, another P5P-dependent enzyme, involves in the biosynthesis of H_2S[13] as previously described in **Fig. 1-2**. The purification of CSE also has been done in rats, mice and human. CSE activity is significantly lower in the liver of 24-month-old mice but it is about 10-times higher in the rat liver than in the liver of full-term human infants and over four times higher than in the adult human liver[84]. CSE activity is lower in guinea pig tissues than in rat tissues: five-fold lower in the liver and 18-fold lower in the kidney[85]. In the rat liver, the activity is low during fetal development, but increases rapidly during the last three days of gestation[86]. As rats mature, total CSE activity in the liver increases, peaking at 24 months of age and then decreasing to the same level found in five-week-old rats[87]. In contrast, CSE mRNA can be detected from the 19th gestational week onwards and the mRNA levels are similar to those of adult liver samples[88]. The most plausible explanation for this discrepancy is the post-transcriptional regulation of CSE gene expression. A very low level of activity has been described in the rat brain compared to in other tissues[89]. Same as CBS, CSE activity also has been measured in various regions of the developing rat brain. CSE activity increases during development, reaching the adult level in postnatal week 2. However, increases enzyme activity clearly increases less in the cerebellum (about 1.8-fold) than in the other regions (about 4-fold). The CSE content in

various regions of the 3-week-old rat brain estimated by immunoblotting is consistent with the enzyme activity; the enzyme level is lower in the cerebellum than in the other regions[90]. Small amounts of CSE mRNA have been detected in the brain[91]. In contrast to the liver and kidney, H_2S production in brain seems to be unrelated to cystathionase activity. CSE inhibitors, D, L-propargylglycine (PAG) and β-cyano-L-alanine (β-CNA), do not suppress the production of H_2S in the brain[9] although they effectively suppress H_2S production in the liver and kidney[13]. However, the effect of treating tissue homogenates with SAM, a specific activator of CBS, did not suggest that CBS plays a greater relative role in the catalysis of cysteine desulfhydration in the kidney than in the liver[13]. The subcellular distribution of CSE has been studied in the rat liver and kidney[92] which was mainly detected in the cytosolic fractions in the both tissues.

1.3.4 Physiological roles of H_2S and underlying mechanisms

Although H_2S was looked at as a toxic gas without any physiological function for quite long time, the possibility of H_2S as a physiological factor cannot be ignored since it has been found and present in mammalian tissues at relatively high level and biosynthesized by endogenous enzymes. Recent studies have contributed significantly to our understanding of the physiological roles of H_2S in the cardiovascular and nervous systems. In addition, H_2S may have a proinflammatory role in some forms of inflammation[93;94] , such as experimental pancreatitis and associated lung injury. Studies of mRNA signal and prophylactic CSE blockage suggested that CSE but not CBS was involved in this proinflammatory pathway although the mechanism is still unclear[95].

H_2S was firstly identified as a vasodilator because of its relaxant effect on smooth muscle. Notably in tissue studies, the activity and expression of CBS were found lacking

in human internal mammary arteries, saphenous veins, coronary arteries and aortic arteries but CSE mRNA expression in the ileum, portal vein, and thoracic aorta.[96]. These studies suggested that CSE but not CBS may play a major role in generating H_2S in cardiovascular tissues under physiological conditions. Further studies on inhibitors further confirmed that the production of H_2S in portal vein and thoracic aorta was catalyzed by CSE, whereas that in ileum was catalyzed by both CSE and CBS. In rat aortic tissues, rabbit ileum, and rabbit vas deferens, the relaxation mediating by H_2S occurred in a dose-related manner[97;98]. Zhao et al.[99] demonstrated in rats that H_2S decreased blood pressure and relaxed aortic tissues by directly opening K_{ATP} channels in vascular smooth muscle cells.

In nervous system, the first and most important evidence for the physiological role of H_2S came from the measurement of endogenous sulfide levels in rats, mice and human brain samples[100]. The study by Awata et al. in 1995[101] provided the enzymatic mechanisms for this endogenous H_2S in rat brain: activities of CBS and CSE were detected in six different brain regions. Data showed that the activity of CBS was about 30-fold greater than that of CSE. The transcriptional expression of CBS in rat brain (hippocampus, cerebellum, cerebral cortex, and brainstem) was later confirmed using Northern blot analysis but CSE mRNA was undetected[9]. The reduced H_2S production after the inhibition of CBS further pinpointed CBS to be the major endogenous enzyme for H_2S production in brain, in contrast to the cardiovascular system.

Several recent publications explored the mechanism(s) by which H_2S formation by brain CBS can be controlled. It is now clear that brain CBS (like NOS) activity is both Ca^{2+} and calmodulin dependent[102]. This enzyme might be achieved by the influx of Ca^{2+}

following depolarization to control neuronal H_2S production. Such "short-term" control mechanism suggests that H_2S, like NO, might act as a neurotransmitter. On the other hand, CBS activity is probably regulated by S-adenosyl-L-methionine (SAM) in a "longer-term" manner since changes on brain SAM levels also affect brain H_2S formation. For example, recent studies showed that female mouse brain contained less H_2S than brains from male, age-matched animals, which perhaps implies a role for sex hormones in the control of central H_2S formation[103]. Furthermore, castration of mice decreased the levels of testosterone, SAM and H_2S in the brain whereas a single injection of testosterone in female mice increased brain levels of SAM and H_2S to those found in male animals. Together, these results suggest that testosterone can manipulate brain H_2S levels indirectly by regulating the local concentration of SAM.

At physiological concentrations, H_2S was found to induce long-term potentiation (LTP) in the hippocampus[9;104]. Mechanistic studies have revealed that H_2S increases cAMP levels in neuronal and glial cell lines and primary neuron cultures and also hyperpolarizes CA1 and dorsal raphe neurons most probably by activating K_{ATP} channels. In addition, H_2S interferes with glutamate-mediated neurotransmission by an action on the NMDA receptor. For example, both direct electrical stimulation and glutamate application increase H_2S production from mouse cerebral cells and NaHS (H_2S donor) facilitates hippocampal LTP by increasing the sensitivity of NMDA receptors following a rise in intracellular cAMP.

The mechanisms underlying H_2S signaling are still unclear. Putative mechanisms are summarized in **Fig. 1-4** (see review by Moore PK in 2003)[94]. The first possible target of H_2S signaling is Ca^{2+} -dependent K_{ATP} channel[105]. The vasorelaxant property of H_2S on

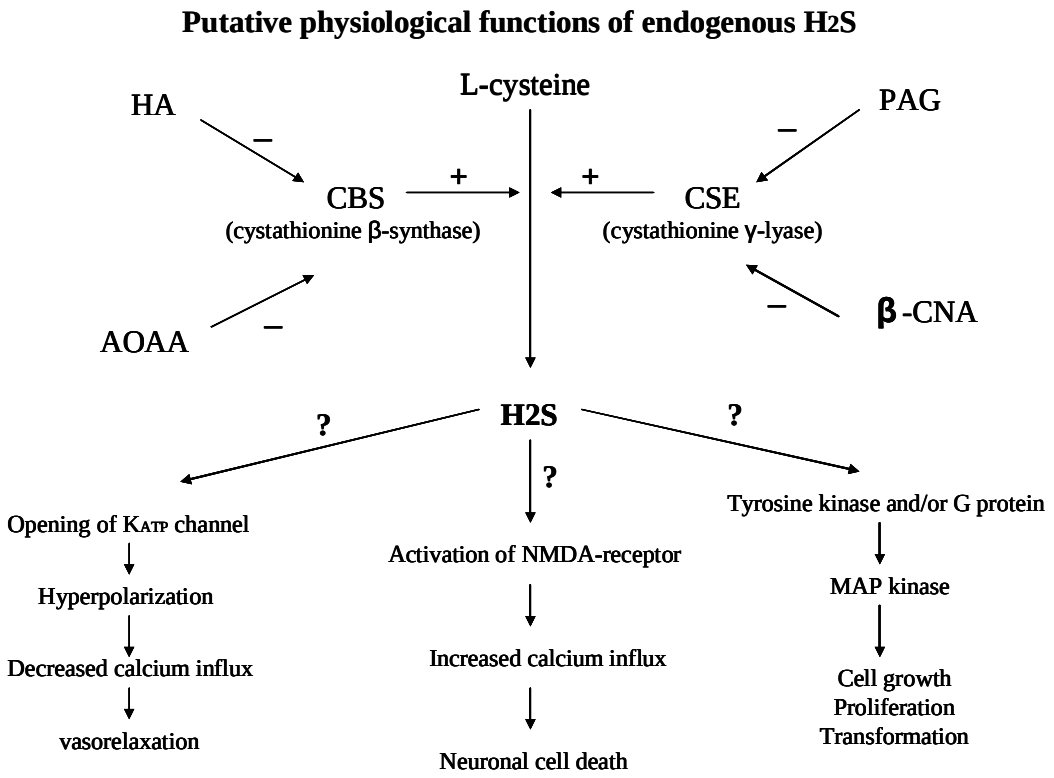


Fig. 1-4 Putative mechanisms underlying H_2S physiological functions.

H_2S may act as a gasotransmitter by (1) opening Ca^{2+} -dependent K_{ATP} channel, (2) activation of NMDA receptor or (3) regulating Tyrosine kinase and/or G protein. A lot of studies have done to investigating the role of H_2S and further studies are necessary for clearance.

the rat aorta tissues was attenuated by Ca^{2+}-dependent-K^+ channel blockers or in Ca^{2+} free bath solution[106]. Additionally, as potential H_2S donors, both sodium hydrosulfide (NaHS) and L-Cys produced significant dose-dependent decreases in isolated uterine spontaneous contractility[107]. In addition, CBS contains a calmodulin-binding sequence in its C-terminal domain, and it appears that this sequence suppresses desulfhydration activity in the absence of Ca^{2+}/calmodulin. Furthermore, NaHS induced a dose – dependent hyperpolarization and reduced input resistance of CA1 neurons or dorsal raphe neurons. Changes in K^+ ion flux were identified to be the main ionic basis for these effects of NaHS, and K_{ATP} channels in neurons were speculated as the specific targets.

N-methyl-D-aspartate (NMDA) receptors may be another target of H_2S signaling. Physiological concentrations of H_2S specifically potentiate the activity of the N-methyl-D-aspartate receptor, and induce long-term potentiation in the hippocampus[9;104]. In the presence of a weak tetanic stimulation, NaHS at 10-120 μM facilitated the induction of long-term potentiation in rat hippocampal slices by enhancing the NMDA-induced inward current. Activation of the cAMP-dependent protein kinase pathway is likely to mediate the interaction of H_2S and NMDA receptors. The 3rd putative mechanism of H_2S signaling was mentioned by a few studies: H_2S may change the activity of MAP kinase by regulating Tyrosine kinase and/or G protein, which may be involved in cell growth, proliferation or transformation[9;94].

1.3.5 Roles of endogenous H_2S in CNS diseases

Recent publications help us to notice the role of H_2S in CNS diseases. That H_2S promotes glutamate-mediated transmission via NMDA receptors might also have implications for neurodegenerative diseases in which excessive activation of NMDA

receptors is involved[108].

In the brain of Alzheimer's disease patients[109], reduced CBS activity, elevated Hcy, and a reduced level of SAM were found when compared with the brains of age-matched normal individuals[110]. These observations on patients with Alzheimer's disease are consistent with a role of CBS in H_2S production. Reduced brain H_2S concentration can reflect a higher turnover perhaps by binding to and enhancing glutamate-mediated transmission via NMDA receptors. In this way, H_2S might contribute to the neuronal loss associated with this disease. H_2S also was reported to modulate hypothalamo-pituitary-adrenal axis function: given NaHS dose-dependently decreased KCl-stimulated corticotrophin-releasing hormone (CRH) in isolated rat hypothalami; SAM inhibited stress-related glucocorticoid increase in vivo[111]. The possibility that H_2S can induce LTP is also very important because this event is fundamental for several physiological processes including both memory and hyperalgesia[112].

1.4 Stroke research

Stroke is caused by an acute loss of focal cerebral functions due to either spontaneous hemorrhage or inadequate cerebral blood supply to a part of the brain as a result of low blood flow, thrombosis, or embolism associated with diseases of the blood vessels, heart, or blood. The recommended standard WHO stroke definition is: a focal (or at times global) neurological impairment of sudden onset, and lasting more than 24 hours (or leading to death) and of presumed vascular origin. Clinically, signs of a stroke vary, but often include the abrupt onset of weakness or numbness in the face or the limbs on the left or right side of the body, loss of vision, acute headache, difficulty producing or understanding speech, physical instability, and dizziness.

1.4.1 Epidemiology

As shown in WHO report of 2006, stroke is the 3rd leading causes of death after cancer and heart disease. On average, every 45 seconds someone in the United States has a stroke; each year about 700,000 people experience a new or recurrent stroke (American Heart & Stroke Association, Stroke fact 2006). These trends will remain so in the future both in developing and developed countries. As strokes are considered as a significant cause of death especially in elderly populations, hypoxic ischemia is also a common cause of damage to the fetal and neonatal brain. Neonatal stroke occurred in approximately 1 in 4,000 to 1 in 10,000 newborns, and more than 80% involve the vascular territory supplied by the middle cerebral artery[(113)].

In addition to its life-threatening properties, stroke is also a major cause of disability in the elderly and often requires long-term institutionalization. According to WHO estimates, 15 million people worldwide suffer a stroke annually; 5 million are left permanently disabled, which is placing a burden on both family and modern society. Such stroke burden is projected to rise from around 38 million dollars globally in 1990 to 61 million dollars in 2020 (Atlas of heart disease and stroke, WHO, Sept. 2004). Unfortunately, no effective stroke therapy exists beyond thrombolysis, which is safe and effective for only a limited population of stroke patients. Therefore, any success in stroke research is more pronounced and meaningful not only to researches but also to human beings.

1.4.2 Classification

Normally, stroke can be classified into ischemic and hemorrhagic based on major mechanisms causing brain damage. Cerebral ischemia is characterized as cessation of

blood flow resulting in insufficient oxygen and glucose delivery to affected areas. The effects of ischemia are fairly rapid because the brain does not store glucose, the chief energy substrate and is incapable of anaerobic metabolism. There are several superficially defined subgroups and types of brain ischemia characterizing a broad spectrum of ischemic conditions in clinical and experimental situations. Clinically, 80% of the stroke cases are ischemic resulting either from atherothrombotic brain infarctions (ABI) or emboli of the cerebral vasculature. Additionally, global ischemia, an important type of ischemic brain insult resulting from the collapse of systemic circulation after cardiac arrest, leads to transitory hypoperfusion of the brain and brain damage at various brain areas[(114)]. Finally, perinatal asphyxia can cause cerebral hypoxic/ischemic injury, which results in severe neurological sequelae and death[(115)]. Non-traumatic intracerebral and subarachnoidal hemorrhage represents approximately 10% to 15% of all strokes. Intracerebral hemorrhage normally originates from deep penetrating vessels and induces injury to brain tissue by disrupting connecting pathways and causing local pressure injury. In either ischemia or hemorrhage, destructive biochemical substances released from a variety of sources play an important role in brain tissue damage, including various neurotransmitters.

1.4.3 Risk factors

Risk factors are traits and lifestyle habits that increase the risk of disease. Numerous epidemiological studies have identified that hypertension, coexisting cardiac diseases, diabetes and hyperlipidemia are among the most important biological risk factors for stroke. Elevated plasma levels of homocysteine or circulating fibrinogen, obesity and recent infection also could be minor risk for stroke. Likewise, there is compelling

evidence that lifestyle factors including smoking, alcohol consumption and lack of physical activity are significant factors for stroke risk. Most of them can be modified, treated or controlled but some cannot. Non-modifiable risk factors for stroke are age, race, sex, genetic factors and geography[116;117]. Randomized trials have established the effectiveness of treatments target modifiable risk factors in stroke prevention. These strategies include anti-hypertension, carotid surgery, glucose control, treatment of hyperlipidemia, anti-thrombotic and anti-platelet therapy, and cessation of tobacco smoking[118].

1.4.4 Therapeutic strategies

The two fundamental approaches to acute stroke therapy are recovering blood perfusion and neuroprotection. Intravenous recombinant tissue plasminogen activator (rtPA) initiated within 3 hours of stroke onset remains the only approved and validated therapy for acute ischemic stroke, and combined use of intravenous/intra-arterial thrombolysis, mechanical thrombolysis, anticoagulant, and anti-platelet to maximize reperfusion are potentially attractive approaches[119]. Neuroprotective agents are being developed targeting many aspects of the ischemic cascade in an attempt to prolong the viability of neurons subjected to ischemia. Any approach on neuroprotection will depend on the intensive understanding of pathophysiological event happening after stroke. Anti-edema agents, glutamate/NMDA receptor antagonists, calcium/sodium channel antagonists, free radical scavenging, and anti-inflammatory agents are all at various stages of clinical development (*Clinical Pharmacology of Cerebral Ischemia 1997, edit by G.J. ter Horst & J. Korf*). However, clinical trials of neuroprotective therapies shown to be effective in animal models have to date been uniformly negative, probably because of the

complexity of the disease or inadequacies of trial design[120].

1.4.5 Research failures

Although the success of rt-PA has revolutionized acute stroke management and proved that stroke is a treatable disease and numerous neuroprotective agents have been found to reduce infarct size in animal models, translation of neuroprotective benefits from the laboratory bench to the emergency room has not been successful. Early success in the preclinical studies may have prematurely pushed numerous agents into clinical trials. Translating bench success to the proof of clinical efficacy and safety has been frustrating. Lack of satisfactory animal models resembling the human disease, and discrepancies between preclinical studies and clinical trials have proven costly. Reasons for the failures have led to intense discussion for the last several years. The discrepancies between preclinical studies and clinical trials may be the cause of some of the problems encountered previously, including (1) outcome measures, (2) functional assessment, (3) pre-morbid conditions , (4) therapeutic windows and (5) drug-dosing schedules[121]. These problems may limit the success of neuroprotective trail but give lessons for the future works. By learning from our past mistakes, we may be able to have more successful studies in the future.

1.5 Cerebral ischemia

There are three main well-accepted mechanisms causing ischemic strokes: (1) thrombosis, (2) embolism and (3) global ischemia (hypotensive) stroke. All are characterized as insufficient oxygen and glucose delivery to brain tissues and cells because of cessation of blood flow. Ischemic conditions challenge brain tissues in several

ways. Even a short lasting obstruction of cerebral arteries or hypoperfusion of the brain may cause quickly irreversible brain damage. However, brain damage may further evolve for a relatively long period after acute insult. The concept of “penumbra”, salvageable tissues surrounding the center of the irreversibly damaged brain tissues in experimental settings has fueled extensive research aimed to establish a mechanism or mechanisms behind ischemic brain pathology and to find potential treatments for stroke. Indeed, several targets for neuroprotection including excitatory amino acid, calcium overload, enzymes, free radicals, gene expression, apoptosis and inflammation have been explored[(122)].

Considerable experimental evidence and recent discoveries involving the biochemical cascade of events that occurs during cerebral ischemia have resulted in an improved understanding of the pathogenesis of ischemic stroke. These discoveries point to the pathophysiological role of an intricate common pathway of neuronal injury that involves overactivation of excitatory amino acid receptors, inordinate intracellular calcium fluxes, activation of catabolic enzymes, and production of free radicals. According to the time course of ischemic cascade, cellular and molecular events can roughly be divided to acute and delayed cascades; both contribute to the final outcome. However, both cascades may share some features and therefore have some overlapping temporally and spatially. For example, inflammation was supposed to contribute to both early brain injury but also to the delayed cascades of brain injury after stroke. In addition, inflammation has been recognized not only as a detrimental response following stroke but also as a course of action involved in the brain recovery following ischemic insult[(123)]. Altogether, all of these cascades comprise of a vast array of functional and morphological events that take place

in the brain immediately or even months after the ischemic insult. These processes are influenced by a variety of neurotransmitters, neuromodulators, growth factors, and alterations in gene expression[124].

1.5.1 Vulnerability of brain tissues to ischemia

Brain represents only about 2.5% of human body weight but it consumes 15% of energy generated in the body. Moreover, brain is particularly susceptible to ischemia compared with other organs. Complete interruption of blood flow to the brain for only 5 minutes triggers the death of vulnerable neurons in several brain regions, whereas 20–40 minutes of ischemia is required to trigger death of cells in heart or kidney. The prominent vulnerability of brain tissues to ischemic damage relates to its high metabolic rate and its exclusive dependence on glucose as an energy source. Most energy utilized in the brain is used by neurons to maintain ionic gradients, which are important for normal cellular function. The brain's heightened vulnerability to ischemia may involve other mechanism in addition to energetic considerations. Normal intrinsic cell-cell and intracellular signaling mechanisms, responsible for information processing may become harmful under ischemic conditions. These processes may hasten energy failure and enhance the final pathways underlying ischemic cell death in all tissues.

1.5.2 Mechanisms underlying the acute brain ischemia

Once acute brain ischemia happening, very complex events will induce the brain damage. Several mechanisms were well accepted as contributors to the damage after acute brain ischemia. These cascades were simply summarized by Dirnagl U. [125](1999) in **Fig. 1-5;** the details of events will be described here.

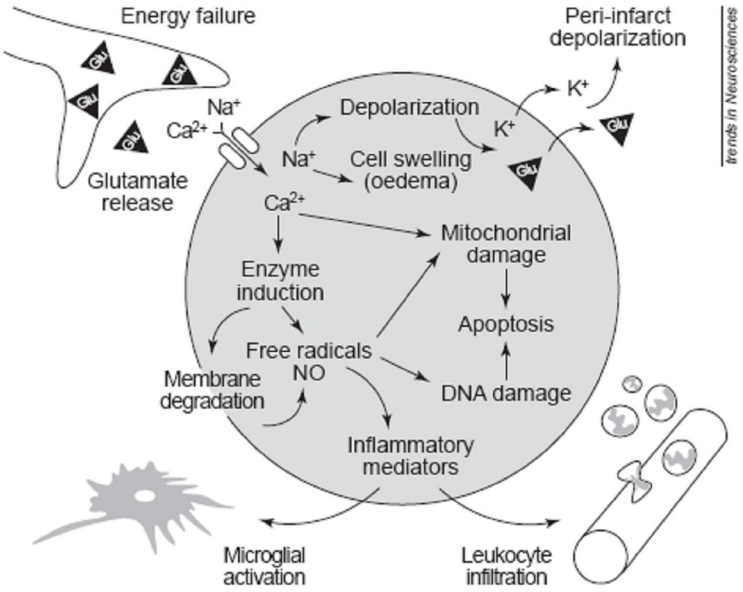


Fig. 1-5 Simplified overview of pathophysiologic mechanisms in the focally ischaemic brain (cited from Dirnagl U. 1999).

Energy failure leads to the depolarization of neurons. Activation of specific glutamate receptors dramatically increases intracellular Ca^{2+}, Na^{+}, Cl^{-} levels while K^{+} is released into the extracellular space. Diffusion of glutamate (Glu) and K^{+} in the extracellular space can propagate a series of spreading waves of depolarization (peri-infarct depolarization). Water shifts to the intracellular space via osmotic gradients and cells swell (edema). The universal intracellular messenger Ca^{2+} over-activates numerous enzyme systems (proteases, lipases, endonucleases, etc.). Free radicals are generated, which damage membranes (lipolysis), mitochondria and DNA, in turn triggering caspase-mediated cell death (apoptosis). Free radicals also induce the formation of inflammatory mediators, which activate microglia and lead to the invasion of blood-borne inflammatory cells (leukocyte infiltration) via up-regulation of endothelial adhesion molecules.

1.5.2.1 Depolarization and energy breakdown

The responds of brain tissue to acute energy breakdown are various temporally and spatially. Within seconds of cerebral ischemia, local cortical activity ceases which were detected by electroencephalography (EEG). This massive shutdown of neural activity is induced by K^+ efflux from neurons, mediated by the opening of voltage-dependent K^+ channels initially and by ATP-dependent K^+ channels later; overall leading to transient plasma membrane hyperpolarization. A few minutes later, despite this energy sparing response, an abrupt and dramatic redistribution of ions occurs across the plasma membrane, associated with membrane depolarization (efflux of K^+ and influx of Na^+, Cl^-, and Ca^{2+}). This "anoxic depolarization" results in the excessive release of neurotransmitters, simultaneously, the energy consuming processes such as reuptake of excitatory amino acids are compromised leading to accumulation of excitatory transmitters, in particular, glutamate, which promotes spatial spread of cellular depolarization further, depletion of energy stores, and advancement of injury cascades.

This region of the brain is often referred as ischemic core, which cannot be saved by any pharmacological interventions. However, the area surrounding the ischemic core, often referred as the penumbra, is also suffering from reduced blood flow to 20%-60% of its normal perfusion level, and is at very high risk of tissue damage[126]. ATP levels are maintained at near-normal level but electrical neuronal functions are interrupted. The neuronal dysfunction is manifested by decreased voltage leading to abnormal EEG finding and failure to detect sensory evoked potentials even though ionic gradients and membrane pumps are generally maintained and functional. As a result, cells composing the penumbra

are at this point viable but at a high risk to death. Also, post-ischemic glucose utilization in the forebrain, except in the hippocampus, was depressed below control values and either remained low (neocortex, striatum) or gradually rose to normal (white matter) by 48 hr after the insult.

Acute breakdown in energy metabolism also results in disturbed protein metabolism and has been observed in response to decreased cerebral blood flow in animal models. For example, it has been shown that protein synthesis decreases in response to even slight reductions in CBF[127;128] in the rodent brain. In the fetal brain, protein synthesis was found to recover faster than adult brain from hypoxic/ischemic insults suggesting developmental differences in response to hypoxic/ischemic insults[129]. It should be noted, however, that hypoxic or ischemic conditions do not inhibit all protein synthesis. Increased production of several transcription factors and heat shock protein can be observed during reperfusion[130]. Interestingly, inhibitors of protein synthesis, such as cycloheximide[131] and anisomycin[132] have been found to protect neurons from ischemia, suggesting the involvement of newly synthesized "killer proteins".

1.5.2.2 Excitotoxicity

Excitotoxicity is a phenomenon in which prolonged activation of excitatory amino acid receptors leads to cell death. The excessive release of glutamate activates glutamate receptors, which can be divided functionally into two subgroups: ionotropic and metabotropic receptors. Ionotropic receptors are directly coupled to ion channels whereas metabotropic glutamate receptors are coupled to more complex intermediary compounds, such as G-protein and phospholipase C (PLC) which modulate intracellular second messengers, such as inositol-1, 4, 5-trisphosphate (IP_3), calcium and cyclic

nucleotides[133;134]. The directly coupled ionotropic receptors can be further divided to three subtypes named by their selective chemical agonists: N-methyl-D-aspartate (NMDA), α-amino-3-hydroxy-5-methyl-isoxazolepropionate (AMPA) and kainate (KA).

Metabotropic glutamate receptor (mGluR) are classified according to their amino acid sequence homology[135]. Class I receptors are positively coupled to PLC and thereby regulate Ca^{2+} release form IP_3-sensitive internal stores and via diacylglycerol (DAG) and IP_3[136]. Class II and III mGluR are, on the other hand, negatively, coupled to adenylyl cyclase regulating decreasing the level of cAMP[137]. Metabotropic glutamate receptors are found pre-and post synaptically and they may modulate the toxicity of ionotropic glutamate receptors, for example during excitotoxicity[138;139]. However, direct stimulation of mGluRs with the selective agonist 1-aminocyclopentane-1, 3-dicarboxylic acid (ACPD) does not result in neurotoxicity[140].

AMPA receptors belong to the class of ionotropic glutamate receptors. They are as widespread in the CNS as NMDA receptors, have fast gating kinetics, and are involved in the generation of the fast component of excitatory postsynaptic potentials. Structurally, AMPA receptors are composed of subunits termed GluR1-GluR4[141]. All subunits have characteristics of a 900 amino acid long polypeptide chain and are subject to alternative splicing[142]. This alternative splicing allows different expression profiles of different AMPA receptors in mature and developing brain, also splicing directed channel modifications may also explain the functional differences and cell specific distribution of different AMPA receptors. Although most neuronal AMPA receptors show small Ca^{2+} permeability upon glutamate stimulation[143-145], they may modulate Ca^{2+} influx due to AMPA receptor mediated Na^+-dependent depolarization and the subsequent opening of

voltage activated calcium channels (VACCs) leading to release of Mg^{2+} blockade and NMDA receptor activation[146;147].

NMDA ionotropic receptors have an essential role in many functions of CNS, and have been suggested to be involved in glutamate mediated processes such as memory acquisition, cognitive processes and learning[148]. These receptors are also crucial for excitotoxicity, where excessive release of synaptic glutamate and inability of neurons to respond properly, leads to neuronal death[149]. NMDA receptors (NR) are heteromeric structures composed of two subunit types, NR 1 subunit and one of four NR 2 subunits (NR2A-NR2D)[150]. The NMDA receptor channels have fundamental differences compared to AMPA and kainite receptor channels, which is related to their physiological role. NMDA receptors have a high single channel conductance, high Ca^{2+} /Na^{+} permeability ratio, a voltage dependent Mg^{2+} block and high affinity for glutamate (*The NMDA receptor, 2nd edition by Collinridge and Watkins 1994, New York, Oxford University Press*).

The least studied glutamate receptor is the ionotropic kainite receptor. Kainite receptors are difficult to distinguish from AMPA receptors pharmacologically since selective agonists and antagonists are not available[151]. Therefore, kainite/AMPA receptors are often referred together as non-NMDA receptors. Also, even though AMPA and KA receptors share a considerable amount to sequence homology, immunoprecipitation studies do not support the hypothesis that KA and AMPA receptors are composed of the same subunits[152]. Indeed, molecular cloning has revealed that KA receptors are composed of high affinity KA1 and KA2 subunits and low affinity GluR5, GluR6 and GluR7 subunits, which may form functional ion channels[153].

Several brain insults elicit a pronounced release of glutamate characteristic to excitotoxicity[154-156]. Reduction in blood flow and strong membrane depolarization, for example in cerebral ischemia, increase the opening of voltage-dependent ion channels and activation of glutamate receptors in neurons. Excessive glutamate release from the presynaptic membrane leads to excessive accumulation of postsynaptic intracellular calcium. Post-synaptically, NMDA receptors are mainly responsible of the influx of Ca^{2+}. Also, some types of AMPA and KA receptors may also contribute to Ca^{2+} influx, as their coupled ion channels are partially permeable to calcium. Excessive neurotransmitter release leading to Ca^{2+} overload triggers cascades that activate potentially detrimental enzymes, DNA fragmentation, proteolysis and lipolysis[157]. As an example, Ca^{2+} overload leads to activation of phospholipase A which produces free fatty acids including arachidonic acid and platelet-activating factor. Arachidonic acid inhibits glutamate reuptake from the synaptic cleft, which in turn leads to further activation of glutamate receptors, Ca^{2+} accumulation, and further arachidonic acid formation[158]. In addition, platelet-activating factor (PAF) increases the glutamate release from the presynaptic membrane[159]. Arachidonic acid is also a substrate for cyclooxygenase-2 (COX-2) which in induced by ischemia and plays a significant role in the development of delayed ischemic brain damage and brain inflammation[160-162]. Other neurotoxic cascades triggered by excitotoxicity include depolarization of mitochondria, calcium overload of mitochondria, and production of reactive oxygen species (ROS)[163].

Blockade of glutamate receptors has been extensively studied as a treatment for brain ischemia, In cat, the selective NMDA receptor antagonist, MK-801, significantly reduced the ischemic infarct size, supporting the important role of NMDA receptors in mediating

brain tissue injury[164;165]. Similar reports have been obtained from rats even if MK-801 is given early after the insult[166]. Also, several AMPA receptor antagonists have been found to provide protection against ischemic insults[167-169]. Moreover, voltage gated ion channel antagonists, such as phenytoin, carbamazepine and lamotrigine for Na^+ channels[170] and nicarpidine[171] for Ca^{2+} channels have been found protective in focal ischemia as well as brain lesion models induced by electric convulsions. However, although therapeutic approaches aimed against excitotoxicity have been shown to protect in experimental brain ischemia models, the narrow therapeutic time-window, together with severe side effects eliminate the use of currently available glutamate receptor antagonists in humans[172].

1.5.2.3 Necrosis

Necrosis means mortification of tissue. It is a passive pathological event arising from the spontaneous insults such as trauma or ischemia. Microscopically it is characterized by cell, organelle and mitochondrial swelling or dilatation, cytoplasmic vacuolation, breaking of cell membranes, disintegration of organelles, and finally cell bursting[173-175].

Defects in membrane permeability and ion transport proteins as well as impairments in oxidative phosphorylation and depletion of high-energy phosphates are early, causal mechanisms for cellular necrosis. The mitochondria undergo a complex sequence of changes that involves contraction or condensation of the inner membrane and dissipation of metrical granules, inner membrane swelling and cristaeolysis, formation of flocculent aggregates, and then disintegration. Evolution of mitochondrial abnormalities has been demonstrated in various forms of cellular necrosis[176].

In necrotic cells, ribosomes are dispersed from the rough endoplasmic reticulum and polyribosomes disassociated, resulting in many monomeric ribosomes that are found

"free" in the cytoplasm, causing the cytoplasmic matrix to appear dense and granular. Also cisterns of the endoplasmic reticulum and Golgi apparatus have been found to dilate, fragment, and vesiculate, and the plasma membrane can undergo a process called blebbing[177]. Because cellular necrosis results in the liberation of antigenically active denatured intracellular debris, it is accompanied by an inflammatory response, which includes leukocytic infiltration, tissue edema, and ultimately a gross change in the overall histology of the focus of tissue damage due to the formation of a "scar".

Du and colleagues (1996)[178] have reported that 90-min transient focal ischemia in rats caused substantial cortical infarction within 6 h and was fully developed 1 day after the insult. They found also in their study that the majority of the cell death in severe ischemia was necrotic, whereas in a mild (30 min) transient focal ischemia model, characteristics of delayed, apoptotic cell death was more evident[179]. Excitotoxicity caused necrosis of neurons in less than 24 h[180]. In addition, global cerebral ischemia causes acute necrosis of principal striatal neurons in 24 h[181], but delayed degeneration of hippocampal CA1 neurons 2-4 days later[182-184]. In the hippocampus, this neuronal death has been called post-ischemic "delayed neuronal death" by Kirino (1982)[185], as distinct from necrotic death.

1.5.2.4 Reactive oxygen species (ROS)

Oxygen-free radicals have been suggested to be involved in the pathogenesis of cerebral ischemia and reperfusion injury[186]. On reperfusion after an ischemic insult, several molecular events, such as phospholipase activation, lipid peroxidation and dysfunction of the mitochondrial respiratory chain, have been shown to lead to production of free radicals and oxidative stress[187].

Oxidative stress is defined as an increase in intracellular ROS such as H_2O_2, superoxide anion (O^{2-}), or hydroxyl radical ($^{\cdot}OH$). Among the oxygen-free radicals, superoxide (O^{2-}) is a sample which is directly toxic to neurons[188]. Importantly, oxygen-free radicals may cause oxidation of proteins and DNA damage, both of which are hallmarks of ischemic injuries. In addition, there are several lines of evidence suggesting that superoxide (O^{2-}) contributes indirectly to tissue damage by enhancing vasogenic edema and blood brain barrier (BBB) disruption after brain ischemia. The role of superoxide (O^{2-}) in compromised BBB is supported by the fact that endothelial cells are the cellular constituents of BBB, but also the major source of superoxide (O^{2-}) production[189]. This way endothelial cells damage themselves in a self-inflicting manner. Moreover, reperfusion-induced oxidative stress (ROS) increases phagocytic activity of infiltrating peripheral leukocytes (neutrophils and macrophages)[190;191] and resident brain microglial cells after ischemia[192].

Oxidative stress and especially ROS are important activators of certain transcription factors, which control the expression of several stress related genes. Activating protein-1 (AP-1) and nuclear factor kappa-B (NF-κB) are among the most well described[193]. Importantly, these cytokines play an important role in the pathogenesis of brain ischemia and post ischemic inflammation in the brain. Ultimately, early oxidative stress and ROS production not only predisposes delayed mechanisms of brain injury such as disruption of the BBB, vasogenic edema, gene expression and inflammation, but also form a link between acute and delayed mechanisms contributing to brain injury.

1.5.2.5 Brain edema after ischemia

During brain ischemia, a fall in ATP or phosphocreatine levels as a compromise to

energy production from glucose and oxygen in the mitochondria. Since brain tissue mainly relies on oxidative energy production, anaerobic energy production that ensues from ischemia leads to production of lactic acid and pH value falling. Increases of lactic acid and H^+ content attract water and cause following cerebral edema, even though most of the cell edema is thought to develop by a leakage of extracellular Na^+ inside the cells and due to a compromised pumping activity of ATP driven Na^+/K^+ ATPase[194]. The resulting edema can further decrease perfusion in the brain and lead to some detrimental effects, such as increase in intracranial pressure, vascular compression and herniation. Meaningfully, brain edema is one of the most important determinants for whether a patient could survive the first few hours after a stroke in clinical cases[195].

1.5.3 Delayed mechanisms contributing to brain damage

Although a fall in energy homeostasis contributes to acute necrotic cell death during the early phases of ischemia, delayed and selective neuronal death also occurs. This is supported by the observation that restoration of cerebral blood flow can rapidly normalize the ionic disturbances and ATP levels in the brain but delayed cell death cannot be prevented. The classic example of the delayed effects of ischemia is the selective neuronal cell death in the vulnerable CA1 area of the hippocampus, which occurs several days after the ischemic insult[196]. It has been suggested that transient energy depletion and loss of ionic homeostasis trigger the delayed or secondary damaging cellular events that eventually kill vulnerable cells.

1.5.3.1 Apoptosis

Apoptosis is an active, energy consuming process of self-destruction of cells or damaged cells are eliminated. It can be also considered as an intrinsic suicidal program of the cell triggered by a wide array of stimuli. Therefore, apoptosis is physiologically an important way to maintain homeostasis of the organism and apoptotic machinery is evolutionarily conserved[173;197].

Cell death by apoptosis is carried out by several facilitating receptors or factors. These include apoptosis inducing or death receptors (e.g. Apo-1/Fas, Apaf-1), apoptosis initiating factors (AIFs), members of the Bcl-2 protein family and cysteine proteases of the caspase/calpain family[198;199]. The various apoptotic pathways and mechanisms can be roughly divided to caspase-dependent and caspase-independent mechanisms.

Caspases have been widely recognized as the key apoptotic molecules[200;201]. Caspases are cysteine containing enzymes having a pentapeptide motif Gln-Ala-Cys-X-gly, where X is Arg, Gln or Gly[202]. This family of proteases is synthesized as zymogens and various apoptotic upstream signals mediate maturation of these precursors to mature proteases. Upon activation these proteases are cleaved to small (10kD) and large (20kD) subunits from pro-caspase to yield active enzymes, which consists of two small and two large subunits as heterotetramers[203]. Caspases can be divided into 2 categories: initiator caspases (caspase-1,-2,-4,-5,-8,-9,-10 and -14), which are activated by oligomerization-induced autoprocessing, while effector caspases (caspase-3, -6, -7) are activated by initiator caspases or other proteases. Two major pathways have been recognized according to their initiator caspase: death receptor mediated apoptosis involving caspase-8 and mitochondrial pathway where various signals can trigger the release of harmful proteins by mitochondria, especially cytochrome c into the cytoplasm which further promotes

aggregation caspase-9, Apaf-1 and cytochrome c to form the apoptosome. The apoptosome instead cleaves and activates procaspase-3 into active caspase-3, the key effector caspase involved in caspase-mediated apoptosis[204;205].

Although caspase inhibition has been shown to prevent apoptosis, cell death with apoptotic morphology still occurs suggesting caspase-independent apoptosis[206;207]. Cell death by caspase-independent mechanisms has been shown to involve activation of other proteases such as calpain[208], proteasome[209] and serine proteases[210], apoptosis initiating factor (AIF)[211] endonuclease G (EndoG)[212] or Bax mediated apoptosis without caspase activation[213].

The basic apoptosis mechanisms in neurons and other brain cells is the same as in all other cell types. However, apoptosis occurring as a physiological event (i.e. developmentally regulated) can be triggered by mechanisms distinct from those that trigger pathological event. Several lines of evidence have suggested the involvement of apoptosis in cerebral ischemia, although acute cell death in ischemia has been traditionally considered necrotic. Apoptotic neurons are predominantly located within the surrounding tissue (penumbra), and not in the ischemic core. Indeed, many experimental studies of transient cerebral ischemia and cerebral hypoxia/ischemia have demonstrated that neurons in the border zone of infarcts, scattered neurons in the cerebral cortex and striatum, and in the vulnerable CA1 area of the hippocampus become susceptible to TUNEL-staining[214-218], a commonly used indicator of apoptosis.

Ischemia induced apoptotic cell death has been suggested to involve activation of caspases, which has been supported by several studies[219]. Caspase-3 activity and its cleavage from its precursor has been detected in adult rats subjected to cerebral

ischemia[220] as well as in neonatal hypoxia/ischemia model[221;222]. Caspase-3 is a well known effector caspase and its inhibition has been found to prevent caspase activated deoxiribonuclease (CAD) activity and subsequent DNA cleavage[223]. Moreover, inhibitors of caspase activity have been found neuroprotective in transient and permanent focal ischemia models in adult animals[224-228] as well as neonates[229]. In focal ischemia, caspase cleavage products and TUNEL staining have been found to co-localized in neurons starting very early 1-2 hours after severe and 9-12 hours after mild ischemia[230;231]. In global ischemia models, expression of caspase-3 mRNA have been found to be up-regulated[232]. Importantly, there are also indications of increase neuronal procaspase-3 levels in clinical atherothrombotic stroke, although this increase is not associated with activated caspase-3 or cleavage of poly (ADP-ribose) polymerase (PARP)[233]. however clinical cases of cardiac arrest show activated caspase-3 and cleavage product of PARP in cortical neurons as well as macrophages and microglia during 6-9 days after the insult[234].

In addition to caspase-3, several other caspases have been found to be activated or up-regulated in response to ischemic insults. For example, caspase-1 or interleukin-1 converting enzyme (ICE) mRNA has been shown to be up-regulated in response to ischemic insults[235;236]. Importantly, increased expression of caspase-1 substrate pro-interleukin-1 β (pro-IL-1β) and its product IL-1β have been widely established in ischemia models. The important role of caspase-1 in ischemic cell death is supported by the protective effects of dominant negative mutation of caspase-1 in mice after ischemia[237]. Alternatively, mice with knock out caspase-1 show reduced brain damage after ischemic insults[238].

Apoptosis can also occur without caspase activation. Ischemic and excitotoxic insults have been reported to involve caspase-independent apoptotic mechanisms[239;240]. Most of these mechanisms are yet to be established in brain disease models, although their existence has been shown in several other experimental settings.

1.6 Experimental models for cerebral ischemia

A lot of literatures describe different approaches used to study cerebral ischemia in which many experimental models were designed to describe the cellular and molecular events that take place in the brain after ischemic insults.

Primary cultures, co-cultures, cell lines and tissue cultures are widely used methods to study ischemia at cellular level. These in vitro models are relatively simple but powerful tools to study elements in brain ischemia in a highly controlled environment. In vivo animal models have mainly employed mammals such as rodents, gerbils, canines and primates. The most widely used rodent cerebral ischemia models are reviewed further.

1.6.1 in vivo models

The testing of potential therapeutic agents in animal disease models is essential prior to launching any clinical trials. Animal model of cerebral ischemia should satisfy the following criteria [241]:

1. The lesion that causes the ischemic injury should be highly reproducible.
2. Monitoring and maintenance of physiological stability should be readily achievable.
3. The cost involved in inducing the stroke and determining biological outcome should be reasonable and valuable.

4. Biological endpoints should be clearly defined and rigorously measured.
5. The occlusive process and physiologic response should mimic human stroke.
6. It should be devoid of or have minimal complicating side effects.

An animal model for stroke is a living experimental system that contains most of the necessary elements: neurons, glia, brain vasculature and the whole complex physiology of the animal. The four most widely used animal models of cerebral ischemia are global ischemia, transient or permanent focal ischemia, and hypoxic ischemia in neonates[242].

1.6.1.1 Global ischemia

Global brain ischemia is induced by cardiac arrest, which results in cessation of systemic blood circulation, hypotension and hypoperfusion of the brain. Transient global ischemia induces selective ischemic cell damage, but not infarct[243]. In contrast, ischemic stroke in the human often results in infarct. Thus, global models may not provide information completely applicable to most naturally occurring human ischemic stroke. However, global models are useful in investigating specific biochemical and physiologic responses of transient events of low cerebral blood flow, as well as mechanisms associated with the process of selective ischemic cell damage[244].

This animal model has been applied in several mammal species such as gerbils[245;246], rats[247], cats[248], and primates[249]. There are several ways to produce global cerebral ischemia, but the most common methods employ the occlusion of the common cerebral arteries and vertebral arteries for 5-30 minutes. Gerbils have been acknowledged to have ideal vasculature for the global forebrain ischemia studies and the simplest and most popular global ischemia model for screening novel neuroprotectants[250]. It is mainly due to absence of anastomosis between the vertebral and internal carotid arteries which is

necessary to complete the Willis circle. So that bilateral occlusion of the common carotids in this model is sufficient to produce severe global forebrain ischemia[251;252]. This widely used method provides a highly reproducible model to study delayed neuronal cell death in the hippocampus[253]. Delayed neuronal cell death occurs selectively in highly vulnerable brain regions such as the neocortical layers (layers 3, 5 and 6), dorsomedial striatum and, perhaps most importantly, in the CA1 region of the hippocampus[254-256].

In the rat, the 4-vessel (common carotid arteries and anterior vertebral arteries) occlusion model[257] is a more difficult surgical procedure than the 2-vessel (common carotid arteries, CCA) occlusion with hypotension[258], but associated with less variability. Other global ischemia models employ occlusion of the CCAs bilaterally combined with increase in intracranial pressure[259], or controlled asphyxiation[260].

1.6.1.2 Focal models

The focal model is a closer approximation to human thromboembolic stroke[261] and produces a heterogeneous pathology that includes a necrotic core and salvageable penumbra, as well as normal, undamaged tissue in both ipsilateral and contralateral hemispheres[262]. In the necrotic core, the area at the center of the ischemic territory, both neurons and glia die mostly through necrosis. The penumbra surrounding the core is the area said to be “at risk” but can be saved if appropriate interventions are given. It has received the most attention because it is then the most important area in the development of effective stroke therapies.

Focal ischemia induced by physically occluding the middle cerebral artery has been performed in several species, including mouse[263], rat[264], rabbit[265], cat[266], dog[267], and

some primates[268]. Non-human primates are clearly the species most similar to human; however, primate models of middle cerebral artery occlusion (MCAO) are not appropriate for initial testing because of high cost and insufficient reproducibility. Rat is currently the best species to perform MCAO, because it is relatively inexpensive, its cerebrovascular anatomy and physiology resemble that of higher species, and physiologic parameters can be easily monitored. There are several varieties of focal models in rat, many of which are variants of middle cerebral artery occlusion based on various methods of occlusion, including clip[269] (transient), coated or bare thread[270] (permanent or transient), cauterization[271] or clot[272] (permanent), and photothrombosis[273] or endothelin-1[274] (permanent, though some degree of reperfusion occurs).

1.6.1.2.1 Permanent MCAO model

The transcranial approach requires careful removal of a section of the skull and the underlying dura. This allows occlusion of the middle cerebral artery (MCA) with the aid of a microscope. Tamura et al. (1981)[264] developed a subtemporal approach of proximal MCAO at a point near the origin of the lateral striate arteries, which produced infarction of both the cortex and the caudate putamen[264]. The original technique, however, was very invasive and the rats survived only for a few hours. Subsequent modifications preserving the zygoma and the masseter muscle improved the postoperative survival for several days and eventually the subtemporal approach emerged as the standard technique of focal cortical ischemia in rats[275-277]. Berderson et al. [278] observed that occlusion of the MCA from its origin medial to the olfactory bulb to its junction with the inferior cerebral vein resulted in a 100% infarction of the frontoparietal cortex. Menzies et al. [279] further expanded and refined the Bederson modification by extending the thermocoagulatory

occlusion to a greater length of the main MCA trunk and to all major branches of the MCA from near its origin to beyond its distal junction with the inferior cerebral vein. So that larger and more uniform and reproducible lesions were obtained, probably related to the effective compromise of the distal cortical interarterial anastomoses. Transcranial procedure involveds the opening of the skull, it thus affects the intracranial pressure and may reduce the edema that an intact skull would otherwise cause. Less invasive alternative noncraniectomy models are the suture model[280], thrombus model[281], endothelin model[282], and photothrombotic model[283]. The occlusion of the MCA is caused either by filaments, heterologous or autologous clots, or endothelin administrated via the common carotid artery. The photosensitive dye excited by light at a specific wavelength injures the endothelium of blood vessels, which set up a nidus for thrombosis. Importantly, in this model no reperfusion or blood reflow occurs to the core of the infarct after the occlusion of the MCA, which is not the case in the transient focal ischemia model. A permanent model of ischemia may also be of clinical significance since it has been reported that a considerable proportion of the human stroke case are not associated with reperfusion during the first 24 hours after clinical stroke[284].

1.6.1.2.2 Transient MCAO model

The transient occlusion models have all the features of the permanent models, as well as the additional complication of reperfusion injury. Recirculation was accomplished by removing the clip[269] or pulling the thread out of the artery[285]. Reperfused ischemic tissues are tissues at risk and best represents stroke in man after spontaneous or therapeutic thrombolysis. The clip models are transcranial and require removal of part of the skull and dura which may affect the intracranial pressure and edema formation. The

intraluminal thread (coated/uncoated) models are less invasive, but experimenters need to visualize the position of the filament either *in situ* postmortem or by staining of endothelium after transient occlusions.

Transient middle cerebral artery occlusion (TMCAO) in rodents has been used routinely to model transient focal cerebral ischemia. The essential feature of this model is the temporary reduction of local blood flow in a defined area of the brain. The TMCAO model has received wide popularity, since it is a simple technique and it produces consistent MCA occlusion and recanalization without craniectomy. In this model (**Fig.1-6**), a (nylon) thread is inserted to the internal carotid artery via common carotid artery (CCA) or external carotid artery (ECA)[286] and advanced so that MCA, anterior cerebral artery (ACA) and posterior cerebral artery (PCA) are blocked. In this model, the severity of the insult is controlled by varying the occlusion time. Another essential feature of this model is that ischemic damage, rather paradoxically, progresses for days and even weeks after restoration of blood flow. This phenomenon called “reperfusion injury: is well characterized and is characteristic of this model.

1.7 Objectives

Although the physiological functions of H_2S have been identified in cardiovascular and nervous systems, its role in stroke is not known. This project is mainly based on our recently finding that elevated plasma cysteine (endogenous precursor of H_2S) levels are associated with poor clinical outcome in acute stroke patients[287]. Combining the knowledge from previous studies on the H_2S signaling mechanism, it is reasonable to

propose our hypothesis: H_2S plays an important role in mediating cell damage in cerebral

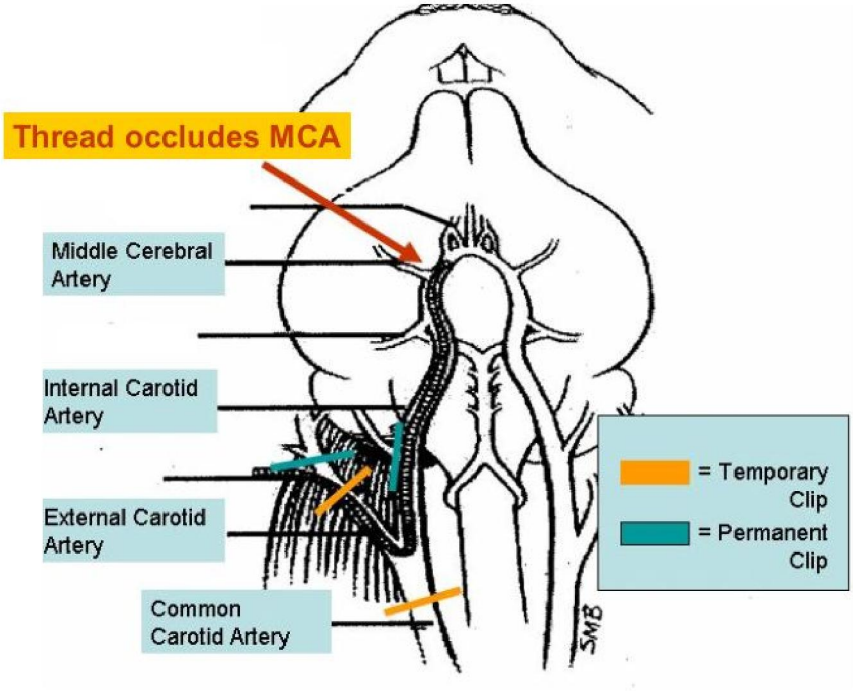


Fig. 1-6 Transient MCAO model by thread occlusion.

In this model, a (nylon) thread is inserted to the internal carotid artery via common carotid artery (CCA) or external carotid artery (ECA) and advanced so that MCA, anterior cerebral artery (ACA) and posterior cerebral artery (PCA) are blocked.

ischemia, via NMDA-receptor activation, which induces excitotoxicity in cells. Therefore we investigated the effects of H_2S and the inhibition of its formation on stroke. Cerebral ischemia was studied in a rat stroke model created by permanent MCAO. The resultant infarct volume, H_2S level changes, acitivity and expression of biosynthesis enzymes were measured 24 h after occlusion. The effects of H_2S were investigated by using its endogenous precursors, exogenous donor and inhibitors of key enzymes for H_2S production. NMDA blocker was also used to investigate if NMDA receptors were involved in this process. This project not only contributes to further understanding on H_2S and also identified a novel therapeutic tharget for the treatment of acute stroke.

2 MATERIALS AND METHODS

2.1 Animals

For in vivo experiments, male Wistar rats (250-280 g) were obtained from University Laboratory Animal Center and housed in groups of 4. All animals were under diurnal lighting conditions and allowed food and water *ad libitum*. All experimental procedures were approved by IACUC (Institutional Animal Care and Use Committee) and performed in accordance with the guidelines set by the National University of Singapore (adapted from Howard-Jones, 1985)[288] and all efforts were made to minimize suffering and the number of rats used.

2.2 Drug treatments

Homocysteine (Hcy), L-cysteine (L-cys), propargylglycine (PAG), aminooxyacetic acid (AOAA), sodium hydrosulfide (NaHS), hydroxylamine (HA), β-cyanoalanine (β-CNA)[9] and MK-801[289] were obtained from Sigma, USA. All drugs were dissolved in saline and administered by intraperitoneal (i.p.) injection at the time points stated in **Table 2-1**. Each compound was administrated with varying dosages at the beginning for optimization. Control rats received corresponding volume of saline only.

Table 2-1 Drugs in using

Drug	Effect	Injection Time point
Hcy	H_2S precursor	50 min before MCAO
L-cys	H_2S precursor	50 min before MCAO
NaHS	H_2S donor	10 min before MCAO
AOAA	CBS inhibitor	60 min before MCAO
HA	CBS inhibitor	60 min before MCAO
PAG	CSE inhibitor	60 min before MCAO
β-CNA	CSE inhibitor	60 min before MCAO
MK 801	NMDA-receptor blocker	60 min before MCAO

2.3 Permanent MCAO model

Permanent focal ischemia was induced by irreversible occlusion of the left middle cerebral artery using a sub-temporal approach based on the model by Chimon & Wong (1998)[269] with modifications. Briefly, rats were anesthetized with chloral hydrate (350mg/kg, i.p., Sigma) and anesthesia was subsequently maintained with a reduced dose (150mg/kg) when necessary. A 2cm skin incision was made at the midline of the left ear and orbit (**Fig. 2-1 (A)**). The underlying skin was separated with membranes and retracted to expose the temporal muscle. The muscle was removed from skull surface and retracted to expose the squamosal bone, especially the point where the zygoma fuses to the squamosal bone. Zygoma was removed with clearance of muscle and tissue and the underside of the temporal bone was exposed by opening with spring-loaded retractors (**Fig. 2-1 (B)**). Under an Olympus stereo zoom microscope; a 2.0-2.5mm diameter hole was created on the squamosal bone by gentle grinding with a dental drill (**Fig. 2-1 (C)**). The dura mater over the MCA was carefully opened and retracted by the tip of a 27-gauge needle (**Fig. 2-1 (D)**). The left MCA thus appeared clearly under the microscope (**Fig. 2-1 (E)**). **Fig. 2-2** shows the typical branching pattern of MCA and the relationship to surrounding anatomical landmarks including the inferior cerebral vein (ICV) and the olfactory tract (OT). The MCA was then occluded by electro-cauterization from the point proximal to its origin and at the point where it intersects the ICV. All branches of the MCA between these 2 points were also cauterized and the artery was then detached to ensure definitive blood flow interruption. The temporal muscle and surrounding soft tissues were put back to original place, and the incision was sutured (**Fig. 2-1 (F)**). During surgery, rectal temperature was maintained at 37± 0.5ºC by means of a rectal probe

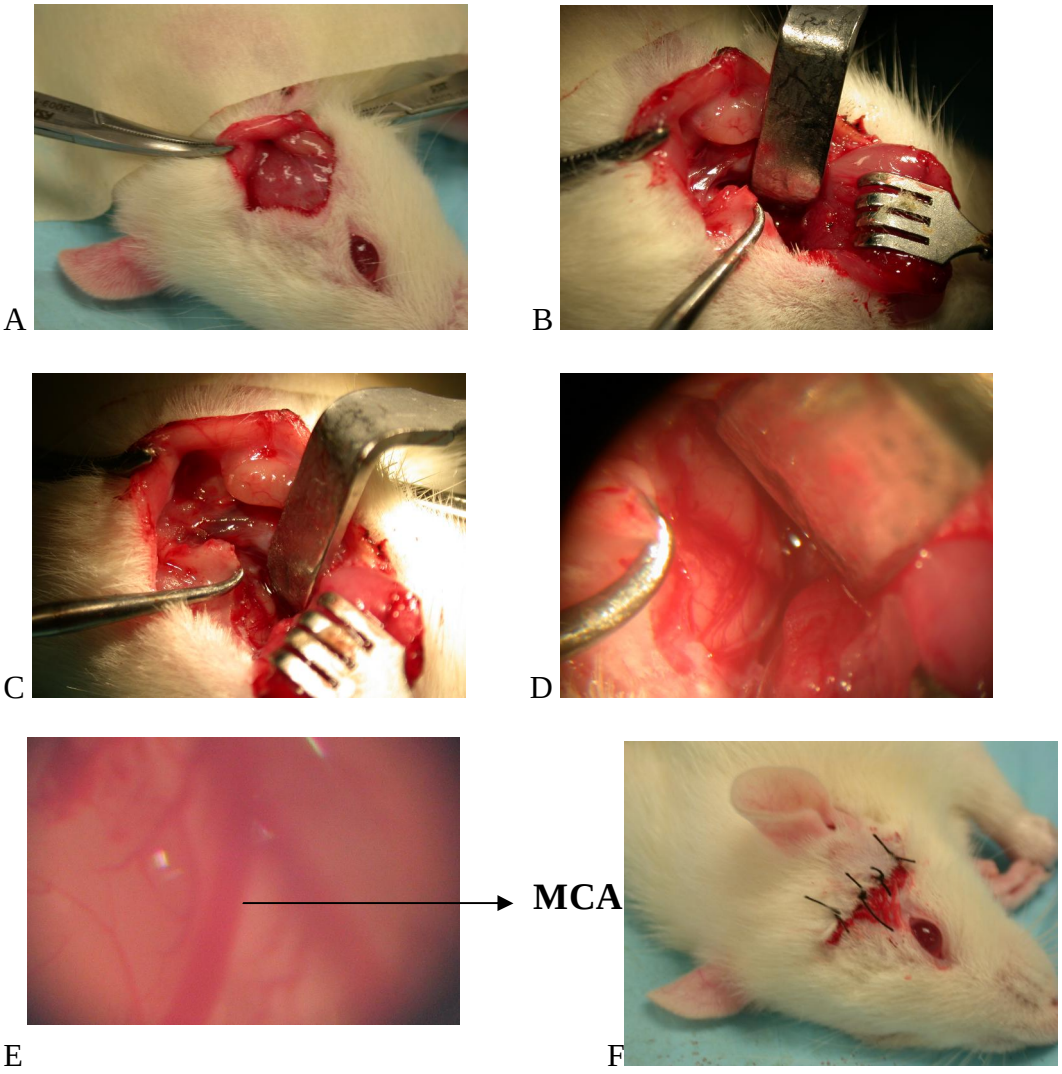


Fig. 2-1 Surgery processes for MCAO model:

(A) Skin incision: expose temporal muscle

(B) Surgery window

(C) Craniectomy window

(D) MCA (middle cerebral artery) under microscope

(E) Open the dura mater over MCA (under microscope: appears blurred because it is covered by saline

(F) After surgery: incision was sutured.

(thermocouple) connected to a heating blanket via an electronic temperature controller. After surgery, the rat was kept warm on the blanket until recovery from anesthesia. Sham-operated control rats under the same surgical procedures only without the occlusion of MCA.

2.4 Measurement of infarct volume

Infarct volume is one of the common indexes for assessing the extent of ischemic brain injury following focal cerebral ischemia. 2, 3, 5-triphenyltetrazolium chloride (TTC, Sigma, USA) staining is a widely used method in quantification of infarct volume in experimental stroke models [(290)]. It is a rapid, convenient, inexpensive and reliable method at 24 h after the onset of ischemia[(291)] and it corresponds closely with other histological methods, such as cresyl violet[(292)].

TTC, which gives a faint yellow when dissolved in solution, is reduced by dehydrogenase of functioning mitochondria to yield a formazan which is purple in colour [(293)]. As infarct brain tissues lacking of dehydrognase, they remain unstained (white) while normal tissues are stained purple, providing visually identification without microscopic examination. A stock solution of 0.4% TTC was prepared by dissolving 0.4g of tetrazolium blue chloride powder (Sigma, USA) in 5ml dimethyl sulphoxide (DMSO) (Sigma, USA) followed by 95ml distilled water, then the suspension was sonicated until the powder had completely dissolved to produce a clear pale-yellow stock solution. The final staining solution was prepared by adding 100ml of the stock solution to 300ml solution buffer (pH7.4) which contained 0.2M Na_2HPO_4, 0.2M NaH_2PO_4 and 8mM $MgCl_2$. This 0.1 % TTC solution could be stored at 4ºC for not more than 3 months.

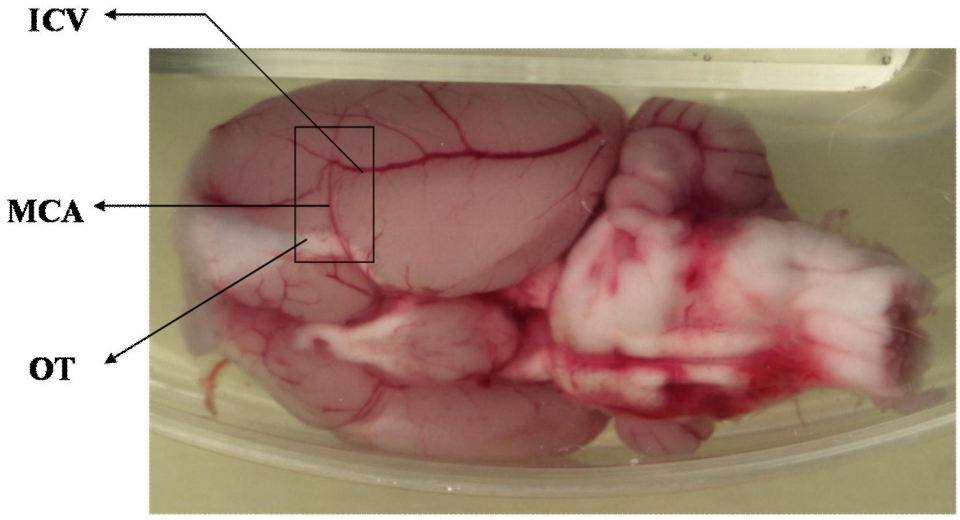


Fig. 2-2 Location of MCA and the surrounding anatomical landmarks.

Inferior cerebral vein (ICV) and the olfactory tract (OT, white). Rectangle highlights the area that usually can be seen through a craniectomy window.

Rats were sacrificed at 24 h after the onset of ischemia. For staining brain sections, the rat brain was quickly removed after decapitation and all of overlying membranes were cleared using a fine forceps. The cerebrum was sectioned into eight 2-mm thick coronal slices using a brain-sectioning block (Zivic Miller, USA) and stained with 0.1% TTC solution at 37ºC for 30 minutes, followed by fixation in a 4% phosphate buffered formaldehyde solution. The fixed coronal slices were arranged in sequence and scanned into computer with 600dpi resolution (**Fig. 2-3**). The infarct volume was measured by using digital imaging software (Olympus Micro Image Lite 4.0 system. **Fig. 2-4**).

The true infarct volume (IV) was calculated by correcting for brain edema and contraction of infarcted tissues (equations 1 and 2)[294]. The measured infarct volume could also be converted by integration to the percentage of true infarct volume of ischemic damage in the contralateral hemisphere (equations 3).

IV (mm^3) = Σ [IA / (1+ASF)] * Thickness ----------equation 1

(Thickness = 2 mm)

ASF (Average swelling factor) = (IH-NIH) / (IH+NIH) / N ----------equation 2

(N is the number of slides from one brain, N=8 in this project)

Percentage of infarct volume = Σ IV / Σ (NIH* Thickness) * 100%---equation 3

2.5 Neurological evaluation after MCAO

Neurological evaluation by movement behavior is another well-used index for assessing the extent of ischemic brain injury after focal cerebral ischemia. Neurological scores were assessed at 24 h, 72 h and on the 7^{th} day after MCAO surgery by a blinded observer. A scale of 0 to 4 [295-297] were used to assess the behavioral changes in the

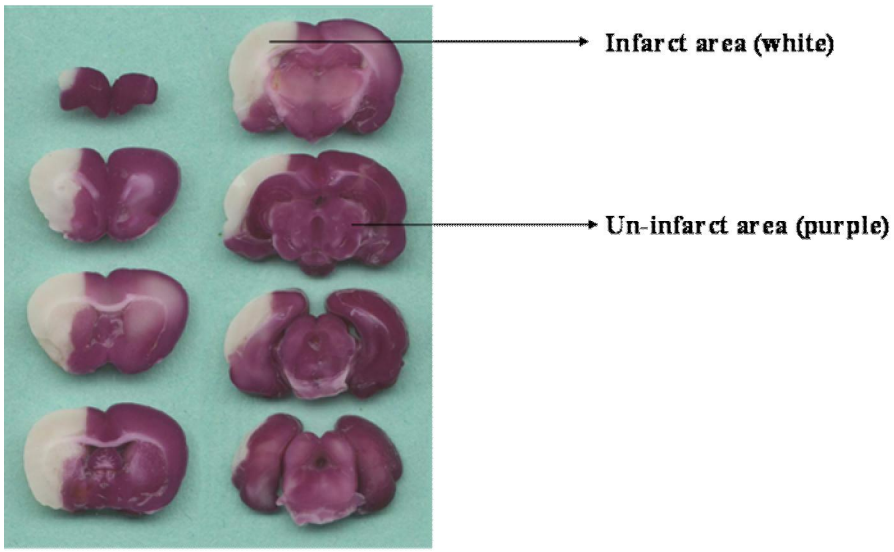
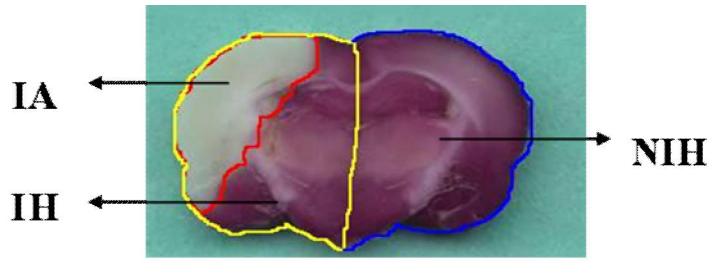


Fig. 2-3 Scanned images of TTC-stained coronal section of a rat brain.

Normal tissue uniformly stained by TTC (right hemisphere, purple). An area of infarction (white area) demonstrated by absence of TTC staining is observed in the left hemisphere at 24 hr after left middle cerebral artery occlusion.



IA: infarct area
IH: infarct hemisphere
NIH: non-infarct hemisphere

Fig. 2-4 Measure infarct volumes with digital imaging software (Olympus Micro Image Lite 4.0 system).

animal after the MCAO surgery (**Table 2-2** and **Fig. 2-5: A-D**). A higher score indicates poorer neurological status. At each time point, the highest score out of three consecutive trials was recorded and the interval between consecutive trials was more than 5 min to avoid animals' boredom. The test consisted of the following maneuvers: firstly, rats were held by the tail 50cm above the ground and their forelimb posture was noted. Normal animals extended both forelimbs toward the floor and were assigned a score of 0. When the forelimb contralateral to the side of the MCAO was consistently flexed when suspended by its tail, the rat was scored 1. Rats were then placed on ground and they were gently held by the tail. Rats that moved spontaneously in all directions but established a mono-directional circling toward the paretic side when given a light jerk of the tail were scored 2. Rats that showed a persistent spontaneous contralateral circling were scored 3. Rats that were inactive but walked only when gently pushed were scored 4.

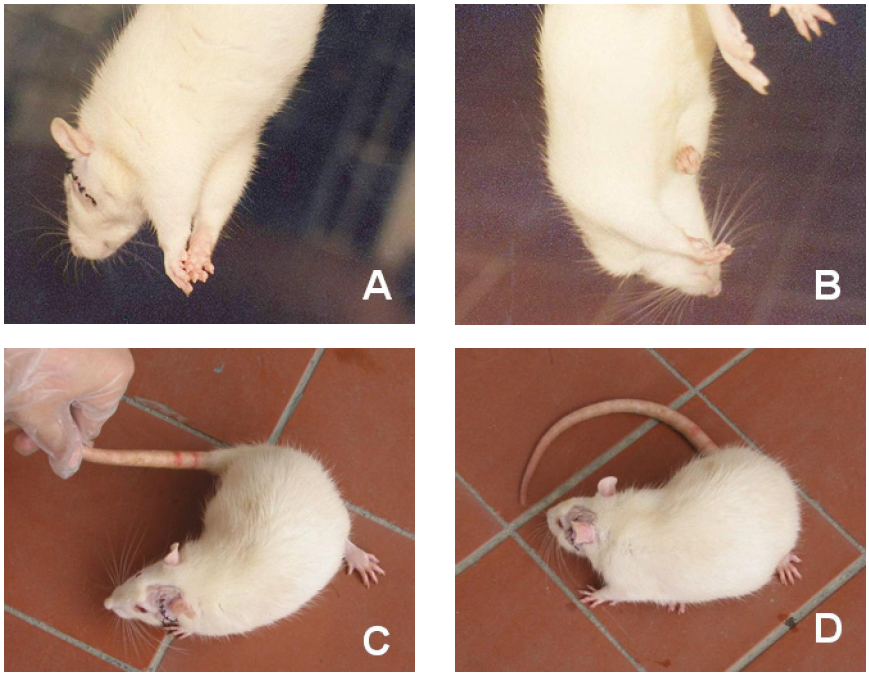


Fig. 2-5 Neurological evaluation after MCAO surgery

(A) animal extended both forelimbs toward the floor and were assigned a score of 0; (B) animal's forelimb contralateral to the side of the MCAO was consistently flexed during the suspension and there was no other abnormality, the rat was scored 1; (C) Spontaneous movement in all directions; contralateral circling only if pulled by tail; (D) Spontaneous contralateral circling.

Table 2-2 Neurological evaluation of rats after MCAO

Score	Neurological Evaluation
0	No apparent deficit (**Figure 2-5:A**)
1	Failure to extend contralateral forepaw fully (**Figure 2-5:B**)
2	Spontaneous movement in all directions; contralateral circling only if pulled by tail (**Figure 2-5: C**)
3	Spontaneous contralateral circling (**Figure 2-5: D**)
4	Inactive, walks only when stimulated

2.6 Measurement of blood pressure

To evaluate if drugs used alter blood pressure, we measure conscious rat blood pressure after loading with the highest dosage used of each drug by a modified tail-cuff method[298]. Briefly, an AD Instruments ML125 NIBP system was used for systolic blood pressure (SBP) measurement and it is a computerized non-invasive (indirect) tail-cuff system (Chart v4.1/Scope v3.6.8 for Windows, AD Instruments) (**Fig. 2-6: A**). Its aim is to record the first appearance of the pulse when it re-enters the tail artery during the deflation cycle of the proximal occlusion cuff. The rat was first kept in a warming cage at 37 ± 1ºC for 5 to 10 minutes. It was then held in a restrainer (**Fig. 2-6: B**) and the tail-cuff was put in place for SBP measurement. The preset maximum cuff pressure for Wistar rats was 200mmHg. Pulse sensor was placed distal to the occluding cuff. Once the pulse had been established, the cuff was inflated to the maximum cuff pressure and then drops gradually back to 40mmHg. The return and increase of the pulse signal could be observed when the pressure dropped to the SBP point. SBP was the pressure at the time when the pulse signal returned to 5% of normal (**Fig. 2-7: A-B**). The measurement was repeated 3 times for each animal and the mean value was recorded.

2.7 Histology

Twenty-four hours after MCAO surgery, rat brain tissues were collected and fixed in 10% neutral formalin, and subsequently processed and embedded in paraffin wax. The specimens were then cut into 5 μm sections, fixed upon the slides, and stained with H & E (Sigma) as a standard method[299]. Briefly, slides were deparaffinized with HistoClear (National Diagnostics), and hydrated by sequential passage through 100% ethanol, 90%

Fig. 2-6 Facilities for rat blood pressure (BP) measurement

(A) AD Instruments ML125 NIBP system was used for systolic blood pressure (SBP) measurement and it is a computerized non-invasive (indirect) tail-cuff system (Chart v4.1/Scope v3.6.8 for Windows, AD Instruments) (B) Rat restrictor: rats were held in this restrictor after warming up.

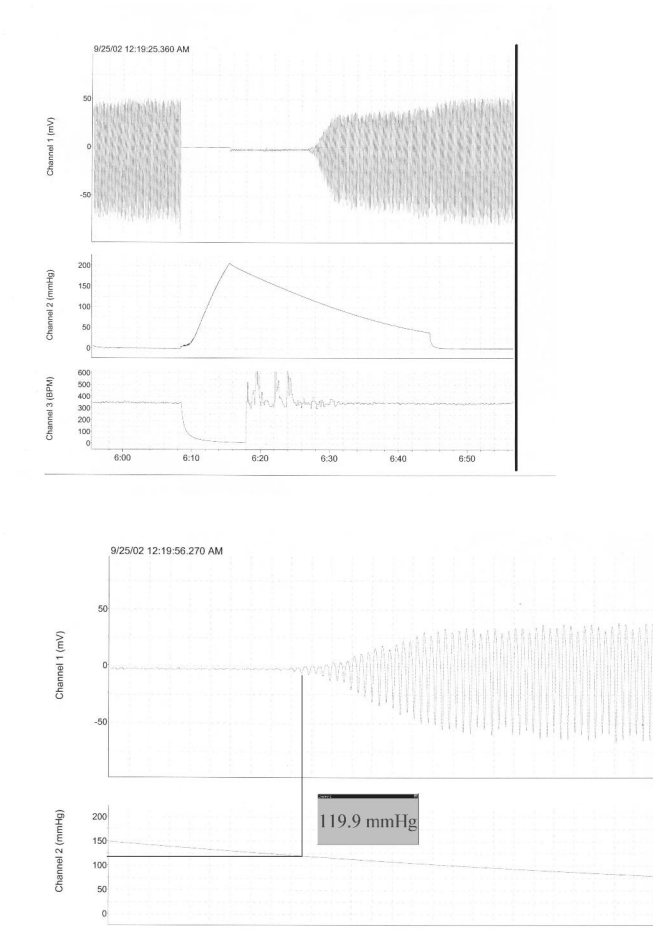


Fig. 2-7 Blood pressure (BP) measuring draft

(A) Computerized recording chart showing the pulse, pressure and heart rate. The pressure (channel 2) begins to drop when the maximum cuff pressure is reached, and continues to drop until it reaches about 40mmHg. The return and increase of the pulse signal (channel 1) can be observed when the pressure dropped to the systolic BP point (indicated with arrow). Heart rate is calculated and showed in channel 3. (B) Pulse signal was observed at the SBP point.

ethanol, 70% ethanol, and distilled water. The sections were then stained with Harris Haematoxylin for 5 min and washed with deionized water before differentiated in 0.1% acid alcohol solution. The stained slides were then washed in tap water for 5 min and counter stained with Eosin (Sigma) for 1 min. Slides were then dehydrated by sequential passage through 70% ethanol, 90% ethanol, 100% ethanol, and finally cleaned with HistoClear. Once dry, the slides were mounted with HistoMount (National Diagnostics).

2.8 Reverse transcription-polymerase chain reaction (RT-PCR)

RT-PCR is a wide-used semi-quantification method to detect the target gene expression.

2.8.1 Total RNA extraction

After decapitation, the ipsilateral cortex was dissected from the rest of the brain on an ice pad. It was weighted and homogenized immediately in 10 volumes (w/v) of ice-cold Trizol reagent (Gibco, BRL) with a Polytron (Janke & Kunkel) and then total RNA was extracted according to manufacturer's instructions. The isolated precipitated RNA was dissolved in diethyl pyrocarbonate (DEPC)-treated water. Total RNA yield was quantified by UV spectrophotometer (UV-1601, Shimadzu) at 260nm wavelength. RNA could be aliquoted and stored at -80ºC.

2.8.2 RT

Total RNA (5 µg) from the cerebral cortex was added to 1 µl of 0.5µg/ul oligo$(dT)_{12}$ and diluted with DEPC-treated water to a final volume of 12 µl. The RNA solution was incubated at 70ºC for 10min and quenched on ice for at least 1 min. 7 µl of the master

mixture was then added to the RNA primer mixture. The master mixture was prepared by adding 2 µl of 10×PCR buffer (M1665, Promega), 2 µl of 25mM $MgCl_2$, 2 µl of 0.1M dithiothreitol (dTT) and 1 µl of 10 mM deoxynucleotide 5'-triphosphate (dNTPs) in the indicated order. Finally, 1 µl (20 U/µl) of AMV reverse transcriptase (Promega) was added to the tube and the mixture was incubated at 42 ºC for 50min. The resulting RT products, complementary DNA (cDNAs), could be stored at -20ºC.

2.8.3 PCR

PCR in exponential phase was performed to allow comparative analysis of the cDNA samples. The PCR was carried out in a total volume of 100 µl containing 50 µl of 2×PCR master mix buffer (M7505, Promega), 1 µl of each primer (20µM), 4 µl of cDNA from the RT reaction, and 44 µl DEPC water. The expression of the housekeeping gene, β-actin, was used as an internal control. The primer sequences and programs used were shown in **Table 2-3 and Table 2-4**, respectively.

2.8.4 Gel analysis

The PCR amplification products were electrophoresed on a 1.5% TAE agarose gel at 100V for 20min (BIO-RAD, PowerPac 300). Both PCR products of CBS or CSE and β-actin of the same sample were run on the same gel simultaneously. 1 KB DNA ladder was used to mark the gene size in each gel. The image was analyzed and quantified by Multi Genius Bio Imaging System (SynGene). The ratio between the target gene and internal control was calculated using the equation below to show the relative expression of target gene in rat brain cortex:

Ratio= BD* of CBS (or CSE)/BD of β-actin----------equation 4

**BD means band density*

2.9 In vitro production of H_2S by plasma and cortical homogenate

In vitro production of H_2S was measured by a modified method which was first introduced by Abe K. et al in 1996[9]. The protocol was modified based on our laboratory condition and optimization in both rat plasma samples and cortical samples. H_2S produced in this assay was trapped in zinc actate solution. The resultant zinc sulfide ($Zn(HS)_2$) can be quantified by adding a special dye, N, N-DPD (N, N-dimethyl-p-phenylenediamine sulfate) in the presence of Fe^{2+} ion.

All assays were done in duplicate. The calibration curve of absorbance versus sulfide concentration was made by using defined concentrations of sodium hydrosulfide (NaHS) solution.

2.9.1 Measurement of H_2S level in rat plasma

Blood samples were collected with heparin-pretreated syringe from heart before animals were decapitated. After being centrifuged at speed of 40,000 g for 5 min, plasma sample was removed as supernant to a fresh tube which could be store at -80 ºC. Each reaction tube contained 75 µl plasma sample mixed with 425 µl dH_2O and 250 µl of 1% zinc actate solution. After adding 10 % TCA (trichloroacetic acid) 250 µl, the reaction solution was incubated in room temperature for 10 min. after incubation, the reaction solution was centrifuged at speed of 14000g for 10 min; the supernate was then transferred to a new tube. The supernate was incubated with 133 µl N, N-DPD (20 mM in 7.2 M HCl) and 133 µl $FeCl_3$ solutions (30 mM in 1.2 M HCl) at room temperature for 15

Table 2-3. Primers for RT-PCR

Gene		Sequence	Product
β-actin	sense	5'- ATCTGGCACCACACCTTCTACAATGAGCTGCG -3'	870 bp
	antisense	5'- CGTCATACTCCTGCTTGCTGATCCACATCTGC -3'	
CBS	sense	5'- ATGCTGCAGAAAGGCTTCAT -3'	559 bp
	antisense	5'- GTGGAAACCAGTCGGTGTCT -3'	
CSE	sense	5'- CGCACAAATTGTCCACAAAC -3'	579 bp
	antisense	5'- GCTCTGTCCTTCTCAGGCAC -3'	

Table 2-4. Programs used for PCR

Name	**Step 1**	**Step 2**	**Step 3**	**cycles**
β-actin	94ºC*30sec	60ºC*2min	72ºC*1min	29
CBS	94ºC*30sec	58ºC*2min	72ºC*1min	32
CSE	94ºC*30sec	58ºC*2min	72ºC*1min	32

min. The absorbance of the resulting solution at 670 nm was measured with a micro plate reader (SUNRISE).

2.9.2 Measurement of H_2S production in cortex

Briefly, the whole cerebral cortex was isolated from adult rats and homogenized in ice-cold 50mM potassium phosphate buffer, pH 8.0, with a Ploytron homogenizer (Heidolph DIAX 900). For each reaction, 1 ml of assay mixture contained: 10 mM L-cysteine, 2mM PDP, 100mM potassium phosphate buffer, pH 8.0, and 12 %(w/v) brain homogenate. Inhibitors (PAG/AOAA/HA/β -CNA) were incubated with tissue homogenates before the enzyme reaction at 37ºC for 5-10 min. Incubations for the enzyme reactions were performed in a transparent plastic bottle with a central tube inside. Center tube were filled with 0.3 ml of 1% (w/v) zinc acetate and a Whatman No. 1 filter paper (about 1.5cm*0.5cm) for trapping evolved H_2S as zinc sulfide. Each bottle was flushed with N_2 for 20 sec and the capped tightly. The reactions were initiated by transferring the flasks from an ice bath to a 37ºC shaking water bath. After 90 min at 37ºC, reactions were stopped by injecting 0.5 ml of 50% (w/v) TCA. Bottles were incubated in the shaking water bath at 37ºC for an additional 60 min to complete trapping of H_2S. After that, to each central tube, 50 µl of 20mM N, N-dimethyl-p-phenylenediamine sulfate (N, N-DPD in 7.2 M HCl) was added, immediately followed by 50 µl of 30 mM $FeCl_3$ in 1.2 M HCl. After 20 min of incubation at room temperature, the absorbance of the resulting solution at 670 mm was measured with a microplate reader (SUNAISE). All assays were done in duplicate. The calibration curve of absorbance versus sulfide concentration was made by using defined concentrations of sodium hydrosulfide (NaHS) solution. A stock solution of NaHS (100 mM) was freshly prepared

by dissolving NaHS compensated with the ratio of $NaHS/H_2O$ immediately before use.

2.10 Protein detection of key enzymes for H_2S endogenous biosynthesis

For investigating the possible changes and locations of 2 key enzymes which are involved in the endogenous pathway of H_2S production, Western blotting and immunohistochemisty were used in this project.

2.10.1 Primary antibody of CBS or CSE

Unfortunately, there are no commercial primary antibodies which are specific to both rat CBS and CSE enzymes. Our collaborator, Dr. Zhu Yi Zhun supplied us polyclonal antibodies which obtained from rabbit serum at 3 months after CBS/CSE protein injection. But their purification and specification did not get any confirmation before which may limit our result of protein expression. Anyway, protein detection test should be done if commercial primary antibodies are available someday. The antigens were synthesized by BioGenes, Germany; peptides sequences as the following:

- CSE 41-55: C-SLATTFKQDSPGQSS
- CBS 314-328: C-RAVVDRWFKSNDDDS

2.10.2 Western blotting

To examine the expression of CBS and CSE in brain tissue, Western blot analysis for these 2 key enzymes were conducted. Brain tissues were homogenized in ice-cold lysis buffer (75 mM Tris-HCl, pH 7.4; 150 mM NaCl; 1% Triton X-100; 10% glycerol; 2 mM Na_3VO_4; 10 μg/ml aprotinin; 10 μg/ml leupeptin; 20 mM NaF; 5 mM PMSF). Lysates were incubated on ice for 30 min before centrifugation (10,000 g at 4°C for 10 min) and

the supernatants were then assayed for protein concentrations using the Biorad protein assay (Bio-Rad, Hercules, CA) according to manufacturer's instructions.. The supernatants were then mixed with sample buffer (125 mM Tris-HCl pH 6.8; 20% glycerol; 10% 2-mercaptoethanol; 4% SDS; 0.025% bromophenol blue) and boiled for 5 min. Proteins (10 µg) were separated by SDS-PAGE and then transferred onto a PVDF (polyvinylidene difluoride) membrane (Bio-Rad, Hercules, CA) using a semi-dry transblotter (ATTO Corp., Tokyo, Japan). The membrane was blocked with 5% non-fat milk solution in Tween 20-Tris-buffered saline (TTBS) (1 M Tris-HCl pH 7.5, 0.9% NaCl, 0.05% Tween 20) for 1.5 hour at room temperature and probed with anti-CBS/anti-CSE polyclonal antibody for another 2 hours. The membrane was then incubated with alkaline phosphatase (AP)-conjugated secondary antibody for 1.5 hour at room temperature, and finally visualized colorimetrically by EGL system for 1 min and exposed to KODAK film.

2.10.3 Immunohistochemistry

Target antigens could be localized in tissue sections by using labeled antibodies as specific reagents through antigen-antibody interactions. This is the principle of immunhistochemistry method to detect specific protein expression.

Rats are perfused transcardially with 4% paraformaldehyde (PFA) in phosphate buffer at 50 mmol/L concentration under deep anesthesia until the color of liver turned from red to pale. After perfusion, the brains were removed, cut into coronal sections, and immediately immersion-fixed in 4% PFA. Coronal sections (30 µm thick) were cut on a vibratome. The brain sections were incubated with a primary antibody diluted with TBS/0.1% Triton X-100 at 4°C overnight. After being washed in TBS/0.1% Triton X-100,

the sections were incubated with a biotinylated secondary antibody for 1 hour at room temperature. They were washed and further incubated with a streptavidin-biotin-peroxidase complex (Vector Laboratories). The peroxidase reaction was carried out via incubation with diaminobenzidine and hydrogen peroxide. Stained brain slides were observed and taken picture under microscope at different amplification lens.

2.11 Statistical analysis

All comparisons were performed by one-way ANOVA followed by post hoc analysis with Bonferroni correction using statistical software SPSS 11.5 for Windows. Data are expressed as mean±SEM. The critical *P* level set for significance is <0.05.

3 RESULTS

3.1 Measurement of infarct volume after MCAO

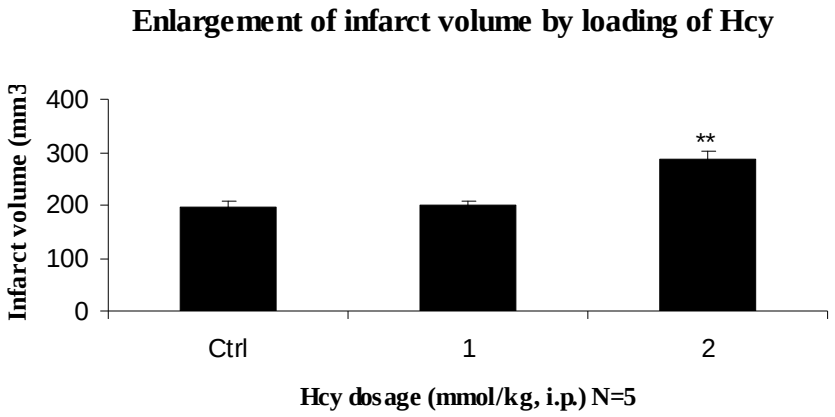
Infarct volume is one of the common indexes for assessing the extent of ischemic brain injury following focal cerebral ischemia. All animals were decapitated at 24 h after MCAO surgery for infarct volume measurement. MCAO causes widespread tissue infarction predominantly in the cortex of the occluded side of the brain.

3.1.1 Dose-dependent enlargement of lesion by H_2S precursors

As the precursors of H_2S, Hcy and L-Cys were preloaded intraperitoneally 50 min before the occlusion of middle cerebral artery. The measurement of infarct volumes showed that both of Hcy (**Fig. 3-1 A**) and L-Cys (**Fig. 3-1 B**) exacerbated brain tissue damages after MCAO surgery by the dose-dependent manner. The mean infarct volume (N=5) was 197±11.5 mm^3 for control rats which receiving saline only. Compared with the control group, 2 mmol/kg of Hcy significantly increased the infarct volume by approximately 45% which mean infarct volume (N=5) was 287±16.6 mm^3. Respectively, 10 mmol/kg of L-Cys significantly increased the infarct volume by approximately 34% which mean infarct volume (N=5) was 264.5±8.6 mm^3. Higher dosages of Hcy (5 mmol/kg) and L-Cys (20 mmol/kg) also were tested but the results could not be used since the mortalities of animals were significantly increased to 80% and 100% respectively. So that 10 mmol/kg of L-cys was used in the following in vivo experiments.

3.1.2 Enlargement of infarct volume by a donor of H_2S, NaHS

A



B

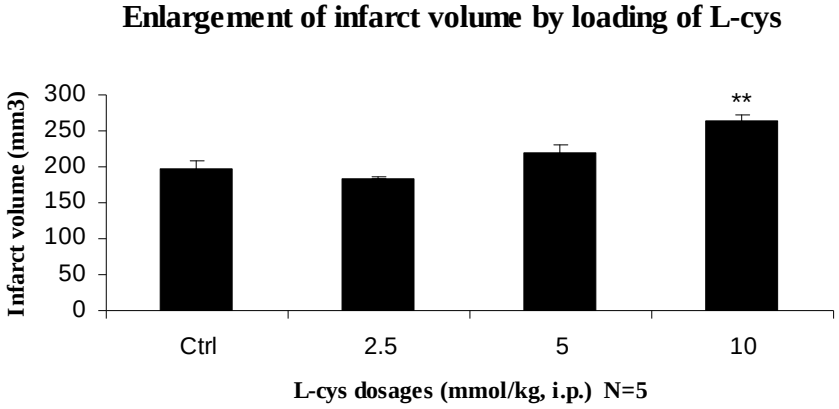


Fig. 3-1 Dose-dependent enlargement of lesion by H_2S precursors loading

The mean infarct volume (N=5) was 197±11.5 mm^3 for control group which receiving saline only. **(A)** Compared with the control group, 2 mmol/kg of Hcy significantly increased the infarct volume by approximately 45% which means infarct volume (N=5) was 287±16.6 mm^3, **P<0.001. **(B)** Respectively, at dosage of 10 mmol/kg, L-Cys significantly increased the infarct volume by approximately 34% which means infarct volume (N=5) was 264.5±8.6 mm^3, **P<0.001.

As a well-known donor of H_2S, NaHS was injected intraperitoneally 10 minutes before MCAO. Administration of NaHS at 0.09 mmol/kg (i.p.) before MCAO had no significant effect on the infarct volume; but at 0.18 mmol/kg, the mean infarct volume (N=5) was 273.3±22.7 mm^3, significantly increased to ~150% of control, 197±11.5 mm^3 (**Fig. 3-2**). NaHS was administered at a sublethal dose[300;301], and no increase in mortality was observed in this group of rats. So that 0.018 mmol/kg of NaHS was used in the following in vivo experiments.

3.1.3 Blockage of MK-801 on enlargement of lesion by L-cys or NaHS loading

For further investigating if NMDA receptor is involved in the neurotoxic effects of L-Cys on brain tissue during cerebral ischemia, the NMDA receptor channel blocker MK-801 was injected intraperitoneally 10 minutes before L-cys administration (**Fig. 3-3**). Coadministration of MK-801 (3 μmol/kg) significantly reduced the proinfarct effect of L-cys at dosage of 10 mmol/kg from 264.5±8.6 mm^3 to 198.4±13.6 mm^3 (N=5). Meanwhile, coadministration of the NMDA receptor channel blocker MK-801 (3 μmol/kg) completely abolished the proinfarct effect of NaHS at dosage of 0.18 mmol/kg, the mean infarct volume of this group (N=5) was 200.9±14.3 mm^3. These results implied that activation of NMDA receptors may be involved in the ischemic cell damage mechanism caused by L-cys or NaHS loading.

3.1.4 Effect of inhibitors of CBS

As the inhibitors of CBS, various dosages of AOAA and HA were administrated intraperitoneally 60 min before the occlusion of middle cerebral artery. Both of them showed ability to reduce infarct volumes in a dosa-dependent manner (**Fig. 3-4)**. Notably,

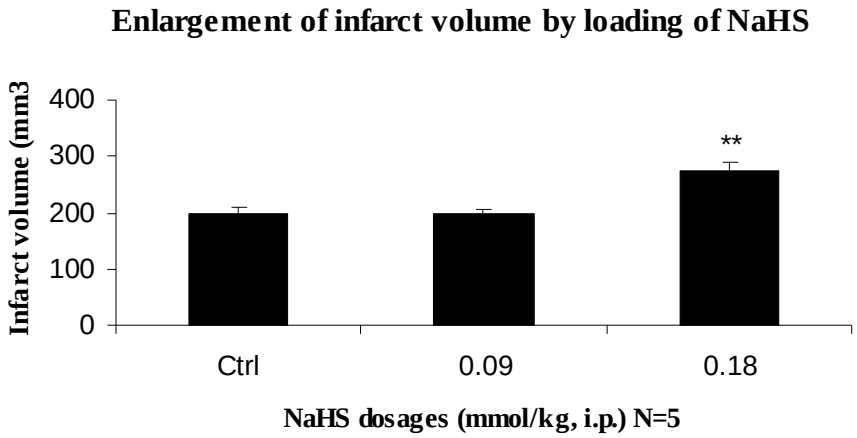


Fig. 3-2 Enlargement of infarct volume by donor of H_2S, NaHS.

The mean infarct volume (N=5) was 197±11.5 mm^3 for control rats which receiving saline only. Administration of NaHS at 0.09 mmol/kg (i.p.) before MCAO had no significant effect on the infarct volume. But at the dosage of 0.18 mmol/kg, NaHS significantly increased the infarct volume to 273.3±22.7 mm^3 (N=5), **P<0.005.

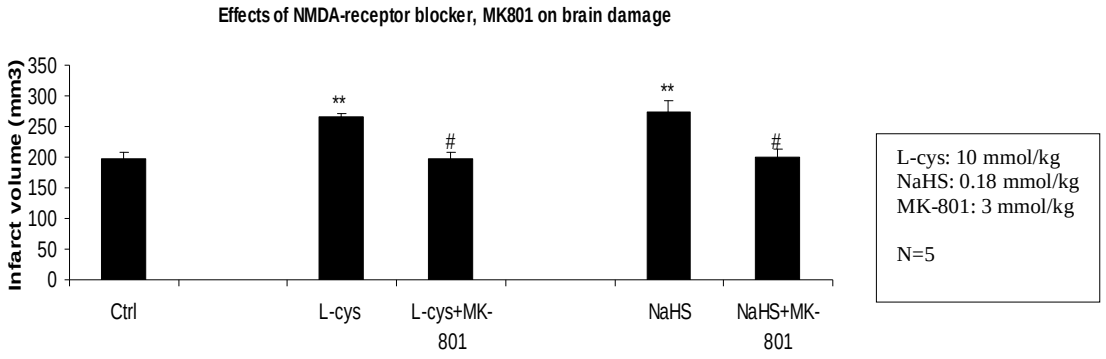


Fig. 3-3 Blockage of MK 801 on enlargement of lesion by L-cys or NaHS loading

Coadministration of MK-801 (3 μmol/kg) significantly reduced the proinfarct effect of L-cys at dosage of 10 mmol/kg from 264.5±8.6 mm^3 to 198.4±13.6 mm^3 (N=5). Meanwhile, coadministration of the NMDA receptor channel blocker MK-801 (3 μmol/kg) completely abolished the proinfarct effect of NaHS at dosage of 0.18 mmol/kg, the mean infarct volume of this group (N=5) was 200.9±14.3 mm^3. These results implied that NMDA receptor may be involved in the neurotoxic effect of H_2S on brain tissue during cerebral ischemia. (**P<0.005 when compared with control group; #P<0.05 when compared with only preloaded with L-cys or NaHS group)

administration of AOAA at 0.025 mmol/kg (i.p.) before MCAO had no significant effect on the infarct volume; but at 0.05 mmol/kg, the mean infarct volume (N=5) was 124.99± 9.68 mm^3, significantly reduced when compared with control group, 197±11.5 mm^3. It is interesting to note that AOAA was not effective at higher doses (ie, 0.1 and 0.5 mmol/kg) which might be because of its toxicity to animals[302;303]. So that 0.05 mmol/kg of AOAA was used in the following in vivo experiments. Similarly, another CBS inhibitor HA was effective at 0.5 to 1.0 mmol/kg. Administration of HA significantly reduced the infarct volume to 133.21±5.0 mm3 at 0.5 mmol/kg and to 112.54±5.67 mm^3 at 1.0 mmol/kg, respectively. The effects of CBS inhibitors on the ischemic lesion suggested the involvement of CBS activity during the cerebral ischemia.

3.1.5 Effect of inhibitors of CSE

Administration of β-CNA and PAG as inhibitors of CSE to rats at 60min before MCAO revealed that both of them were also able to reduce infarct volume in a dose-dependent manner (**Fig. 3-5**). At dosage of 0.5 mmol/kg, intraperitoneal administration of PAG significantly reduced the infarct volume to 164.33±4.62 mm^3, compared with the control group ($P<0.05$). At higher dosages, 1 mmol/kg and 2 mmol/kg of PAG further reduced the infarct volume to 134.96±6.13 mm^3 and 90.24±3.02 mm^3, respectively ($P<0.005$). Similarly, another CSE inhibitor, β-CNA also significantly reduced the infarct volume in a dose-dependent manner but it was effective at higher dosages: no effect at 0.5 mmol/kg, 162.97±10.41 mm^3 at 1 mmol/kg and 147.82±6.99 mm^3 at 2 mmol/kg. . Notably, β-CNA and PAG were effective at much higher doses of 1 or 2 mmol/kg and PAG appeared to be more effective in reducing infarct volume relative to β-CNA than its potency in inhibiting H_2S production suggested in in vitro experiments.

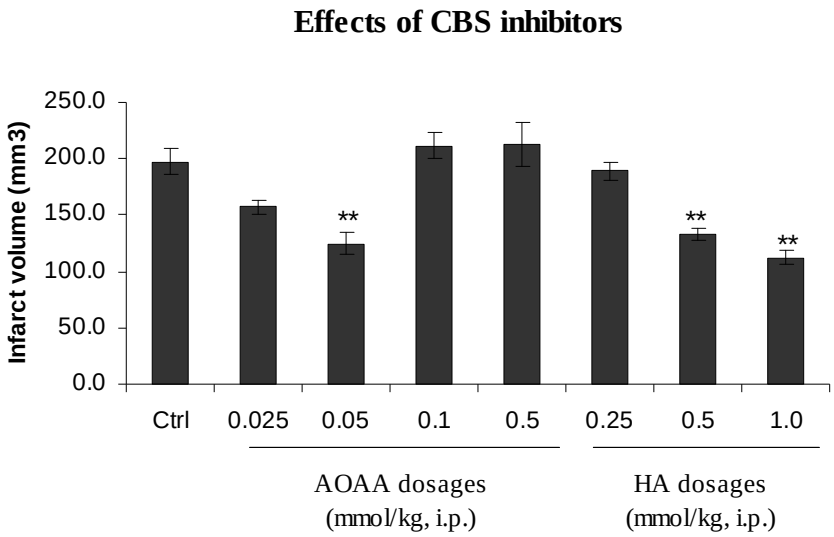


Fig. 3-4 Inhibitors of CBS reduced infarct tissue damages

Both of AOAA and HA were able to reduce infarct volumes in a dosa-dependent manner. Notably, administration of AOAA at 0.025 mmol/kg (i.p.) before MCAO had no significant effect on the infarct volume; but at 0.05 mmol/kg, the mean infarct volume (N=5) was 124.99± 9.68 mm^3, significantly reduced when compared with control group, 197±11.5 mm^3, **P<0.005. Similarly, another CBS inhibitor HA was effective at 0.5 to 1.0 mmol/kg. Administration of HA significantly reduced the infarct volume to 133.21±5.0 mm3 at 0.5 mmol/kg and to 112.54±5.67 mm^3 at 1.0 mmol/kg, respectively (N=5, **P<0.005). It is interesting to note that AOAA was not effective at higher doses (0.1 and 0.5 mmol/kg).

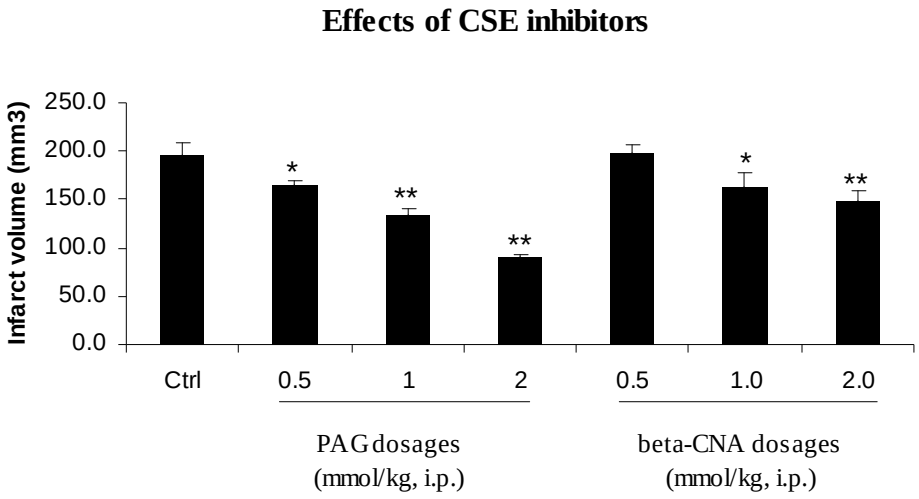


Fig. 3-5 Inhibitors of CSE reduced the ischemic damages in a dose-dependent manner.

At dosage of 0.5 mmol/kg, intraperitoneal administration of PAG significantly reduced the infarct volume to 164.33±4.62 mm^3, compared with the control group (*P<0.05, N=5). At higher dosages, 1 mmol/kg and 2 mmol/kg of PAG further reduced the infarct volume to 134.96±6.13 mm^3 and 90.24±3.02 mm^3, respectively (**P<0.005, N=5). Similarly, another CSE inhibitor, β-CNA also significantly reduced the infarct volume in a dose-dependent manner but it was effective at higher dosages: no effect at 0.5 mmol/kg, 162.97±10.41 mm^3 (*P<0.05, N=5) at 1 mmol/kg and 147.82±6.99 mm^3 (**P<0.005, N=5) at 2 mmol/kg. .

3.1.6 Enlargement of lesion by L-cys loading required the conversion of L-cys to H2S

For further confirming the involvement of H_2S during cerebral ischemia, L-cys was coadministrated with either CBS inhibitor (AOAA) or CSE inhibitor (PAG) at their effective dosages before occlusion of MCA (**Fig. 3-6**). Compared with the L-cys loading group which mean infarct volume was 264.57±6.69 mm^3, preloading of 0.05 mmol/kg of AOAA significantly reduced the tissue lesion caused by L-cys loading to 183.08±10.72 mm^3 whereas preloading of 1 mmol/kg of PAG significantly reduced the tissue lesion to 158.44±9.1 mm^3 as well.

Moreover, the combinatorial effects of AOAA and PAG were also tested in cerebral ischemia rats which were preloaded with L-cys or NaHS (**Fig. 3-7**). Co-loading of AOAA (0.05 mmol/kg) and PAG (1 mmol/kg) significantly reduced the ischemic lesion caused by preloading of L-cys (10mmol/kg) from 264.57±6.69 mm^3 to 126.32±2.19 mm^3 (N=5, **P<0.005). But compared with NaHS pretreated group which mean infarct volume is 273.33±17.57 mm^3, preloading of AOAA and PAG had no significant effect on ischemic lesion (251.88±21.87 mm^3). These results further confirmed that the conversion of L-cys to H_2S was required in the enlargement of lesion by L-cys loading.

3.2 Neurological evaluation after MCAO

Neurological evaluation by movement behavior is another well-used index which is supposed to reflect the extent of ischemic brain injury after focal cerebral ischemia. But its value is a little limited by 2 main reasons: first, animal's behavior may be affected by some unquatitized complex factors such as the health of animal; secondly, the standard score used in this study was commonly used but it only has 5 scores (0-4). Neurological

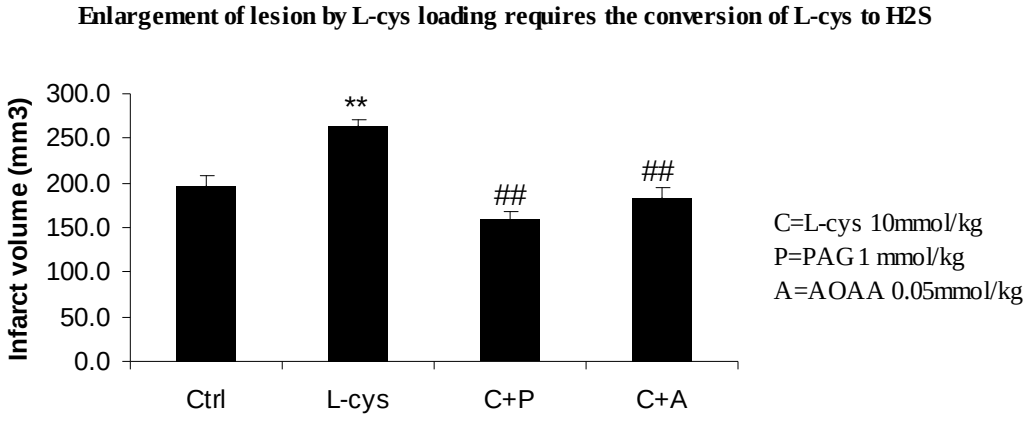


Fig. 3-6 Enlargement of ischemic lesion by L-cys loading requires the conversion of Cys to H_2S.

At their effective dosages, both inhibitors of CBS (AOAA) and CSE (PAG) can significantly decreased lesions caused by L-cys loading in cerebral ischemia. The mean infarct volume (N=5) was 197±11.5 mm^3 for control group which receiving saline only. As previously described, preloading of 10 mmol/kg L-cys significantly increased the infarct volume by approximately 34% which mean infarct volume (N=5) was 264.5±8.6 mm^3, **P<0.001. Pre-treatment of 1 mmol/kg PAG could significantly reduce such enlargement of ischemic lesion to 158.44±9.10 mm^3 (##P<0.001) and 0.05 mmol/kg of AOAA also significantly reduce such enlargement to 183.07±10.72 mm^3 (##P<0.001). These results clued that the requirement of conversion of L-cys to H_2S during cerebral ischemia.

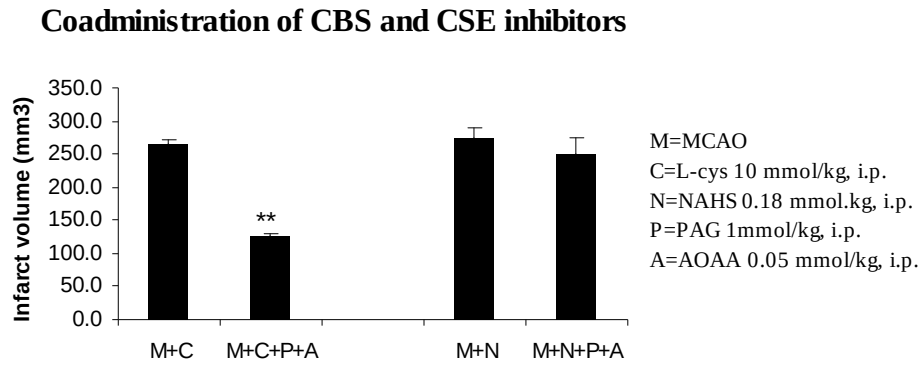


Fig. 3-7 Effects of coadministration of CBS and CSE inhibitors

Co-loading of AOAA (0.05 mmol/kg) and PAG (1 mmol/kg) significantly reduced the ischemic lesion caused by preloading of L-cys (10mmol/kg) from 264.57±6.69 mm^3 to 126.32±2.19 mm^3 (N=5, **P<0.005). But compared with NaHS pretreated group which mean infarct volume is 273.33±17.57 mm^3, preloading of AOAA and PAG had no significant effect on ischemic lesion (251.88±21.87 mm^3).

scores were examined at 24 h, 72 h, 1 week, 2 weeks and 4 weeks after MCAO surgery by a trained and blinded observer. The results of neurological scores of groups were shown at Mean±SEM in **Table 3-1.** As expected, the results showed that neurological scores were parallel with the severity of stroke (infarct volume results). None of the sham-operated animals showed any motor-behavioral abnormalities and the scores were 0 during the assessment (data not shown). Within every group, all animals' behavior was affected by the ischemic lesion after MCAO surgery and developed some of features upon recovery during 4 weeks time (**Fig. 3-8**). Among all groups, the animals preloaded with L-cys showed the highest scores all the time. The animals preloaded with NaHS also showed the highest scores at 24 h after MCAO but their recoveries were faster. The result was in line with the previous infarct volume results, which confirmed that preloaded with either H_2S precursor (L-cys) or donor (NaHS) exacerbated the ischemic brain damages and the inhibitors of CBS and CSE could alleviate such exacerbation.

3.3 Body weight changing

Body weight was another general parameter for animal health that should be related with damage grade. In this study, animals' body weight was recorded synchronously as neurological evaluation, at the day of MCAO surgery, 24 h, 72 h, 1 week, 2 weeks and 4 weeks after MCAO surgery respectively and the results were shown in Table 3-2 as Mean±SEM (N=4 or 5). Within each group except sham group, body weight only reduced significantly at 24 h after MCAO and then increased steadily from ~280g to ~400g over the 4-week period. There were no significant differences among all groups at any time point (**Fig. 3-9**).

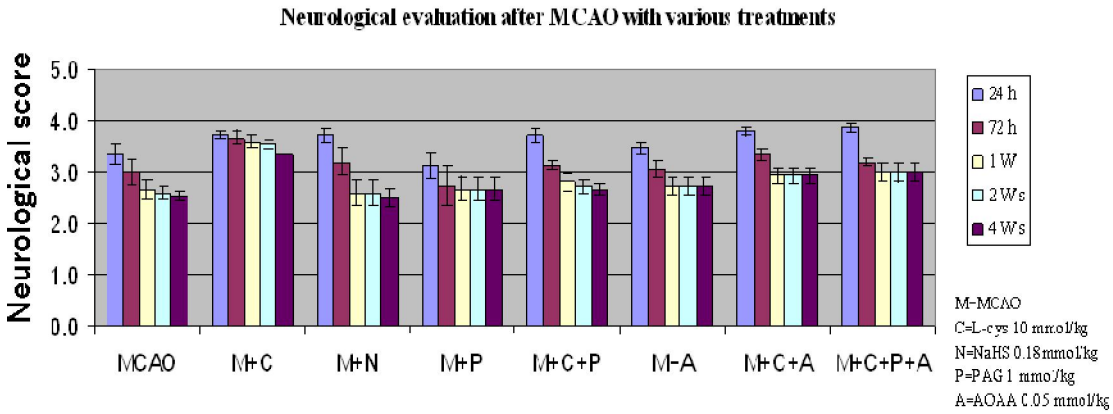


Fig. 3-8 Neurological evaluation after experimental cerebral ischemia

Neurological scores were examined at 24 h, 72 h, 1 week, 2 weeks and 4 weeks after MCAO surgery by a trained and blinded observer. None of the sham-operated animals showed any motor-behavioral abnormalities and the scores were 0 during the assessment (data not shown). Within every group (N=5), all animals' behavior was affected by the ischemic lesion after MCAO surgery and developed some of features upon recovery during 4 weeks time. Among all groups, the animals preloaded with L-cys showed the highest scores all the time. The animals preloaded with NaHS also showed the highest scores at 24 h after MCAO but their recoveries were faster. The result was in line with the previous infarct volume results, which confirmed that preloaded with either H_2S precursor (L-cys) or donor (NaHS) exacerbated the ischemic brain damages and the inhibitors of CBS and CSE could alleviate such exacerbation.

Table 3-1 Neurological scores after MCAO (Mean±SE, N=5)

	MCAO	M+C	M+N	M+P	M+C+P	M+A	M+C+A	M+C+P+A
24 h	3.33 ± 0.183	3.73 ± 0.067	3.73 ± 0.125	3.13 ± 0.249	3.73 ± 0.125	3.47 ± 0.133	3.8 ± 0.082	3.867 ± 0.082
72 h	3.0 ± 0.258	3.67 ± 0.149	3.2 ± 0.271	2.73 ± 0.386	3.13 ± 0.082	3.067 ± 0.163	3.33 ± 0.105	3.2 ± 0.082
1 week	2.67 ± 0.183	3.6 ± 0.125	2.58 ± 0.255	2.67 ± 0.236	2.8 ± 0.17	2.73 ± 0.365	2.93 ± 0.163	3.0 ± 0.183
2 weeks	2.6 ± 0.125	3.53 ± 0.082	2.58 ± 0.255	2.67 ± 0.236	2.73 ± 0.125	2.73 ± 0.163	2.93 ± 0.163	3.0 ± 0.183
4 weeks	2.53 ± 0.082	3.33 ± 0.001	2.50 ± 0.192	2.67 ± 0.236	2.67 ± 0.105	2.73 ± 0.163	2.93 ± 0.163	3.0 ± 0.183

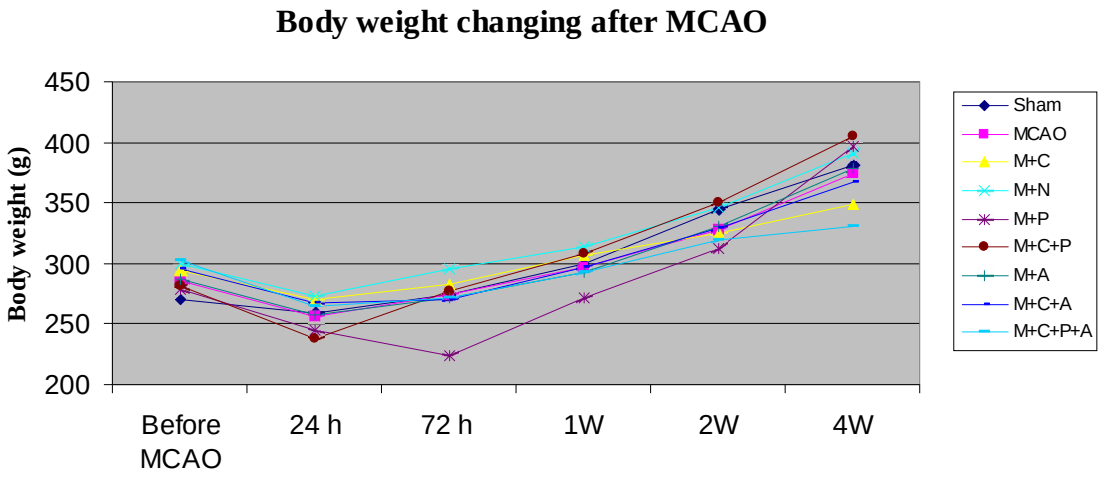


Fig. 3-9 Changing of animals' body weight after MCAO surgery.

All animals lost weight significantly at 24 h after surgery except sham group (N=5, **P<0.005) and then increased steadily from ~280g to ~400g over the 4-week period. There were no significant differences among all groups at any time point (refer to the data in Table 3-2).

Table 3-2 Body weight (g) changing (Mean±SE) after MCAO (N=5)

	sham	MCAO	M+C	M+N	M+P	M+C+P	M+A	M+C+A	M+C+P+A
Before MCAO	269.8±4.97	335.2±11.82	293.8±6.41	300±6.46	278.6±3.85	282±4.21	287.4±4.15	295±14.55	302±12.56
24 h	259.2±5.57	302±9.51	269.6±5.84	273.6±5.54	245.6±8.07	238±6.57	257±8.93	267±13.21	264±11.21
72 h	274.4±7.97	304.2±11.84	283.2±5.82	296±10.05	223.8±9.69	277±7.65	271±12.33	270±5.98	272±7.81
1 W	299.2±13.56	306±16.42	306.8±3.65	314.3±13.62	271±8.6	308±13.03	292.2±12.21	297±8.31	293±6.89
2 Ws	345.3±8.98	312±20.48	343.6±6.45	346.3±19.29	312±9.53	350±11.23	330.8±15.33	329±11.53	319±15.67
4 Ws	351.5±7.5	331.6±37.22	368±20.35	391±39.83	396.6±5.92	405±11.48	378.6±27.02	367±9.01	330±10.88

3.4 Blood pressures (BP) measurement

To exclude complicated effects of physiological parameters on rats during cerebral ischemia, highest doses of each compound were loaded and blood pressure were recorded at different time points after MCAO surgery which will be described as following: BP was recorded immediately before and after compounds loading, immediately after MCAO surgery, 1 week and 4 weeks after MCAO surgery respectively. There was a rapid and transit reduction of systolic BP after MCAO in all groups except sham group, and the reduction were most significant at one day after MCAO. As rats recovered 1 week after MCAO, their systolic BP returned to the similar or a little lower level as before. The results also told that loading of each compound did not significantly affect the blood pressure so that their effects on infarct brain tissue had little relationship with blood pressure (**Table 3-3**).

3.5 Histology

Rats pretreated with saline or Cys (3 per group) were killed 24 hours after MCAO and intracardiac perfusion with 10% formalin was performed. The brains were then removed and processed for conventional histological analysis using the standard hematoxylin & eosin staining. Consistently, histological studies demonstrated extensive primary infarct and edema (note the partial obliteration of the lateral ventricles) in the ipsilateral cerebrum of Cys-pretreated rats (**Fig. 3-10 B**). The ischemic penumbra extended further dorsally in the cortex to include the primary somatosensory cortex (S1) (**Fig. 3-10 B**). Damage in the septodiencephalic region appeared to be most severe, where much of the ipsilateral caudate–putamen nucleus was affected (**Fig. 3-10 B&D**). In the

Table 3-3 Blood pressure (mmHg) measurement (Mean±SE, N=5)

	Before injection	30min after injection	After MCAO	24 h after MCAO
Control	139.3±14.1	142.3±8.0	134.9±12.6	140.3±8.5
L-cys	136.8±8.1	140.6±9.8	135.5±7.0	142.1±6.4
NaHS	138.6±8.6	135.5±7.4	139.1±10.2	137.0±7.6
AOAA	140.8±8.5	139.2±6.8	138.5±5.4	142.9±7.6
HA	140.3±8.5	143.4±10.7	139.6±8.8	138.5±8.3
PAG	141.5±8.9	142.2±8.2	141.0±9.5	136.2±6.6
B-CNA	140.1±10.1	141.5±10.0	142.7±7.1	143.5±7.9

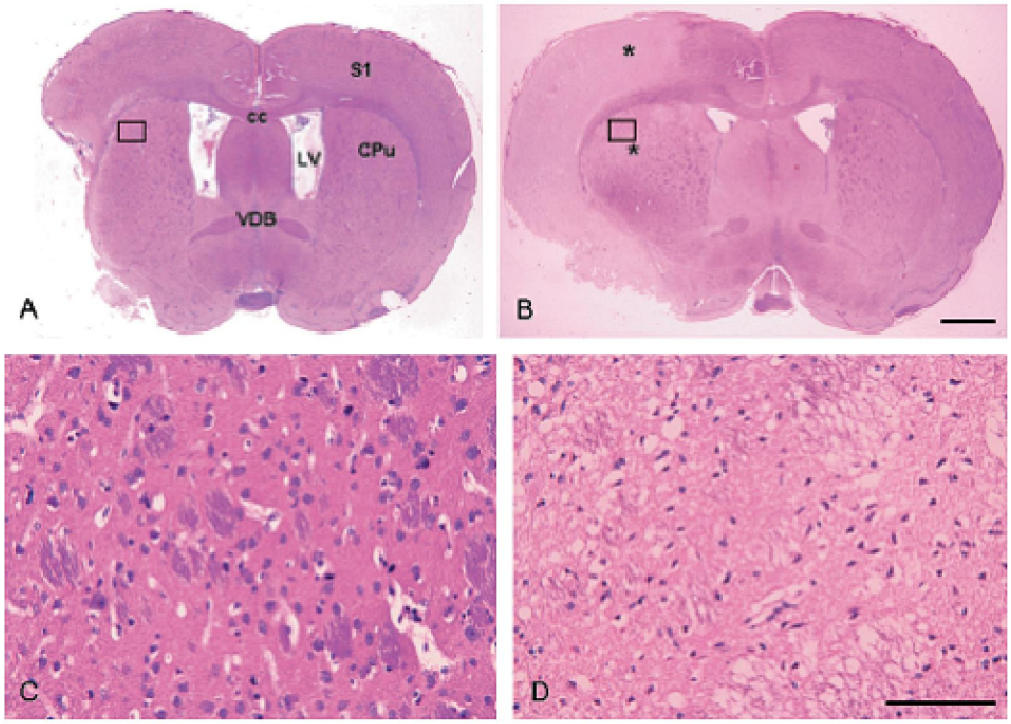


Fig. 3-10 Histology of brain slides after MCAO

Representative brain photomicrographs of rats at the level of septodiencephalic region after middle cerebral artery occlusion with hematoxylin & eosin staining. (A) Saline-pretreated and (B) cysteine pretreated (10 mmol/kg intraperitoneally), panels (C) and (D) are high magnification views of the window indicated in (A) and (B), respectively. (B) Asterisks indicate ischemic penumbra extending to the primary somatosensory cortex (S1) as well as the caudate putamen nucleus (CPu) in the cysteine-pretreated brain. LV=lateral ventricle; VDB=vertical diagonal band; cc=corpus callosum. Scale bars = (A, B) 1 mm; (C, D) 100 mm.

saline-pretreated control rats, the ischemic penumbra extended rostrocaudally from the septodiencephalic to the caudal diencephalic regions of the cerebrum, similar to that observed in the Cys-pretreated group, but the depth of damage and edema were much less, i.e. the caudate–putamen was only marginally involved. Under high magnification, it could be clearly observed that neurons appeared normal, retaining most of the typical histological features (**Fig. 3-10 C**), in contrast to the shrunken and hyperchromatic neurons observed in the Cys-pretreated caudate putamen (**Fig. 3-10 D**).

3.6 Gene detection

All results of in vivo experiments clued to H_2S was involved the brain tissue damage during cerebral ischemia. However, when the cortical gene expression of CBS and CSE were investigated using RT-PCR, no significant difference was observed between sham-operated and MCAO rats (**Fig. 3-11**).

It has been reported that CSE is not expressed at detectable levels or expressed at a barely detectable level in the rat and mouse brain by Northern or Western blot analysis. However in this study, it has to be noted that the expression of CSE mRNA (by RT-PCR) is apparently higher than that of CBS, which is in stark contrast to the data obtained in the in vitro assay. It is possible that CSE is expressed at the mRNA level but not at the protein level. More conclusive studies can be made only when antibodies to both CBS and CSE become available.

3.7 Assessment of H_2S in vitro

The enzyme activity is critical to production in a biological reaction. In this study, the acitivity of two key enzymes, CBS and CSE were assessed via an indirect method which

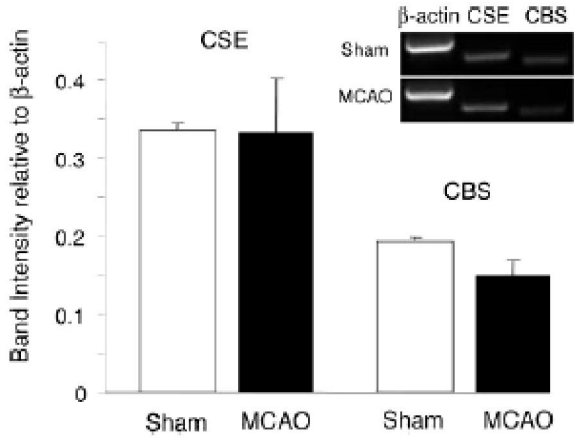


Fig. 3-11 Cortical expression of CBS and CSE mRNA in sham-operated and MCAO rats.

RT-PCR was performed as described in the Methods. No significant difference in expression of either enzyme was detected between sham-operated and MCAO rats (independent sample *t* test). N=3. Inset is representative bands for CBS, CSE, and β-actin RT-PCR products obtained by gel electrophoresis.

was described by Abe[9].

3.7.1 Endogenous production of H_2S in rat cortex

The endogenous level of H_2S in the cerebral cortex almost doubled in the damaged cortex after MCAO (19.37±0.587 nmol/g tissue) when compared with sham-operated controls (10.57±0.956 nmol/g tissue). H_2S levels increased further to 24.66±1.268 nmol/g tissue after L-cys preloading (**Fig. 3-12**). In addition, the H_2S synthesizing activity in cortical homogenates also increased ~147% (2.788±0.28 μM [HS]/g/min) after MCAO when compared with sham-operated group (1.879±0.124 μM [HS]/g/min) (**Fig. 3-13**). These in vitro results confirmed the changing levels of endogenous H_2S after the cerebral ischemia.

3.7.2 Inhibition on H_2S production by CBS and CSE inhibitors

To gain further insight into the role played by H_2S in this stroke model, the inhibitory effects of two CBS inhibitors (AOAA and HA) and two CSE inhibitors (β-CNA and PAG) on the H_2S synthesizing activity in cortical homogenate was studied. All 4 inhibitors inhibited H_2S production in vitro in a dose-dependent manner (**Fig. 3-14**). AOAA exhibited the greatest potency with an IC_{50} value of 12.6 μmol/L, reaching 98% inhibition at a concentration of 0.5 mmol/L (data not show in this figure). The IC_{50} values for the other inhibitors are 0.5 mmol/L (HA), 2.5 mmol/L (β-CNA), and 7.1 mmol/ L (PAG). In addition, the CSE inhibitors achieved only 70% (β-CNA) and 55% (PAG) inhibition at the highest concentration used (10 mmol/L).

AOAA inhibited H_2S production effectively with an IC_{50} value of 12.6 μmol/L and caused almost complete inhibition of H_2S production at 0.5 mmol/L. In contrast, PAG, a

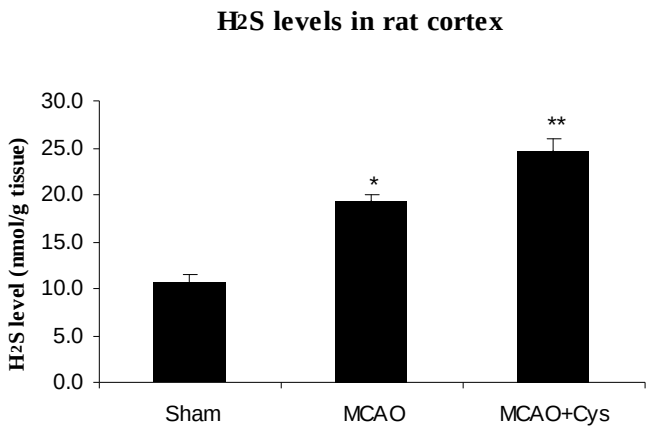


Fig. 3-12 H_2S levels in cortical tissues 24 hours after MCAO with or without Cys loading.

Cys (10 mmol/kg) was injected intraperitoneally 50 minutes before MCAO. N=4. One-way ANOVA: *P<0.001 against the shamoperated control group and **P<0.02 against MCAO group by post hoc analysis with Bonferroni correction.

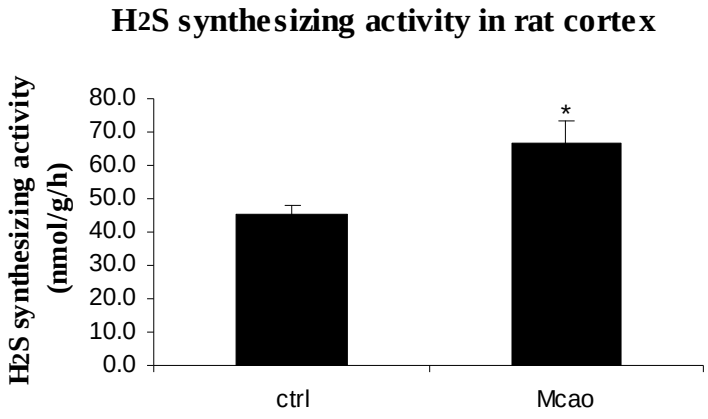


Fig. 3-13 H_2S synthesizing activity in rat cortex tissues.

H_2S synthesizing activity in cortical homogenates also increased ~147% (45.10±2.981 nmol/g/min) after MCAO when compared with sham-operated group (66.91±6.711 nmol/g/min). N=4, **P<0.001 against sham-operated control rats by independent sample *t* test.

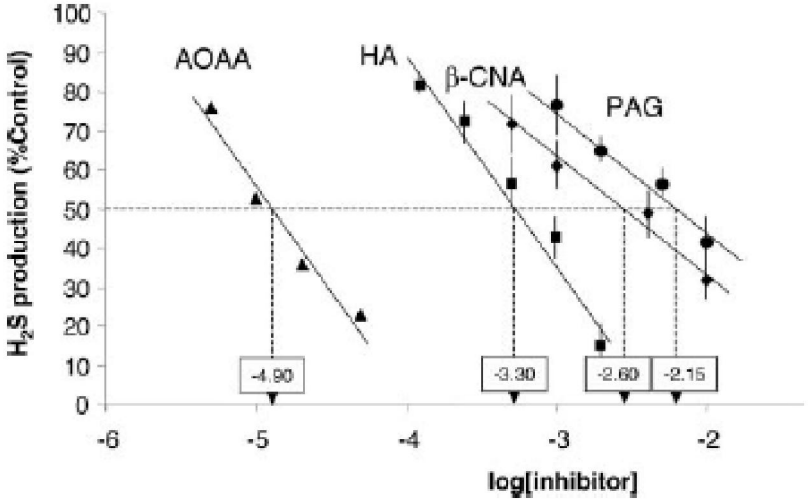


Fig. 3-14 Inhibition of H_2S synthesizing activity in cortical homogenate by inhibitors: [AOAA (▲), HA (■), β-CNA (♦) and PAG (●)]. The H_2S assay was performed as described in the Methods. Each point represents the mean±SEM of 3 independent experiments determined in duplicate. The calculated IC_{50} values were 12.6 μmol/L (AOAA), 0.5 mmol/L (HA), 2.5 mmol/L (β-CNA), and 7.1 mmol/L (PAG).

potent CSE inhibitor, inhibited cortical H_2S production with an IC_{50} value of 7.1 mmol/L, suggesting that PAG may be acting as a low-affinity inhibitor of CBS in this instance rather than as an inhibitor of CSE.

As previously described, all 4 inhibitors reduced the enlargement of MCAO-induced infarct volume by L-cys loading in a dose-dependent manner (**Fig. 3-4 & Fig. 3-5**). The rank order of potency was AOAA, HA, PAG, β-CNA (**Fig. 3-14**). Significantly, the observed potencies of the compounds as H_2S synthesis inhibitors in vitro paralleled their effectiveness in reducing MCAO infarct size in vivo. AOAA, as the most potent inhibitor, significantly reduced infarct volume at a dose of 0.05 mmol/kg. Interestingly, at higher doses, AOAA no longer exhibited any protective effects, probably indicating over inhibition of H_2S formation, leading to detrimental effects, supporting an important neuromodulator role for H_2S in the brain. It was further noted that at doses 0.5 mmol/kg, rats showed an unacceptably high mortality rate (data not shown).

3.8 Protein detection of key enzymes in rat brain

Endogenous production of H_2S in mammalian tissues is mainly dependent on two key enzymes, CBS and CSE. Both acitivity and expression level are important to H_2S synthesis. In this study, the enzymes expressions on protein level were detected by western blotting and immunohistochemistry.

3.8.1 Western blotting

Although no commercial antibodies for both of rat CBS and CSE are available yet, the protein level of CBS and CSE were tested via western blotting with the polyclonal antibodies as a gift from Dr. Zhu Yizhun (Department of Pharmacology, National

University of Singapore). These antibodies were rabbit's serum products at three months after antigens injection. The antigens were synthesized by BioGenes, Germany; peptides sequences as the following:

- CSE 41-55: C-SLATTFKQDSPGQSS
- CBS 314-328: C-RAVVDRWFKSNDDDS

Unfortunately, the results showed that these antibodies were not specific for both rat CBS and CSE proteins because of high level of background and multiple protein bands shown. Therefore, we could not confirm our results of *in vivo* experiments at protein levels.

3.8.2 Immunohistochemistry

In this study, I tried to locate the protein expression of CBS and CSE in rat brain via immunohistochemisty with the same antibodies above. Expression of both CBS and CSE were detected almost everywhere in the brain sections which might because of nonspecific properties of antibodies (**Fig. 3-15 A-D**). Limited with this technological reason, we cannot tell the expression of these two key enzymes and cannot compare the possible changing in normal rat brain with infracted brain. But further works should be finished for testify our hypothesis if the reliable antibodies are available in the future. It would be an important evidence to confirm the role of endogenous H_2S during experimental cerebral ischemia.

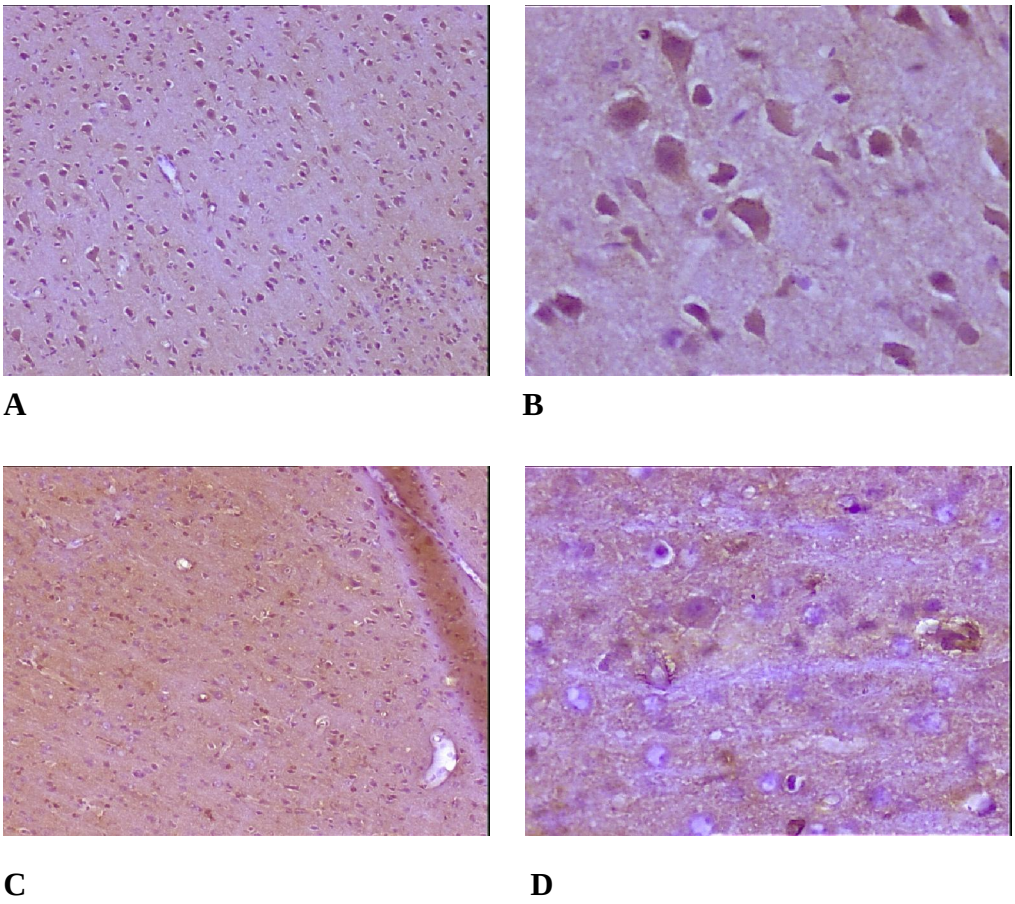


Fig. 3-15 Immunohistochemisty pictures of brain section

(A) CSE antibody (1:2000) under 10X4 magnification

(B) CSE antibody (1:2000) under 10X10 magnification

(C) CBS antibody (1:2000) under 10X4 magnification

(D) CBS antibody (1:2000) under 10X10 magnification

4 Discussions

H_2S is the most potential candidate for next gasotransmitter since its biological functions have been noticed in the nearest decade. This study was designed to clarify the potential role of H_2S as a mediator via NMDA-receptor activation in experimental ischemic model. The results showed that H_2S level was increased in ischemic brain tissue and these increases could be inhibited significantly by the blockers of H_2S endogenerate enzymes. Both of precursors and donor of H_2S exacerbated cerebral ischemic damages and these exacerbations were decreased by NMDA-receptor blocker. This study aimed at providing further evidence on the role of H_2S as the next potential gasotransmitter after NO and CO. Moreover, this study also provided a new therapy method for the cerebral ischemia.

4.1 The physiological functions of H_2S

Gasotransmitter is a novel concept which is defined as small molecule of endogenous gas with important physiological functions[(304)]. The production and metabolism of gasotransmitters are enzymatically regulated, and their effects are not dependent on specific membrane receptors. Following the identification of NO and CO as gasotransmitters, H_2S may be qualified as the third gasotransmitter.

H_2S was looked as only a toxic gas until the detectable levels of H_2S were measured in the circulation and in specific tissues. H_2S is produced endogenously in mammalian tissues from L-cysteine mainly by two pyrodixal-5'-phosphated-dependent enzymes, CBS and CSE. Moreover, the genes that code for specific enzymes responsible for endogenous

H_2S production were determinated and the specific cellular and membrane targets of H_2S were identificated[305].

The physiological functions of H_2S were first noticed in the cardiovascular system. H_2S has been shown to exhibit potent vasodilator activity both in vitro and in vivo[306]. Recent studies have shown that H_2S is generated from vascular smooth muscle cells (SMCs), catalyzed by specific H_2S-generating enzyme[307;308]. At physiologically relevant concentrations, H_2S relaxes vascular tissues; an effect mediated by the activation of ATP-sensitive K^+ (K_{ATP}) channels in vascular SMCs. H_2S directly alters the activity of K_{ATP} channels without the involvement of second messengers[309;310]. At much lower concentrations, H_2S greatly enhanced the smooth muscle relaxation induced by NO in the thoracic aorta which suggested the synergy of H_2S with NO. Another study by Bian et al.[311] suggested that endogenous H_2S contributed to cardioprotection induced by ischemic preconditioning, which effect may involve protein kinase C and K_{ATP} channels.

Besides of the physiological functions in the cardiovascular system, H_2S also may have a potential role in inflammation. Increased plasma H_2S levels were detected in both humans with septic shock and mice with lipopolysaccharied-induced inflammation. The donor of H_2S resulted in marked histological signs of inflammation but the CSE inhibitor exhibited marked anti-inflammatory activity[312]. These finding suggested the enhanced formation of H_2S contributed to the pathophysiology of the organ injury in endotoxemia[313;314]. Moreover, Bhatia and colleagues suggested the important proinflammatory role of H_2S because that the CSE inhibitor significantly reduced the severity of pancreatitis and associated lung injury[315].

4.2 The effects of H_2S in central nerve system

4.2.1 Neurons

Much progress has been made in the past decade in elucidating the roles of H_2S at physiological and pathological conditions at the cellular level. H_2S, at physiological levels, was first shown to selectively stimulate NMDA receptor-mediated currents. This stimulation facilitates the induction of hippocampal LTP, but only in the presence of a weak titanic stimulation. H_2S alone does not induce LTP thus suggesting that H_2S mainly facilitates LTP in active synapses[9;104]. The underlying mechanism by which H_2S potentiates NMDA receptor function remains unknown, though one plausible route may be through redox modulation of thiol groups scattered along the extracellular domains of neuronal NMDA receptors, which are sensitive to oxidizing/reducing agents. There are many endogenous (e.g. pyrroloquinoline quinine, lipoic acid, reactive free radical oxygen species, glutathione, dihydrolipoic acid) as well as exogenous molecules (cyanide, flupirtine) that are capable of oxidizing and reducing NMDA receptor[316] leading to the attenuation and potentiation, respectively, of receptor responses. Therefore, H_2S may activate NMDA receptor by virtue of its reducing property. One plausible redox modulatory site is the Cys pair (Cys744 and Cys798) located on the extracellular domains of the NR1 subunit[317].

Intracellularly, H_2S enhances NMDA receptor-mediated response via cAMP production. Exogenous H_2S increases the production of cAMP in primary cultures of rat cerebral and cerebellar neurons, or in some neuronal and glial cell lines[104]. cAMP activates cAMP-dependent protein kinase (PKA) during the initiation and late phase of LTP[318;319]. The activated PKA may, in turn, phosphorylate NMDA receptor subunits

NR1, NR2A and NR2B at specific site so as to enhance NMDA currents which is essential for LTP induction[320;321]. As such, cAMP may regulate LTP by phosphorylation of NMDA receptors. Besides this, H_2S also decreases the time required to respond to NMDA, i.e. increases the sensitivity of NMDA receptor to its ligand, in a dose-dependent manner via the cAMP pathway.

More recently, it has also been shown that H_2S upregulates γ-aminobutyric acid (GABA) B receptor ($GABA_BR$), a G protein-coupled receptor located at pre- and post-synaptic sites[322]. 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. H_2S has been shown to hyperpolarize neurons in the CA1 and dorsal raphe nucleus by increasing K^+ efflux probably via ATP-dependent K^+ (K_{ATP}) channels[10]. At pre-synaptic sites, $GABA_BR$ regulates the release of neurotransmitters, such as GABA and Glu, by inhibiting the voltage-sensitive Ca^{2+} channels. Upregulation of $GABA_BR$ expression by H_2S implies that H_2S may play a part in maintaining the excitation/inhibition balance in brain.

In addition to its mediator role, H_2S has been shown to protect the neurons from oxidative stress in both extracellular and intracellular microenvironment. It is well established that reduced GSH is an important antioxidant defense in the brain. It protects the brain by scavenging free radicals and other reactive species, removing hydrogen peroxide and lipid peroxides, preventing oxidation of biomolecules[323]. H_2S shares similar neuroprotective properties with GSH with a comparable potency *in vitro.* This has been demonstrated by the ability of H_2S in (i) inhibiting hypochlorous acid-mediated oxidative damage[324]; and (ii) inhibiting peroxynitrite-mediated protein nitration and

cytotoxicity[325]. In addition, H_2S readily scavenges H_2O_2, an important source of oxidative stress in most cells *in vitro*[326]. Although intracellular GSH levels in neurons (and glia) are in the mM concentration range, its extracellular levels in the brain is virtually zero[324;327;328]. Thus, the extracellular environment has a high dependency on other homeostatically regulated non-glutathione antioxidants, such as ascorbic acid, to scavenge free-radicals[329]. As such, H_2S may serve as another important candidate of endogenous antioxidant in the brain extracellular microenvironment due to its high endogenous production, readily diffusible property and high antioxidant potency comparable to that of GSH.

H_2S increases the production of reduced GSH[330] in neurons. NaHS treatment alone is able to increase the amount of GSH by enhancing the activity of γ-glutamylcysteine synthetase (γ-GCS) and up-regulating Cys (rate-limiting substrate of GSH synthesis) transport. This increase in glutathione content was shown to protect neurons from oxytosis, a form of programmed cell death caused by oxidative stress, triggered by high concentrations of Glu. H_2S also protects cells of an immortalized mouse hippocampal cell line from oxidative Glu toxicity by activating ATP-dependent K^+ (K_{ATP}) and Cl^- channels, in addition to increasing the levels of glutathione. Through these two mechanisms, H_2S is able to provide complete protection against Glu-induced cell death in different types of nerve cells, increasing cell viability to a level similar to neurons not treated with Glu [331;332].

4.2.2 Glia

H_2S serves an important neuromodulator role in glia cells. Astrocytes, a major type of glia cells, play an important role in maintaining neuronal excitability, regulating brain pH

homeostasis, and uptaking various neurotransmitters, including Glu around its synapses[333]. More importantly, a sufficiently large increase in $[Ca^{2+}]_i$ in an astrocyte is capable of inducing and propagating a spreading wave of increased intracellular calcium termed "calcium wave" in adjacent astrocytes[334]. In contrast to neurons which transmit signals via generating action potential, astrocytes and other glial cells communicate with each other via calcium signaling[335]. It provides a basis for astrocytes to act as a syncytium for possibly modulating neuronal and vascular function, suggesting the integral modulatory role of glia cells in synaptic transmission[333;335]. Exogenous H_2S elicits calcium waves in primary cultures of astrocytes and hippocampal slices[336]. This calcium wave triggered by H_2S is also preceded by an increase in intracellular calcium concentration ($[Ca^{2+}]_i$) which occurs via calcium influx through calcium channels on the plasma membrane and to a lesser extent via calcium release from intracellular calcium store. In brain slices, this increase in $[Ca^{2+}]_i$ caused by H_2S spreads to the neighbouring astrocyte population and triggers a calcium wave.

In contrast to astrocytes, microglial cells serve as the local macrophage population which could be activated upon foreign challenge in a similar analogy as peripheral macrophages[337]. Microglia has been proposed to play a role in the progression of neuronal diseases such as Alzheimer's disease (AD)[338] and Parkinson's disease[339]. We recently found that exogenous H_2S application has been shown to increase $[Ca^{2+}]_i$ of microglia reversibly in a dose-dependent manner[340]. Exogenous H_2S triggers a calcium influx via plasma membrane and calcium release from intracellular store. This influx is partly dependent on activation of adenylyl cyclase and independent of the phospholipase C – protein kinase C – inositol triphosphate pathway. Moreover, inhibiting the synthesis

of endogenous H_2S significantly decreased $[Ca^{2+}]_i$, suggesting that endogenous H_2S may have a positive tonic influence on $[Ca^{2+}]_i$ homeostasis. Besides its role as a second messenger, calcium ions serve as an integrator to control microglial behavior under resting and activated conditions, with elevated basal calcium concentration being a characteristic of activated microglia cells after lipopolysaccharide (LPS) challenge[341]. With the readily diffusible property of H_2S, it would be logical to anticipate that H_2S might play a role in activating neighboring microglial cells by elevating their basal calcium level.

Despite evidences showing the neuroprotective role of H_2S on neurons, no study has been conducted on the protective effect of H_2S on glial population to date. Various studies have demonstrated that GSH is localized preferentially in glia[342;343], with average intracellular levels of 4 mM in glia and 2.5 mM in neurons[344]. In view of the effect of H_2S in increasing GSH content in neurons, H_2S might exert a similar or even more potent effect on glial cells to increase antioxidative GSH.

Based on the known actions of H_2S described above, the potential physiological functions of H_2S in the brain may include calcium homeostasis, potentiation of LTP, suppression of oxidative stress, and modulation of neurotransmission, which has been summarized in **Fig. 4-1**.

4.2.3 CNS diseases

Up to date, only two CNS diseases characterized by alterations of H_2S metabolism were described: firstly, H_2S levels were found to be decreased by 55% in brains of 13 patients with Alzheimer's disease, compared with controls[345]. Interestingly, no change in brain L-cysteine concentration or in CBS expression was detected in the patients with Alzheimer's disease. However, the concentration of SAM in brains from Alzheimer's

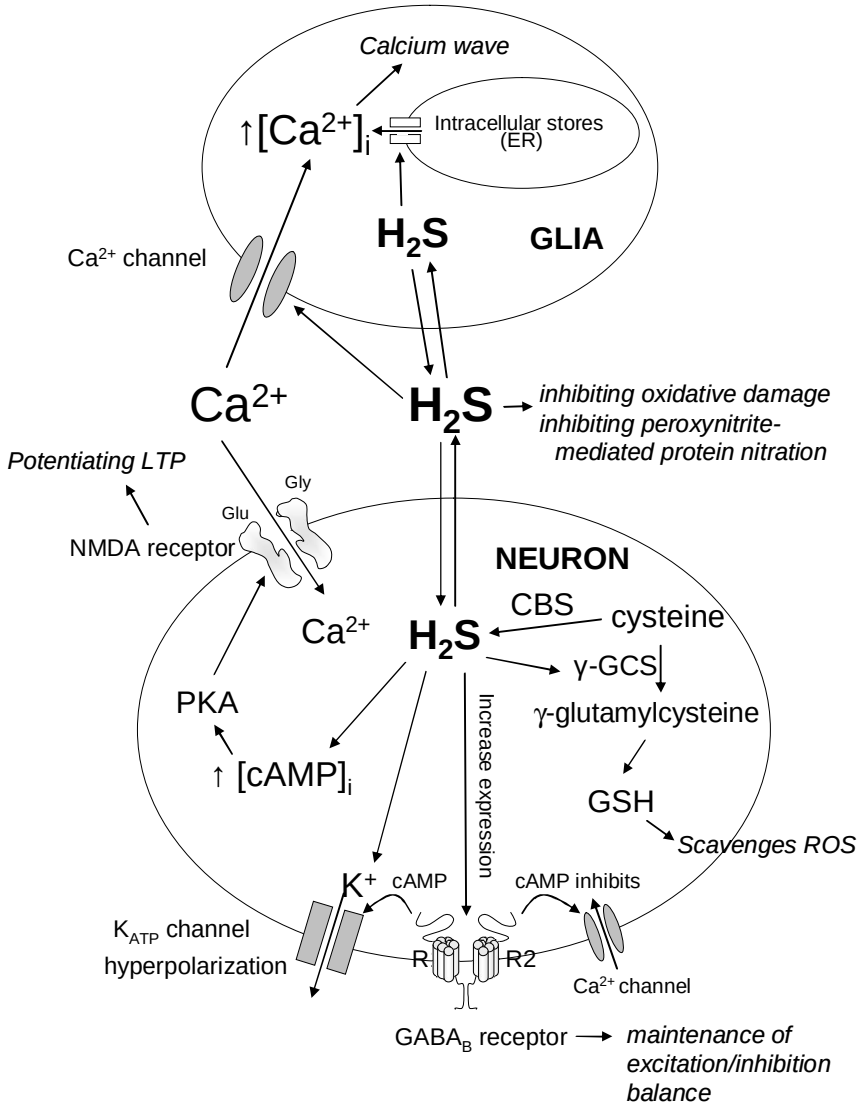


Fig. 4-1 Physiological functions of H2S in CNS (Reviewed by Qu K et al. 2007, accepted)

Potential physiological functions of H_2S in the brain may include calcium homeostasis, potentiation of LTP, suppression of oxidative stress, and modulation of neurotransmission.

disease sufferers was also less (reduced by 70%) compared with controls, which suggests that a deficiency in SAM might underlie the lack of H_2S in these samples. Reduced brain H_2S concentration can reflect a higher turnover perhaps by binding to and enhancing glutamate-mediated transmission via NMDA receptors. In this way, H_2Smight contribute to the neuronal loss associated with this disease.

The second central nerver system disease related to H_2S level is Down syndrome. a metabolite of H_2S, urinary thiosulfate and erythrocyte sulfhaemoglobin levels were both significantly increased in subjects with Down syndrome when compared with diet-matched controls[346]. The significance of this observation remains to be determined but again is suggestive of a role for this gas in CNS disease.

4.3 The role of H_2S as a mediator in cerebral ischemia

Although several recent publications demonstrated the role of H_2S in CNS, no study focused the cerebral ischemia yet. This study is the continuous work following the clinical findings that high level of plasma cysteine indicated poor clinical outcome in acute stroke patients. Cysteine is known to cause neuronal cell death and has been reported to be elevated in brain ischemia but it was first time to correlate plasma levels of cysteine with long-term clinical outcome at 3 months in acute stroke[299]. In this clinical cases study, 36 patients were assessed at 24 to 48 hours for early deterioration from stroke progression or complications (secondary outcome measure) and at 90 days after stroke onset using the modified Rankin scale (primary outcome measure). A significant association was found between plasma cysteine levels and patient outcome, with lowest levels in patients who had good outcome, intermediate levels in patients with poor outcome but were alive, and highest levels in deceased patients. Meanwhile, other excitatory amino acids such as

methionine, glutamate, aspartate, and glycine levels did not show any such association. These were consistent with the failures of clinical trials of a variety of NMDA receptor antagonists or modulators of the NMDA receptor to stroke patients to date [347]. In contrast, long-term clinical outcome appeared to be related to the plasma levels of cysteine. Further more, multiple regression analysis showed that cysteine remained an independent predictor of outcome since elevation on cysteine levels did not correlate with baseline NIHSS scores or with stroke subtypes. Thus, cysteine may provide additional prognostic information to physical examination. The observed increase in cysteine levels in patients who had poorer outcome might be the result of increased release after the stroke. If so, the present findings raise the possibility that these amino acids are involved in the pathophysiology of acute stroke. On the other hand, it is entirely possible that the cysteine levels were raised because of other comorbidities that may be present even before the stroke. Although raised homecystein levels may in principle lead to increased cysteine levels, it has been reported that plasma Cys remained unchanged in some patients with stroke with hyperhomocysteinemia[348]. Those clinical findings encouraged us to investigate the role and the mechanism of cysteine during stroke brain damage. The following works were finished on rat permanent cerebral ischemia model.

In this study, dose-dependent administration of both cysteine and its precursor, homocysteine increased the infarct volume by approximately 45% and 34% respectively in a rat experimental cerebral ischemia model (**Fig. 3-1 A&B**). Furthermore, the effect of cysteine was abolished by inhibitors of the enzyme cystathionine b-synthase (CBS) and cystathionine gamma-lyase (CSE) that convert cysteine to hydrogen sulfide (H_2S), indicating that this novel neuromodulators may be acting as a mediator of ischemic brain

damage (**Fig. 3-4 & Fig. 3-5**). Raised plasma cysteine in patients with stroke may reflect increased production of H_2S in the brain and thus predispose to poor outcome in clinical stroke. Inhibition of H_2S formation may therefore be a novel approach in acute stroke therapy. In the animal study, the plasma concentration of Cys achieved at the Cys loading dose used (10 mmol/kg) was likely to be much higher than that obtained in patient plasma, approximately 60 to 80 mmol/L. However, cross-species extrapolation is not meaningful here. For many drugs, it is known that human requires a much lower dose (on a unit body weight basis) than rats for the same effect.

Cysteine is known to be toxic to neurons. It causes neuronal death when given orally to infant mice[(349)] and has also been shown to be important in the pathology of brain injury in immature animals after hypoxic–ischemic brain injury[(350)]. Using a rat hippocampal slice preparation, Cys was shown to be innocuous under normal conditions but causes toxicity to neurons deprived of glucose, oxygen, or both[(351)]. Extracellular levels of Cys have also been found to be markedly elevated after ischemic brain injury caused by carotid artery ligation in Mongolian gerbils[(352)]. Thus, elevation in extracellular Cys may occur during brain ischemia and contribute to the pathophysiology of ischemic brain injury. Our animal model data support this view and suggest that elevated plasma cysteine may be responsible for worse outcome in clinical stroke. In addition, homocysteine, another precursor of H_2S, is also strongly linked with increased risk of acute stroke. Homocysteine may be converted by the action of cystathionine b-synthase (CBS, EC 4.2.1.22) to cystathionine, which, in turn, is acted on by cystathionine g-lyase (CSE, EC 4.4.1.1) to form Cys. Thus, increased homocysteine may lead to increased Cys and H_2S production. Moreover, it has been reported that CBS may condense Cys and homocysteine

to form cystathionine and H_2S[17]. These strongly suggest a possible association and interaction of the actions of Cys and homocysteine in stroke through the production of H_2S.

The toxicity of Cys has been shown to be mediated through the NMDA receptor and can be blocked by various NMDA antagonists[60;353], although Cys is not an agonist on the NMDA receptors. Interestingly, Cys is the precursor of H_2S, a novel neuromodulator[9] that can enhance NMDA receptor function[104]. It is, therefore, possible that high Cys may be translated into increased production of H_2S, which mediates tissue injuries through the NMDA receptors. This is strongly supported by the present observation that inhibition of the conversion from Cys to H_2S, the proinfarct effect of Cys, was completely abolished (**Fig. 3-4 & Fig. 3-5**). Cys has been shown previously to increase infarct volume after MCAO in a dose-dependent manner[299]. This effect of Cys was abolished by AOAA, a CBS inhibitor, suggesting that Cys exerts its effect after conversion to H_2S via the action of CBS in the brain. We show here that NaHS, an H_2S donor, is also able to enhance the destructive effects of cerebral ischemia, leading to a marked increase in the extent of tissue damage. At a dose of 0.18 mmol/kg of NaHS (**Fig 3-2**), the effective dose of H_2S is about 0.06 mmol/kg based on a yield of approximately 30%[354]; this is equivalent to only 0.6% of the effective dose of Cys at 10 mmol/kg. This is therefore consistent with the possibility that Cys increased the cerebral infarct by production of H_2S.

Moreover, the effects of both Cys and NaHS were abolished by MK-801 pretreatment (**Fig. 3-3**), confirming that H_2S acts most likely by an effect via NMDA receptors. It has been reported previously that physiological concentrations of H_2S enhances NMDA receptor function through activation of adenylyl cyclase. Increased production of cAMP,

observed in primary cultures of both neuronal and glial cells, may lead to phosphorylation of the NMDA receptor subunits at specific sites by protein kinase A, resulting in the activation of NMDA receptor-mediated excitatory postsynaptic current[104]. Thus, in cerebral ischemia, H_2S may enhance the NMDA receptor mediated excitotoxicity of glutamate. Together with the observed increase in the endogenous level of both H_2S and H_2S synthesizing activity in the MCAO damaged cortex (**Fig. 3-12 & Fig. 3-13**), these various observations strongly suggest that H_2S plays an important role in tissue damage in the ischemic brain, possibly through enhancement of NMDA receptor-mediated calcium overload. Another possibility is that H_2S influences the ischemic infarction by altering cerebral blood flow. However, because H_2S causes vasodilation and vasodilators are generally cerebroprotective, leading to reduced infarct size[355]; this possible mechanism is much less likely. The ability of cortical tissue to increase production of H_2S very quickly after MCAO or Cys loading suggests that the enzyme responsible for this conversion is not saturated by its substrate in vivo. Abe and Kimura[9] noted that CBS inhibitors including AOAA completely inhibited the production of H_2S in rat whole brain homogenates, whereas CSE inhibitors, including PAG, were ineffective at a concentration of 2 mmol/L. Abe and Kimura also concluded that CBS is the predominant enzyme responsible for H_2S production in the brain. It has also been reported that CBS is localized in most areas of the adult mouse brain but predominantly in the cell bodies and neuronal processes of Purkinje cells and Ammon's horn neurons[356]. Consistently, our present results (**Fig. 3-14**) also suggest a predominantly CBS-catalyzed production of H_2S in the cerebral cortex. AOAA inhibited H_2S production effectively with an IC50 value of 12.6 μmol/L and caused almost complete inhibition of H_2S production at 0.5 mmol/L. In

contrast, PAG, a potent CSE inhibitor, inhibited cortical H_2S production with an IC50 value of 7.1 mmol/L, suggesting that PAG may be acting as a low-affinity inhibitor of CBS in this instance rather than as an inhibitor of CSE. It has been reported that CSE is not expressed at detectable levels or expressed at a barely detectable level in the rat and mouse brain by Northern or Western blot analysis. However, it has to be noted that the expression of CSE mRNA (by RT-PCR) is apparently higher than that of CBS (**Fig. 3-11**), which is in stark contrast to the data obtained in the in vitro assay. It is possible that CSE is expressed at the mRNA level but not at the protein level. More conclusive studies can be made only when antibodies to both CBS and CSE become available.

All 4 inhibitors used reduced the MCAO-induced infarct volume in a dose-dependent manner. Significantly, the observed potencies of the compounds as H_2S synthesis inhibitors in vitro paralleled their effectiveness in reducing MCAO infarct size in vivo. AOAA, as the most potent inhibitor, significantly reduced infarct volume at a dose of 0.05 mmol/kg. Interestingly, at higher doses, AOAA no longer exhibited any protective effects, probably indicating over inhibition of H_2S formation, leading to detrimental effects, supporting an important neuromodulator role for H_2S in the brain. It was further noted that at doses 0.5 mmol/kg, rats showed an unacceptably high mortality rate. The toxicity of higher dosages of AOAA was consistent with other studies which mentioned that AOAA might result selective neuronal loss in the rat cortex[357] and AOAA may produce excitotoxic lesion by impairment of intracellular energy metabolism and its ability to block the mitochondrial malate-aspartate shunt[358].

In the mammalian CNS, H_2S is formed from the amino acid cysteine by the action of cystathionine β-synthase (CBS) with serine as the by-product. Based on both in vivo and

in vitro works in this study, it is first time but reasonable to consider the role of H_2S as a mediator during experimental cerebral ischemia. It provided further knowledge on the effects of H_2S in CNS. The results suggested endogenous synthase of H_2S in the brain is mainly catalyzed by CBS, which is consistent with the previous works. Combined with recent publications which probed that the mechanism(s) by which H_2S formation by brain CBS can be controlled. Such work can provide important clues to the potential biological roles of this gas in the CNS. It is now clear that brain CBS activity is both Ca^{2+} and calmodulin dependent, suggesting that 'short-term' control of neuronal H_2S production by this enzyme might be achieved by the influx of Ca^{2+} following depolarization. The existence of such a control mechanism suggests that H_2S, like NO, might act as a neurotransmitter.

4.4 Conclusion and prospect

It is first time to suggest the possible role of H_2S during cerebral ischemia and the underlying mechanism via present results. Inhibition of H_2S synthesis should be investigated further for its potential as a novel neuroprotective stroke therapy. It is also a development to further understand the role of H_2S as a potential gasotransmitter in central nerve system.

The value of this study was limited by the disappointing results of gene expression and protein expression of CBS and CSE in brain tissues. Besides of the technological limitation, it was also because of the complexity of endogenous biosynthesis and metabolism of H_2S in mammalian. A lot of potential factors may be involved but unknown and this blank is still waiting to be filled. It will be more valuable if we could investigate the protein level changes of two key enzymes for H_2S biosynthesis during the

cerebral ischemia. In addition, as CBS is a calcium and calmodulin dependent enzyme, the biosynthesis of H_2S should be acutely controlled by the intracellular concentration of calcium. Further works should be finished in in vitro hypoxia model to investigate the possible effect of H_2S to various cell types in central nerve system and the underlying molecular mechanisms. The calcium levels and NMDA receptor's involvement should be noticed in in vitro experiments. It will tell a complete story about the role of H_2S during cerebral ischemia.

Sufficient evidence has accumulated in support of H_2S acting as a signaling molecule in the mammalian CNS. This field is still in its infancy and much will be learnt in the near future about the central roles play by H_2S in health and disease as interest on this molecule grows among neuroscientists. One area that is of particular interest concerns the crosstalk between H_2S and NO in the CNS. It is obvious that the two systems have much in common. For instance, it is well established that NMDA receptor activation leads to Ca^{2+} influx and the increase in $[Ca]_i$ stimulates neuronal NOS activity to produce NO[359]. In addition, it has been shown that the cytoplasmic domain of NR1 subunit of NMDA receptor is physically associated with neuronal NOS via the postsynaptic density protein PSD95[360]. NMDA receptor activity can also be downregulated when NO modifies the thiol group of a critical Cys residue located on the extracellular domain of NR2A subunit via S-nitrosylation[361]. Adding H_2S to this picture would no doubt multiply the level of complexity with regard to the regulation of NMDA receptor function by many-fold. Perhaps, H_2S is a key to fully understand NO functions in the CNS, and vice versa.

5 REFERENCE LISTS

(1) LOEWI O. On the background of the discovery of neurochemical transmission. J Mt Sinai Hosp N Y 1957; 24(6):1014-1016.

(2) Werman R. Criteria for identification of a central nervous system transmitter. Comp Biochem Physiol 1966; 18(4):745-766.

(3) Amara SG, Fontana AC. Excitatory amino acid transporters: keeping up with glutamate. Neurochem Int 2002; 41(5):313-318.

(4) Foster AC, Kemp JA. Glutamate- and GABA-based CNS therapeutics. Curr Opin Pharmacol 2006; 6(1):7-17.

(5) de WD. The neuropeptide concept. Prog Brain Res 1987; 72:93-108.

(6) Wang R. Two's company, three's a crowd: can H2S be the third endogenous gaseous transmitter? FASEB J 2002; 16(13):1792-1798.

(7) Goodwin LR, Francom D, Dieken FP, Taylor JD, Warenycia MW, Reiffenstein RJ et al. Determination of sulfide in brain tissue by gas dialysis/ion chromatography: postmortem studies and two case reports. J Anal Toxicol 1989; 13(2):105-109.

(8) Warenycia MW, Goodwin LR, Benishin CG, Reiffenstein RJ, Francom DM, Taylor JD et al. Acute hydrogen sulfide poisoning. Demonstration of selective uptake of sulfide by the brainstem by measurement of brain sulfide levels. Biochem Pharmacol 1989; 38(6):973-981.

(9) Abe K, Kimura H. The possible role of hydrogen sulfide as an endogenous neuromodulator. J Neurosci 1996; 16(3):1066-1071.

(10) Reiffenstein RJ, Hulbert WC, Roth SH. Toxicology of hydrogen sulfide. Annu Rev Pharmacol Toxicol 1992; 32:109-134.

(11) Beauchamp RO, Jr., Bus JS, Popp JA, Boreiko CJ, Andjelkovich DA. A critical review of the literature on hydrogen sulfide toxicity. Crit Rev Toxicol 1984; 13(1):25-97.

(12) Burnett WW, King EG, Grace M, Hall WF. Hydrogen sulfide poisoning: review of 5 years' experience. Can Med Assoc J 1977; 117(11):1277-1280.

(13) Stipanuk MH, Beck PW. Characterization of the enzymic capacity for cysteine desulphhydration in liver and kidney of the rat. Biochem J 1982; 206(2):267-277.

(14) Erickson PF, Maxwell IH, Su LJ, Baumann M, Glode LM. Sequence of cDNA for rat cystathionine gamma-lyase and comparison of deduced amino acid sequence with related Escherichia coli enzymes. Biochem J 1990; 269(2):335-340.

(15) CAVALLINI D, MONDOVI B, DE MC, SCIOSCIA-SANTORO A. The mechanism of desulphhydration of cysteine. Enzymologia 1962; 24:253-266.

(16) Julian D, Statile JL, Wohlgemuth SE, Arp AJ. Enzymatic hydrogen sulfide production in marine invertebrate tissues. Comp Biochem Physiol A Mol Integr Physiol 2002; 133(1):105-115.

(17) Chen X, Jhee KH, Kruger WD. Production of the neuromodulator H2S by cystathionine beta-synthase via the condensation of cysteine and homocysteine. J Biol Chem 2004; 279(50):52082-52086.

(18) Lee JI, Londono M, Hirschberger LL, Stipanuk MH. Regulation of cysteine dioxygenase and gamma-glutamylcysteine synthetase is associated with hepatic cysteine level. J Nutr Biochem 2004; 15(2):112-122.

(19) Stipanuk MH, Dominy JE, Jr., Lee JI, Coloso RM. Mammalian cysteine metabolism: new insights into regulation of cysteine metabolism. J Nutr 2006; 136(6 Suppl):1652S-1659S.

(20) Droge W, Eck HP, Gmunder H, Mihm S. Modulation of lymphocyte functions and immune responses by cysteine and cysteine derivatives. Am J Med 1991; 91(3C):140S-144S.

(21) Ji C, Kaplowitz N. Hyperhomocysteinemia, endoplasmic reticulum stress, and alcoholic liver injury. World J Gastroenterol 2004; 10(12):1699-1708.

(22) Hosoya K, Tomi M, Ohtsuki S, Takanaga H, Saeki S, Kanai Y et al. Enhancement of L-cystine transport activity and its relation to xCT gene induction at the blood-brain barrier by diethyl maleate treatment. J Pharmacol Exp Ther 2002; 302(1):225-231.

(23) Guebel DV, Torres NV. Dynamics of sulfur amino acids in mammalian brain: assessment of the astrocytic-neuronal cysteine interaction by a mathematical hybrid model. Biochim Biophys Acta 2004; 1674(1):12-28.

(24) Wang XF, Cynader MS. Astrocytes provide cysteine to neurons by releasing glutathione. J Neurochem 2000; 74(4):1434-1442.

(25) El-Khairy L, Ueland PM, Refsum H, Graham IM, Vollset SE. Plasma total cysteine as a risk factor for vascular disease: The European Concerted Action Project. Circulation 2001; 103(21):2544-2549.

(26) El-Khairy L, Ueland PM, Nygard O, Refsum H, Vollset SE. Lifestyle and cardiovascular disease risk factors as determinants of total cysteine in plasma: the Hordaland Homocysteine Study. Am J Clin Nutr 1999; 70(6):1016-1024.

(27) Ozkan Y, Ozkan E, Simsek B. Plasma total homocysteine and cysteine levels as cardiovascular risk factors in coronary heart disease. Int J Cardiol 2002; 82(3):269-277.

(28) Hankey GJ, Eikelboom JW. Homocysteine levels in patients with stroke: clinical relevance and therapeutic implications. CNS Drugs 2001; 15(6):437-443.

(29) Hankey GJ, Eikelboom JW. Homocysteine and stroke. Curr Opin Neurol 2001; 14(1):95-102.

(30) Beyer K, Lao JI, Carrato C, Rodriguez-Vila A, Latorre P, Mataro M et al. Cystathionine beta synthase as a risk factor for Alzheimer disease. Curr Alzheimer Res 2004; 1(2):127-133.

(31) Heafield MT, Fearn S, Steventon GB, Waring RH, Williams AC, Sturman SG. Plasma cysteine and sulphate levels in patients with motor neurone, Parkinson's and Alzheimer's disease. Neurosci Lett 1990; 110(1-2):216-220.

(32) Drennan CL, Huang S, Drummond JT, Matthews RG, Lidwig ML. How a protein binds B12: A 3.0 A X-ray structure of B12-binding domains of methionine synthase. Science 1994; 266(5191):1669-1674.

(33) Selhub J. Homocysteine metabolism. Annu Rev Nutr 1999; 19:217-246.

(34) Vitvitsky V, Dayal S, Stabler S, Zhou Y, Wang H, Lentz SR et al. Perturbations in homocysteine-linked redox homeostasis in a murine model for hyperhomocysteinemia. Am J Physiol Regul Integr Comp Physiol 2004; 287(1):R39-R46.

(35) Vitvitsky V, Thomas M, Ghorpade A, Gendelman HE, Banerjee R. A functional transsulfuration pathway in the brain links to glutathione homeostasis. J Biol Chem 2006; 281(47):35785-35793.

(36) Tsai MY, Welge BG, Hanson NQ, Bignell MK, Vessey J, Schwichtenberg K et al. Genetic causes of mild hyperhomocysteinemia in patients with premature occlusive coronary artery diseases. Atherosclerosis 1999; 143(1):163-170.

(37) Wang XL, Duarte N, Cai H, Adachi T, Sim AS, Cranney G et al. Relationship between total plasma homocysteine, polymorphisms of homocysteine metabolism related enzymes, risk factors and coronary artery disease in the Australian hospital-based population. Atherosclerosis 1999; 146(1):133-140.

(38) Matthias D, Becker CH, Riezler R, Kindling PH. Homocysteine induced arteriosclerosis-like alterations of the aorta in normotensive and hypertensive rats following application of high doses of methionine. Atherosclerosis 1996; 122(2):201-216.

(39) Jacobsen DW. Homocysteine and vitamins in cardiovascular disease. Clin Chem 1998; 44(8 Pt 2):1833-1843.

(40) Kruman II, Culmsee C, Chan SL, Kruman Y, Guo Z, Penix L et al. Homocysteine elicits a DNA damage response in neurons that promotes apoptosis and hypersensitivity to excitotoxicity. J Neurosci 2000; 20(18):6920-6926.

(41) Streck EL, Bavaresco CS, Netto CA, Wyse AT. Chronic hyperhomocysteinemia provokes a memory deficit in rats in the Morris water maze task. Behav Brain Res 2004; 153(2):377-381.

(42) Bleich S, Degner D, Sperling W, Bonsch D, Thurauf N, Kornhuber J. Homocysteine as a neurotoxin in chronic alcoholism. Prog Neuropsychopharmacol Biol Psychiatry 2004; 28(3):453-464.

(43) Sai X, Kawamura Y, Kokame K, Yamaguchi H, Shiraishi H, Suzuki R et al. Endoplasmic reticulum stress-inducible protein, Herp, enhances presenilin-mediated generation of amyloid beta-protein. J Biol Chem 2002; 277(15):12915-12920.

(44) Pohlandt F. Cystine: a semi-essential amino acid in the newborn infant. Acta Paediatr Scand 1974; 63(6):801-804.

(45) Gaull G, Sturman JA, Raiha NC. Development of mammalian sulfur metabolism: absence of cystathionase in human fetal tissues. Pediatr Res 1972; 6(6):538-547.

(46) Horowitz JH, Rypins EB, Henderson JM, Heymsfield SB, Moffitt SD, Bain RP et al. Evidence for impairment of transsulfuration pathway in cirrhosis. Gastroenterology 1981; 81(4):668-675.

(47) Meister A, Anderson ME. Glutathione. Annu Rev Biochem 1983; 52:711-760.

(48) Steranka LR, Rhind AW. Effect of cysteine on the persistent depletion of brain monoamines by amphetamine, p-chloroamphetamine and MPTP. Eur J Pharmacol 1987; 133(2):191-197.

(49) Nishiuch Y, Sasaki M, Nakayasu M, Oikawa A. Cytotoxicity of cysteine in culture media. In Vitro 1976; 12(9):635-638.

(50) Anderson ME, Meister A. Intracellular delivery of cysteine. Methods Enzymol 1987; 143:313-325.

(51) Karlsen RL, Grofova I, Malthe-Sorenssen D, Fonnum F. Morphological changes in rat brain induced by L-cysteine injection in newborn animals. Brain Res 1981; 208(1):167-180.

(52) Olney JW, Zorumski C, Price MT, Labruyere J. L-cysteine, a bicarbonate-sensitive endogenous excitotoxin. Science 1990; 248(4955):596-599.

(53) Olney JW, Ho OL. Brain damage in infant mice following oral intake of glutamate, aspartate or cysteine. Nature 1970; 227(5258):609-611.

(54) Saez G, Thornalley PJ, Hill HA, Hems R, Bannister JV. The production of free radicals during the autoxidation of cysteine and their effect on isolated rat hepatocytes. Biochim Biophys Acta 1982; 719(1):24-31.

(55) Nath KA, Salahudeen AK. Autoxidation of cysteine generates hydrogen peroxide: cytotoxicity and attenuation by pyruvate. Am J Physiol 1993; 264(2 Pt 2):F306-F314.

(56) Puka-Sundvall M, Eriksson P, Nilsson M, Sandberg M, Lehmann A. Neurotoxicity of cysteine: interaction with glutamate. Brain Res 1995; 705(1-2):65-70.

(57) BIRNBAUM SM, WINITZ M, GREENSTEIN JP. Quantitative nutritional studies with water-soluble, chemically defined diets. III. Individual amino acids as sources of non-essential nitrogen. Arch Biochem Biophys 1957; 72(2):428-436.

(58) Shapre LG, Olney JW, Ohlendorf C, Lyss A, Zimmerman M, Gale B. Brain damage and associated behavioral deficits following the administration of L-cysteine to infant rats. Pharmacol Biochem Behav 1975; 3(2):291-298.

(59) Wang XF, Cynader MS. Pyruvate released by astrocytes protects neurons from copper-catalyzed cysteine neurotoxicity. J Neurosci 2001; 21(10):3322-3331.

(60) Janaky R, Varga V, Hermann A, Saransaari P, Oja SS. Mechanisms of L-cysteine neurotoxicity. Neurochem Res 2000; 25(9-10):1397-1405.

(61) Julian D, Statile JL, Wohlgemuth SE, Arp AJ. Enzymatic hydrogen sulfide production in marine invertebrate tissues. Comp Biochem Physiol A Mol Integr Physiol 2002; 133(1):105-115.

(62) Goodwin LR, Francom D, Dieken FP, Taylor JD, Warenycia MW, Reiffenstein RJ et al. Determination of sulfide in brain tissue by gas dialysis/ion chromatography: postmortem studies and two case reports. J Anal Toxicol 1989; 13(2):105-109.

(63) Stipanuk MH. Metabolism of sulfur-containing amino acids. Annu Rev Nutr 1986; 6:179-209.

(64) Kraus J, Packman S, Fowler B, Rosenberg LE. Purification and properties of cystathionine beta-synthase from human liver. Evidence for identical subunits. J Biol Chem 1978; 253(18):6523-6528.

(65) Skovby F, Kraus JP, Rosenberg LE. Biosynthesis and proteolytic activation of cystathionine beta-synthase in rat liver. J Biol Chem 1984; 259(1):588-593.

(66) Kraus JP, Rosenberg LE. Cystathionine beta-synthase from human liver: improved purification scheme and additional characterization of the enzyme in crude and pure form. Arch Biochem Biophys 1983; 222(1):44-52.

(67) Skovby F, Kraus JP, Rosenberg LE. Biosynthesis and proteolytic activation of cystathionine beta-synthase in rat liver. J Biol Chem 1984; 259(1):588-593.

(68) Kraus JP, Rosenberg LE. Cystathionine beta-synthase from human liver: improved purification scheme and additional characterization of the enzyme in crude and pure form. Arch Biochem Biophys 1983; 222(1):44-52.

(69) Skovby F, Kraus JP, Rosenberg LE. Biosynthesis and proteolytic activation of cystathionine beta-synthase in rat liver. J Biol Chem 1984; 259(1):588-593.

(70) Brown FC, Gordon PH. Cystathionine synthase from rat liver: partial purification and properties. Can J Biochem 1971; 49(5):484-491.

(71) Kraus J, Packman S, Fowler B, Rosenberg LE. Purification and properties of cystathionine beta-synthase from human liver. Evidence for identical subunits. J Biol Chem 1978; 253(18):6523-6528.

(72) Kimura H, Nakagawa H. Studies on cystathionine synthetase characteristics of purified rat liver enzyme. J Biochem (Tokyo) 1971; 69(4):711-723.

(73) Quere I, Paul V, Rouillac C, Janbon C, London J, Demaille J et al. Spatial and temporal expression of the cystathionine beta-synthase gene during early human development. Biochem Biophys Res Commun 1999; 254(1):127-137.

(74) Awata S, Nakayama K, Suzuki I, Sugahara K, Kodama H. Changes in cystathionine gamma-lyase in various regions of rat brain during development. Biochem Mol Biol Int 1995; 35(6):1331-1338.

(75) Eto K, Ogasawara M, Umemura K, Nagai Y, Kimura H. Hydrogen sulfide is produced in response to neuronal excitation. J Neurosci 2002; 22(9):3386-3391.

(76) Eto K, Kimura H. A novel enhancing mechanism for hydrogen sulfide-producing activity of cystathionine beta-synthase. J Biol Chem 2002; 277(45):42680-42685.

(77) Miles EW, Kraus JP. Cystathionine beta-synthase: structure, function, regulation, and location of homocystinuria-causing mutations. J Biol Chem 2004; 279(29):29871-29874.

(78) Quere I, Paul V, Rouillac C, Janbon C, London J, Demaille J et al. Spatial and temporal expression of the cystathionine beta-synthase gene during early human development. Biochem Biophys Res Commun 1999; 254(1):127-137.

(79) Allsop J, Watts RW. Methionine adenosyltransferase, cystathionine beta-synthase and cystathionine gamma-lyase activity of rat liver subcellular particles, human blood cells and mixed white cells from rat bone marrow. Clin Sci Mol Med Suppl 1975; 48(6):509-513.

(80) Mudd SH, Skovby F, Levy HL, Pettigrew KD, Wilcken B, Pyeritz RE et al. The natural history of homocystinuria due to cystathionine beta-synthase deficiency. Am J Hum Genet 1985; 37(1):1-31.

(81) Mudd SH, Skovby F, Levy HL, Pettigrew KD, Wilcken B, Pyeritz RE et al. The natural history of homocystinuria due to cystathionine beta-synthase deficiency. Am J Hum Genet 1985; 37(1):1-31.

(82) Cruysberg JR, Boers GH, Trijbels JM, Deutman AF. Delay in diagnosis of homocystinuria: retrospective study of consecutive patients. BMJ 1996; 313(7064):1037-1040.

(83) Mudd SH, Skovby F, Levy HL, Pettigrew KD, Wilcken B, Pyeritz RE et al. The natural history of homocystinuria due to cystathionine beta-synthase deficiency. Am J Hum Genet 1985; 37(1):1-31.

(84) Zlotkin SH, Anderson GH. The development of cystathionase activity during the first year of life. Pediatr Res 1982; 16(1):65-68.

(85) Wrobel M, Ubuka T, Yao WB, Abe T. L-cysteine metabolism in guinea pig and rat tissues. Comp Biochem Physiol B Biochem Mol Biol 1997; 116(2):223-226.

(86) Heinonen K. Studies on cystathionase activity in rat liver and brain during development. Effects of hormones and amino acids in vivo. Biochem J 1973; 136(4):1011-1015.

(87) Nakata K, Kawase M, Ogino S, Kinoshita C, Murata H, Sakaue T et al. Effects of age on levels of cysteine, glutathione and related enzyme activities in livers of mice and rats and an attempt to replenish hepatic glutathione level of mouse with cysteine derivatives. Mech Ageing Dev 1996; 90(3):195-207.

(88) Levonen AL, Lapatto R, Saksela M, Raivio KO. Human cystathionine gamma-lyase: developmental and in vitro expression of two isoforms. Biochem J 2000; 347 Pt 1:291-295.

(89) Ogasawara Y, Isoda S, Tanabe S. Tissue and subcellular distribution of bound and acid-labile sulfur, and the enzymic capacity for sulfide production in the rat. Biol Pharm Bull 1994; 17(12):1535-1542.

(90) Awata S, Nakayama K, Suzuki I, Sugahara K, Kodama H. Changes in cystathionine gamma-lyase in various regions of rat brain during development. Biochem Mol Biol Int 1995; 35(6):1331-1338.

(91) Erickson PF, Maxwell IH, Su LJ, Baumann M, Glode LM. Sequence of cDNA for rat cystathionine gamma-lyase and comparison of deduced amino acid sequence with related Escherichia coli enzymes. Biochem J 1990; 269(2):335-340.

(92) Ogasawara Y, Isoda S, Tanabe S. Tissue and subcellular distribution of bound and acid-labile sulfur, and the enzymic capacity for sulfide production in the rat. Biol Pharm Bull 1994; 17(12):1535-1542.

(93) Li L, Bhatia M, Zhu YZ, Zhu YC, Ramnath RD, Wang ZJ et al. Hydrogen sulfide is a novel mediator of lipopolysaccharide-induced inflammation in the mouse. FASEB J 2005; 19(9):1196-1198.

(94) Moore PK, Bhatia M, Moochhala S. Hydrogen sulfide: from the smell of the past to the mediator of the future? Trends Pharmacol Sci 2003; 24(12):609-611.

(95) Bhatia M, Wong FL, Fu D, Lau HY, Moochhala SM, Moore PK. Role of hydrogen sulfide in acute pancreatitis and associated lung injury. FASEB J 2005; 19(6):623-625.

(96) Hosoki R, Matsuki N, Kimura H. The possible role of hydrogen sulfide as an endogenous smooth muscle relaxant in synergy with nitric oxide. Biochem Biophys Res Commun 1997; 237(3):527-531.

(97) Teague B, Asiedu S, Moore PK. The smooth muscle relaxant effect of hydrogen sulphide in vitro: evidence for a physiological role to control intestinal contractility. Br J Pharmacol 2002; 137(2):139-145.

(98) Zhao W, Wang R. H(2)S-induced vasorelaxation and underlying cellular and molecular mechanisms. Am J Physiol Heart Circ Physiol 2002; 283(2):H474-H480.

(99) Zhao W, Zhang J, Lu Y, Wang R. The vasorelaxant effect of H(2)S as a novel endogenous gaseous K(ATP) channel opener. EMBO J 2001; 20(21):6008-6016.

(100) Goodwin LR, Francom D, Dieken FP, Taylor JD, Warenycia MW, Reiffenstein RJ et al. Determination of sulfide in brain tissue by gas dialysis/ion chromatography: postmortem studies and two case reports. J Anal Toxicol 1989; 13(2):105-109.

(101) Awata S, Nakayama K, Suzuki I, Sugahara K, Kodama H. Changes in cystathionine gamma-lyase in various regions of rat brain during development. Biochem Mol Biol Int 1995; 35(6):1331-1338.

(102) Eto K, Ogasawara M, Umemura K, Nagai Y, Kimura H. Hydrogen sulfide is produced in response to neuronal excitation. J Neurosci 2002; 22(9):3386-3391.

(103) Eto K, Kimura H. The production of hydrogen sulfide is regulated by testosterone and S-adenosyl-L-methionine in mouse brain. J Neurochem 2002; 83(1):80-86.

(104) Kimura H. Hydrogen sulfide induces cyclic AMP and modulates the NMDA receptor. Biochem Biophys Res Commun 2000; 267(1):129-133.

(105) Zhao W, Zhang J, Lu Y, Wang R. The vasorelaxant effect of H(2)S as a novel endogenous gaseous K(ATP) channel opener. EMBO J 2001; 20(21):6008-6016.

(106) Zhao W, Wang R. H(2)S-induced vasorelaxation and underlying cellular and molecular mechanisms. Am J Physiol Heart Circ Physiol 2002; 283(2):H474-H480.

(107) Sidhu R, Singh M, Samir G, Carson RJ. L-cysteine and sodium hydrosulphide inhibit spontaneous contractility in isolated pregnant rat uterine strips in vitro. Pharmacol Toxicol 2001; 88(4):198-203.

(108) Sattler R, Tymianski M. Molecular mechanisms of glutamate receptor-mediated excitotoxic neuronal cell death. Mol Neurobiol 2001; 24(1-3):107-129.

(109) Eto K, Asada T, Arima K, Makifuchi T, Kimura H. Brain hydrogen sulfide is severely decreased in Alzheimer's disease. Biochem Biophys Res Commun 2002; 293(5):1485-1488.

(110) Clarke R, Smith AD, Jobst KA, Refsum H, Sutton L, Ueland PM. Folate, vitamin B12, and serum total homocysteine levels in confirmed Alzheimer disease. Arch Neurol 1998; 55(11):1449-1455.

(111) Dello RC, Tringali G, Ragazzoni E, Maggiano N, Menini E, Vairano M et al. Evidence that hydrogen sulphide can modulate hypothalamo-pituitary-adrenal axis function: in vitro and in vivo studies in the rat. J Neuroendocrinol 2000; 12(3):225-233.

(112) Silva AJ. Molecular and cellular cognitive studies of the role of synaptic plasticity in memory. J Neurobiol 2003; 54(1):224-237.

(113) Ashwal S, Pearce WJ. Animal models of neonatal stroke. Curr Opin Pediatr 2001; 13(6):506-516.

(114) Horn M, Schlote W. Delayed neuronal death and delayed neuronal recovery in the human brain following global ischemia. Acta Neuropathol (Berl) 1992; 85(1):79-87.

(115) Johnston MV, Trescher WH, Ishida A, Nakajima W. Neurobiology of hypoxic-ischemic injury in the developing brain. Pediatr Res 2001; 49(6):735-741.

(116) Sacco RL, Wolf PA, Gorelick PB. Risk factors and their management for stroke prevention: outlook for 1999 and beyond. Neurology 1999; 53(7 Suppl 4):S15-S24.

(117) Gorelick PB. New horizons for stroke prevention: PROGRESS and HOPE. Lancet Neurol 2002; 1(3):149-156.

(118) Straus SE, Majumdar SR, McAlister FA. New evidence for stroke prevention: scientific review. JAMA 2002; 288(11):1388-1395.

(119) Fisher M, Brott TG. Emerging therapies for acute ischemic stroke: new therapies on trial. Stroke 2003; 34(2):359-361.

(120) Muir KW. Heterogeneity of stroke pathophysiology and neuroprotective clinical trial design. Stroke 2002; 33(6):1545-1550.

(121) Cheng YD, Al-Khoury L, Zivin JA. Neuroprotection for Ischemic Stroke: Two Decades of Success and Failure. Neurorx 2004; 1(1):36-45.

(122) Dirnagl U, Iadecola C, Moskowitz MA. Pathobiology of ischaemic stroke: an integrated view. Trends Neurosci 1999; 22(9):391-397.

(123) Barone FC, Feuerstein GZ. Inflammatory mediators and stroke: new opportunities for novel therapeutics. J Cereb Blood Flow Metab 1999; 19(8):819-834.

(124) Albers GW. Potential therapeutic uses of N-methyl-D-aspartate antagonists in cerebral ischemia. Clin Neuropharmacol 1990; 13(3):177-197.

(125) Dirnagl U, Iadecola C, Moskowitz MA. Pathobiology of ischaemic stroke: an integrated view. Trends Neurosci 1999; 22(9):391-397.

(126) Ginsberg MD, Pulsinelli WA. The ischemic penumbra, injury thresholds, and the therapeutic window for acute stroke. Ann Neurol 1994; 36(4):553-554.

(127) Hossmann KA. Disturbances of cerebral protein synthesis and ischemic cell death. Prog Brain Res 1993; 96:161-177.

(128) Nedergaard M, Jakobsen J, Diemer NH. Autoradiographic determination of cerebral glucose content, blood flow, and glucose utilization in focal ischemia of the rat brain: influence of the plasma glucose concentration. J Cereb Blood Flow Metab 1988; 8(1):100-108.

(129) Berger R, Djuricic B, Jensen A, Hossmann KA, Paschen W. Ontogenetic differences in energy metabolism and inhibition of protein synthesis in hippocampal slices during in vitro ischemia and 24 h of recovery. Brain Res Dev Brain Res 1996; 91(2):281-291.

(130) Ikeda T, Ikenoue T, Xia XY, Xia YX. Important role of 72-kd heat shock protein expression in the endothelial cell in acquisition of hypoxic-ischemic tolerance in the immature rat. Am J Obstet Gynecol 2000; 182(2):380-386.

(131) Goto K, Ishige A, Sekiguchi K, Iizuka S, Sugimoto A, Yuzurihara M et al. Effects of cycloheximide on delayed neuronal death in rat hippocampus. Brain Res 1990; 534(1-2):299-302.

(132) Shigeno T, Yamasaki Y, Kato G, Kusaka K, Mima T, Takakura K et al. Reduction of delayed neuronal death by inhibition of protein synthesis. Neurosci Lett 1990; 120(1):117-119.

(133) Nicotera P, Lipton SA. Excitotoxins in neuronal apoptosis and necrosis. J Cereb Blood Flow Metab 1999; 19(6):583-591.

(134) Matute C, Alberdi E, Ibarretxe G, Sanchez-Gomez MV. Excitotoxicity in glial cells. Eur J Pharmacol 2002; 447(2-3):239-246.

(135) Conn PJ, Pin JP. Pharmacology and functions of metabotropic glutamate receptors. Annu Rev Pharmacol Toxicol 1997; 37:205-237.

(136) Masu M, Tanabe Y, Tsuchida K, Shigemoto R, Nakanishi S. Sequence and expression of a metabotropic glutamate receptor. Nature 1991; 349(6312):760-765.

(137) Tanabe Y, Nomura A, Masu M, Shigemoto R, Mizuno N, Nakanishi S. Signal transduction, pharmacological properties, and expression patterns of two rat metabotropic glutamate receptors, mGluR3 and mGluR4. J Neurosci 1993; 13(4):1372-1378.

(138) Nicoletti F, Bruno V, Copani A, Casabona G, Knopfel T. Metabotropic glutamate receptors: a new target for the therapy of neurodegenerative disorders? Trends Neurosci 1996; 19(7):267-271.

(139) Yu SP, Sensi SL, Canzoniero LM, Buisson A, Choi DW. Membrane-delimited modulation of NMDA currents by metabotropic glutamate receptor subtypes 1/5 in cultured mouse cortical neurons. J Physiol 1997; 499 (Pt 3):721-732.

(140) Durkin JP, Tremblay R, Buchan A, Blosser J, Chakravarthy B, Mealing G et al. An early loss in membrane protein kinase C activity precedes the excitatory amino acid-induced death of primary cortical neurons. J Neurochem 1996; 66(3):951-962.

(141) Hollmann M, Hartley M, Heinemann S. Ca2+ permeability of KA-AMPA--gated glutamate receptor channels depends on subunit composition. Science 1991; 252(5007):851-853.

(142) Sommer B, Keinanen K, Verdoorn TA, Wisden W, Burnashev N, Herb A et al. Flip and flop: a cell-specific functional switch in glutamate-operated channels of the CNS. Science 1990; 249(4976):1580-1585.

(143) Brorson JR, Bleakman D, Chard PS, Miller RJ. Calcium directly permeates kainate/alpha-amino-3-hydroxy-5-methyl-4- isoxazolepropionic acid receptors in cultured cerebellar Purkinje neurons. Mol Pharmacol 1992; 41(4):603-608.

(144) Jonas P, Racca C, Sakmann B, Seeburg PH, Monyer H. Differences in Ca2+ permeability of AMPA-type glutamate receptor channels in neocortical neurons caused by differential GluR-B subunit expression. Neuron 1994; 12(6):1281-1289.

(145) Moudy AM, Yamada KA, Rothman SM. Rapid desensitization determines the pharmacology of glutamate neurotoxicity. Neuropharmacology 1994; 33(8):953-962.

(146) Lu YM, Yin HZ, Chiang J, Weiss JH. Ca(2+)-permeable AMPA/kainate and NMDA channels: high rate of Ca2+ influx underlies potent induction of injury. J Neurosci 1996; 16(17):5457-5465.

(147) Carriedo SG, Yin HZ, Lamberta R, Weiss JH. In vitro kainate injury to large, SMI-32(+) spinal neurons is Ca2+ dependent. Neuroreport 1995; 6(6):945-948.

(148) Mori H, Mishina M. Structure and function of the NMDA receptor channel. Neuropharmacology 1995; 34(10):1219-1237.

(149) Lipton SA, Rosenberg PA. Excitatory amino acids as a final common pathway for neurologic disorders. N Engl J Med 1994; 330(9):613-622.

(150) Sucher NJ, Awobuluyi M, Choi YB, Lipton SA. NMDA receptors: from genes to channels. Trends Pharmacol Sci 1996; 17(10):348-355.

(151) Bleakman D. Kainate receptor pharmacology and physiology. Cell Mol Life Sci 1999; 56(7-8):558-566.

(152) Puchalski RB, Louis JC, Brose N, Traynelis SF, Egebjerg J, Kukekov V et al. Selective RNA editing and subunit assembly of native glutamate receptors. Neuron 1994; 13(1):131-147.

(153) Hollmann M, Heinemann S. Cloned glutamate receptors. Annu Rev Neurosci 1994; 17:31-108.

(154) Benveniste H, Drejer J, Schousboe A, Diemer NH. Elevation of the extracellular concentrations of glutamate and aspartate in rat hippocampus during transient cerebral ischemia monitored by intracerebral microdialysis. J Neurochem 1984; 43(5):1369-1374.

(155) Jorgensen MB, Diemer NH. Selective neuron loss after cerebral ischemia in the rat: possible role of transmitter glutamate. Acta Neurol Scand 1982; 66(5):536-546.

(156) Shimada N, Graf R, Rosner G, Heiss WD. Differences in ischemia-induced accumulation of amino acids in the cat cortex. Stroke 1990; 21(10):1445-1451.

(157) Dirnagl U, Iadecola C, Moskowitz MA. Pathobiology of ischaemic stroke: an integrated view. Trends Neurosci 1999; 22(9):391-397.

(158) Katsuki H, Okuda S. Arachidonic acid as a neurotoxic and neurotrophic substance. Prog Neurobiol 1995; 46(6):607-636.

(159) Bazan NG, Rodriguez de Turco EB, Allan G. Mediators of injury in neurotrauma: intracellular signal transduction and gene expression. J Neurotrauma 1995; 12(5):791-814.

(160) Planas AM, Soriano MA, Rodriguez-Farre E, Ferrer I. Induction of cyclooxygenase-2 mRNA and protein following transient focal ischemia in the rat brain. Neurosci Lett 1995; 200(3):187-190.

(161) Adams J, Collaco-Moraes Y, de BJ. Cyclooxygenase-2 induction in cerebral cortex: an intracellular response to synaptic excitation. J Neurochem 1996; 66(1):6-13.

(162) Miettinen S, Fusco FR, Yrjanheikki J, Keinanen R, Hirvonen T, Roivainen R et al. Spreading depression and focal brain ischemia induce cyclooxygenase-2 in cortical

neurons through N-methyl-D-aspartic acid-receptors and phospholipase A2. Proc Natl Acad Sci U S A 1997; 94(12):6500-6505.

(163) Nicotera P, Lipton SA. Excitotoxins in neuronal apoptosis and necrosis. J Cereb Blood Flow Metab 1999; 19(6):583-591.

(164) Park CK, Nehls DG, Graham DI, Teasdale GM, McCulloch J. Focal cerebral ischaemia in the cat: treatment with the glutamate antagonist MK-801 after induction of ischaemia. J Cereb Blood Flow Metab 1988; 8(5):757-762.

(165) Ozyurt E, Graham DI, Woodruff GN, McCulloch J. Protective effect of the glutamate antagonist, MK-801 in focal cerebral ischemia in the cat. J Cereb Blood Flow Metab 1988; 8(1):138-143.

(166) Park CK, Nehls DG, Graham DI, Teasdale GM, McCulloch J. The glutamate antagonist MK-801 reduces focal ischemic brain damage in the rat. Ann Neurol 1988; 24(4):543-551.

(167) Kawasaki-Yatsugi S, Yatsugi S, Takahashi M, Toya T, Ichiki C, Shimizu-Sasamata M et al. A novel AMPA receptor antagonist, YM872, reduces infarct size after middle cerebral artery occlusion in rats. Brain Res 1998; 793(1-2):39-46.

(168) Shimizu-Sasamata M, Kano T, Rogowska J, Wolf GL, Moskowitz MA, Lo EH. YM872, a highly water-soluble AMPA receptor antagonist, preserves the hemodynamic penumbra and reduces brain injury after permanent focal ischemia in rats. Stroke 1998; 29(10):2141-2148.

(169) Graham SH, Chen J, Lan JQ, Simon RP. A dose-response study of neuroprotection using the AMPA antagonist NBQX in rat focal cerebral ischemia. J Pharmacol Exp Ther 1996; 276(1):1-4.

(170) Rataud J, Debarnot F, Mary V, Pratt J, Stutzmann JM. Comparative study of voltage-sensitive sodium channel blockers in focal ischaemia and electric convulsions in rodents. Neurosci Lett 1994; 172(1-2):19-23.

(171) Kittaka M, Giannotta SL, Zelman V, Correale JD, DeGiorgio CM, Weiss MH et al. Attenuation of brain injury and reduction of neuron-specific enolase by nicardipine in systemic circulation following focal ischemia and reperfusion in a rat model. J Neurosurg 1997; 87(5):731-737.

(172) Dirnagl U, Iadecola C, Moskowitz MA. Pathobiology of ischaemic stroke: an integrated view. Trends Neurosci 1999; 22(9):391-397.

(173) Jellinger KA. General aspects of neurodegeneration. J Neural Transm Suppl 2003;(65):101-144.

(174) Kerr JF, Wyllie AH, Currie AR. Apoptosis: a basic biological phenomenon with wide-ranging implications in tissue kinetics. Br J Cancer 1972; 26(4):239-257.

(175) Wyllie AH, Kerr JF, Currie AR. Cell death: the significance of apoptosis. Int Rev Cytol 1980; 68:251-306.

(176) Martin LJ, Al-Abdulla NA, Brambrink AM, Kirsch JR, Sieber FE, Portera-Cailliau C. Neurodegeneration in excitotoxicity, global cerebral ischemia, and target deprivation: A perspective on the contributions of apoptosis and necrosis. Brain Res Bull 1998; 46(4):281-309.

(177) Kerr JF, Gobe GC, Winterford CM, Harmon BV. Anatomical methods in cell death. Methods Cell Biol 1995; 46:1-27.

(178) Du C, Hu R, Csernansky CA, Hsu CY, Choi DW. Very delayed infarction after mild focal cerebral ischemia: a role for apoptosis? J Cereb Blood Flow Metab 1996; 16(2):195-201.

(179) Du C, Hu R, Csernansky CA, Hsu CY, Choi DW. Very delayed infarction after mild focal cerebral ischemia: a role for apoptosis? J Cereb Blood Flow Metab 1996; 16(2):195-201.

(180) Portera-Cailliau C, Price DL, Martin LJ. Excitotoxic neuronal death in the immature brain is an apoptosis-necrosis morphological continuum. J Comp Neurol 1997; 378(1):70-87.

(181) Petito CK, Pulsinelli WA. Sequential development of reversible and irreversible neuronal damage following cerebral ischemia. J Neuropathol Exp Neurol 1984; 43(2):141-153.

(182) Petito CK, Pulsinelli WA. Sequential development of reversible and irreversible neuronal damage following cerebral ischemia. J Neuropathol Exp Neurol 1984; 43(2):141-153.

(183) Pulsinelli WA, Brierley JB, Plum F. Temporal profile of neuronal damage in a model of transient forebrain ischemia. Ann Neurol 1982; 11(5):491-498.

(184) Kirino T. Delayed neuronal death in the gerbil hippocampus following ischemia. Brain Res 1982; 239(1):57-69.

(185) Kirino T. Delayed neuronal death in the gerbil hippocampus following ischemia. Brain Res 1982; 239(1):57-69.

(186) Barone FC, Feuerstein GZ. Inflammatory mediators and stroke: new opportunities for novel therapeutics. J Cereb Blood Flow Metab 1999; 19(8):819-834.

(187) Chan PH. Oxygen radicals in focal cerebral ischemia. Brain Pathol 1994; 4(1):59-65.

(188) Patel M, Day BJ, Crapo JD, Fridovich I, McNamara JO. Requirement for superoxide in excitotoxic cell death. Neuron 1996; 16(2):345-355.

(189) Terada LS, Willingham IR, Rosandich ME, Leff JA, Kindt GW, Repine JE. Generation of superoxide anion by brain endothelial cell xanthine oxidase. J Cell Physiol 1991; 148(2):191-196.

(190) Benjelloun N, Renolleau S, Represa A, Ben-Ari Y, Charriaut-Marlangue C. Inflammatory responses in the cerebral cortex after ischemia in the P7 neonatal Rat. Stroke 1999; 30(9):1916-1923.

(191) Mabuchi T, Kitagawa K, Ohtsuki T, Kuwabara K, Yagita Y, Yanagihara T et al. Contribution of microglia/macrophages to expansion of infarction and response of oligodendrocytes after focal cerebral ischemia in rats. Stroke 2000; 31(7):1735-1743.

(192) Kreutzberg GW. Microglia: a sensor for pathological events in the CNS. Trends Neurosci 1996; 19(8):312-318.

(193) Lavrovsky Y, Chatterjee B, Clark RA, Roy AK. Role of redox-regulated transcription factors in inflammation, aging and age-related diseases. Exp Gerontol 2000; 35(5):521-532.

(194) Siesjo BK. Mechanisms of ischemic brain damage. Crit Care Med 1988; 16(10):954-963.

(195) Dirnagl U, Iadecola C, Moskowitz MA. Pathobiology of ischaemic stroke: an integrated view. Trends Neurosci 1999; 22(9):391-397.

(196) Kirino T. Delayed neuronal death in the gerbil hippocampus following ischemia. Brain Res 1982; 239(1):57-69.

(197) Reed JC. Mechanisms of apoptosis. Am J Pathol 2000; 157(5):1415-1430.

(198) Reed JC. Mechanisms of apoptosis. Am J Pathol 2000; 157(5):1415-1430.

(199) Yuan J, Yankner BA. Apoptosis in the nervous system. Nature 2000; 407(6805):802-809.

(200) Hengartner MO. The biochemistry of apoptosis. Nature 2000; 407(6805):770-776.

(201) Leist M, Jaattela M. Four deaths and a funeral: from caspases to alternative mechanisms. Nat Rev Mol Cell Biol 2001; 2(8):589-598.

(202) Earnshaw WC, Martins LM, Kaufmann SH. Mammalian caspases: structure, activation, substrates, and functions during apoptosis. Annu Rev Biochem 1999; 68:383-424.

(203) Thornberry NA, Lazebnik Y. Caspases: enemies within. Science 1998; 281(5381):1312-1316.

(204) Antonsson B. Bax and other pro-apoptotic Bcl-2 family "killer-proteins" and their victim the mitochondrion. Cell Tissue Res 2001; 306(3):347-361.

(205) Wang X. The expanding role of mitochondria in apoptosis. Genes Dev 2001; 15(22):2922-2933.

(206) Lorenzo HK, Susin SA, Penninger J, Kroemer G. Apoptosis inducing factor (AIF): a phylogenetically old, caspase-independent effector of cell death. Cell Death Differ 1999; 6(6):516-524.

(207) Carmody RJ, Cotter TG. Oxidative stress induces caspase-independent retinal apoptosis in vitro. Cell Death Differ 2000; 7(3):282-291.

(208) Squier MK, Miller AC, Malkinson AM, Cohen JJ. Calpain activation in apoptosis. J Cell Physiol 1994; 159(2):229-237.

(209) Hirsch T, Dallaporta B, Zamzami N, Susin SA, Ravagnan L, Marzo I et al. Proteasome activation occurs at an early, premitochondrial step of thymocyte apoptosis. J Immunol 1998; 161(1):35-40.

(210) Hughes FM, Jr., Evans-Storms RB, Cidlowski JA. Evidence that non-caspase proteases are required for chromatin degradation during apoptosis. Cell Death Differ 1998; 5(12):1017-1027.

(211) Susin SA, Lorenzo HK, Zamzami N, Marzo I, Snow BE, Brothers GM et al. Molecular characterization of mitochondrial apoptosis-inducing factor. Nature 1999; 397(6718):441-446.

(212) Li LY, Luo X, Wang X. Endonuclease G is an apoptotic DNase when released from mitochondria. Nature 2001; 412(6842):95-99.

(213) Jorgensen MB, Diemer NH. Selective neuron loss after cerebral ischemia in the rat: possible role of transmitter glutamate. Acta Neurol Scand 1982; 66(5):536-546.

(214) Endres M, Kaps M, Moskowitz MA. [Apoptosis and ischemic infarct]. Nervenarzt 1998; 69(6):459-464.

(215) Charriaut-Marlangue C, Margaill I, Represa A, Popovici T, Plotkine M, Ben-Ari Y. Apoptosis and necrosis after reversible focal ischemia: an in situ DNA fragmentation analysis. J Cereb Blood Flow Metab 1996; 16(2):186-194.

(216) Cheng Y, Deshmukh M, D'Costa A, Demaro JA, Gidday JM, Shah A et al. Caspase inhibitor affords neuroprotection with delayed administration in a rat model of neonatal hypoxic-ischemic brain injury. J Clin Invest 1998; 101(9):1992-1999.

(217) Clemens JA, Stephenson DT, Dixon EP, Smalstig EB, Mincy RE, Rash KS et al. Global cerebral ischemia activates nuclear factor-kappa B prior to evidence of DNA fragmentation. Brain Res Mol Brain Res 1997; 48(2):187-196.

(218) Gillardon F, Bottiger B, Schmitz B, Zimmermann M, Hossmann KA. Activation of CPP-32 protease in hippocampal neurons following ischemia and epilepsy. Brain Res Mol Brain Res 1997; 50(1-2):16-22.

(219) Love S. Apoptosis and brain ischaemia. Prog Neuropsychopharmacol Biol Psychiatry 2003; 27(2):267-282.

(220) Namura S, Zhu J, Fink K, Endres M, Srinivasan A, Tomaselli KJ et al. Activation and cleavage of caspase-3 in apoptosis induced by experimental cerebral ischemia. J Neurosci 1998; 18(10):3659-3668.

(221) Han BH, D'Costa A, Back SA, Parsadanian M, Patel S, Shah AR et al. BDNF blocks caspase-3 activation in neonatal hypoxia-ischemia. Neurobiol Dis 2000; 7(1):38-53.

(222) Arvin KL, Han BH, Du Y, Lin SZ, Paul SM, Holtzman DM. Minocycline markedly protects the neonatal brain against hypoxic-ischemic injury. Ann Neurol 2002; 52(1):54-61.

(223) Luo Y, Cao G, Pei W, O'Horo C, Graham SH, Chen J. Induction of caspase-activated deoxyribonuclease activity after focal cerebral ischemia and reperfusion. J Cereb Blood Flow Metab 2002; 22(1):15-20.

(224) Hara H, Fink K, Endres M, Friedlander RM, Gagliardini V, Yuan J et al. Attenuation of transient focal cerebral ischemic injury in transgenic mice expressing a mutant ICE inhibitory protein. J Cereb Blood Flow Metab 1997; 17(4):370-375.

(225) Hara H, Friedlander RM, Gagliardini V, Ayata C, Fink K, Huang Z et al. Inhibition of interleukin 1beta converting enzyme family proteases reduces ischemic and excitotoxic neuronal damage. Proc Natl Acad Sci U S A 1997; 94(5):2007-2012.

(226) Loddick SA, Rothwell NJ. Neuroprotective effects of human recombinant interleukin-1 receptor antagonist in focal cerebral ischaemia in the rat. J Cereb Blood Flow Metab 1996; 16(5):932-940.

(227) Krupinski J, Lopez E, Marti E, Ferrer I. Expression of caspases and their substrates in the rat model of focal cerebral ischemia. Neurobiol Dis 2000; 7(4):332-342.

(228) Schulz JB, Weller M, Matthews RT, Heneka MT, Groscurth P, Martinou JC et al. Extended therapeutic window for caspase inhibition and synergy with MK-801 in the treatment of cerebral histotoxic hypoxia. Cell Death Differ 1998; 5(10):847-857.

(229) Cheng Y, Deshmukh M, D'Costa A, Demaro JA, Gidday JM, Shah A et al. Caspase inhibitor affords neuroprotection with delayed administration in a rat model of neonatal hypoxic-ischemic brain injury. J Clin Invest 1998; 101(9):1992-1999.

(230) Fink K, Zhu J, Namura S, Shimizu-Sasamata M, Endres M, Ma J et al. Prolonged therapeutic window for ischemic brain damage caused by delayed caspase activation. J Cereb Blood Flow Metab 1998; 18(10):1071-1076.

(231) Schulz JB, Weller M, Matthews RT, Heneka MT, Groscurth P, Martinou JC et al. Extended therapeutic window for caspase inhibition and synergy with MK-801 in the treatment of cerebral histotoxic hypoxia. Cell Death Differ 1998; 5(10):847-857.

(232) Ni B, Wu X, Su Y, Stephenson D, Smalstig EB, Clemens J et al. Transient global forebrain ischemia induces a prolonged expression of the caspase-3 mRNA in rat hippocampal CA1 pyramidal neurons. J Cereb Blood Flow Metab 1998; 18(3):248-256.

(233) Love S, Barber R, Wilcock GK. Neuronal death in brain infarcts in man. Neuropathol Appl Neurobiol 2000; 26(1):55-66.

(234) Love S, Barber R, Srinivasan A, Wilcock GK. Activation of caspase-3 in permanent and transient brain ischaemia in man. Neuroreport 2000; 11(11):2495-2499.

(235) Bhat RV, DiRocco R, Marcy VR, Flood DG, Zhu Y, Dobrzanski P et al. Increased expression of IL-1beta converting enzyme in hippocampus after ischemia: selective localization in microglia. J Neurosci 1996; 16(13):4146-4154.

(236) Yrjanheikki J, Tikka T, Keinanen R, Goldsteins G, Chan PH, Koistinaho J. A tetracycline derivative, minocycline, reduces inflammation and protects against focal cerebral ischemia with a wide therapeutic window. Proc Natl Acad Sci U S A 1999; 96(23):13496-13500.

(237) Friedlander RM, Gagliardini V, Hara H, Fink KB, Li W, MacDonald G et al. Expression of a dominant negative mutant of interleukin-1 beta converting enzyme in transgenic mice prevents neuronal cell death induced by trophic factor withdrawal and ischemic brain injury. J Exp Med 1997; 185(5):933-940.

(238) Schielke GP, Yang GY, Shivers BD, Betz AL. Reduced ischemic brain injury in interleukin-1 beta converting enzyme-deficient mice. J Cereb Blood Flow Metab 1998; 18(2):180-185.

(239) Zhan RZ, Wu C, Fujihara H, Taga K, Qi S, Naito M et al. Both caspase-dependent and caspase-independent pathways may be involved in hippocampal CA1 neuronal death because of loss of cytochrome c From mitochondria in a rat forebrain ischemia model. J Cereb Blood Flow Metab 2001; 21(5):529-540.

(240) Cao G, Minami M, Pei W, Yan C, Chen D, O'Horo C et al. Intracellular Bax translocation after transient cerebral ischemia: implications for a role of the mitochondrial apoptotic signaling pathway in ischemic neuronal death. J Cereb Blood Flow Metab 2001; 21(4):321-333.

(241) Hossmann KA. Experimental models for the investigation of brain ischemia. Cardiovasc Res 1998; 39(1):106-120.

(242) Lipton P. Ischemic cell death in brain neurons. Physiol Rev 1999; 79(4):1431-1568.

(243) Pulsinelli WA, Brierley JB, Plum F. Temporal profile of neuronal damage in a model of transient forebrain ischemia. Ann Neurol 1982; 11(5):491-498.

(244) McAuley MA. Rodent models of focal ischemia. Cerebrovasc Brain Metab Rev 1995; 7(2):153-180.

(245) Levine S, Payan H. Effects of ischemia and other procedures on the brain and retina of the gerbil (Meriones unguiculatus). Exp Neurol 1966; 16(3):255-262.

(246) Kirino T. Delayed neuronal death in the gerbil hippocampus following ischemia. Brain Res 1982; 239(1):57-69.

(247) Pulsinelli WA, Brierley JB. A new model of bilateral hemispheric ischemia in the unanesthetized rat. Stroke 1979; 10(3):267-272.

(248) Cervantes M, Gonzalez-Vidal MD, Ruelas R, Escobar A, Morali G. Neuroprotective effects of progesterone on damage elicited by acute global cerebral ischemia in neurons of the caudate nucleus. Arch Med Res 2002; 33(1):6-14.

(249) Myers RE, Yamaguchi S. Nervous system effects of cardiac arrest in monkeys. Preservation of vision. Arch Neurol 1977; 34(2):65-74.

(250) Levine S, Sohn D. Cerebral ischemia in infant and adult gerbils. Relation to incomplete circle of Willis. Arch Pathol 1969; 87(3):315-317.

(251) Levine S, Payan H. Effects of ischemia and other procedures on the brain and retina of the gerbil (Meriones unguiculatus). Exp Neurol 1966; 16(3):255-262.

(252) Levine S, Sohn D. Cerebral ischemia in infant and adult gerbils. Relation to incomplete circle of Willis. Arch Pathol 1969; 87(3):315-317.

(253) Kirino T. Delayed neuronal death in the gerbil hippocampus following ischemia. Brain Res 1982; 239(1):57-69.

(254) Kirino T. Delayed neuronal death in the gerbil hippocampus following ischemia. Brain Res 1982; 239(1):57-69.

(255) Kirino T, Sano K. Selective vulnerability in the gerbil hippocampus following transient ischemia. Acta Neuropathol (Berl) 1984; 62(3):201-208.

(256) Kirino T, Tamura A, Sano K. Delayed neuronal death in the rat hippocampus following transient forebrain ischemia. Acta Neuropathol (Berl) 1984; 64(2):139-147.

(257) Pulsinelli WA, Brierley JB. A new model of bilateral hemispheric ischemia in the unanesthetized rat. Stroke 1979; 10(3):267-272.

(258) Ginsberg MD, Busto R. Rodent models of cerebral ischemia. Stroke 1989; 20(12):1627-1642.

(259) Ljunggren B, Ratcheson RA, Siesjo BK. Cerebral metabolic state following complete compression ischemia. Brain Res 1974; 73(2):291-307.

(260) Hendrickx HH, Rao GR, Safar P, Gisvold SE. Asphyxia, cardiac arrest and resuscitation in rats. I. Short term recovery. Resuscitation 1984; 12(2):97-116.

(261) Traystman RJ. Animal models of focal and global cerebral ischemia. ILAR J 2003; 44(2):85-95.

(262) Bolander HG, Persson L, Hillered L, d'Argy R, Ponten U, Olsson Y. Regional cerebral blood flow and histopathologic changes after middle cerebral artery occlusion in rats. Stroke 1989; 20(7):930-937.

(263) Backhauss C, Karkoutly C, Welsch M, Krieglstein J. A mouse model of focal cerebral ischemia for screening neuroprotective drug effects. J Pharmacol Toxicol Methods 1992; 27(1):27-32.

(264) Tamura A, Graham DI, McCulloch J, Teasdale GM. Focal cerebral ischaemia in the rat: 1. Description of technique and early neuropathological consequences following middle cerebral artery occlusion. J Cereb Blood Flow Metab 1981; 1(1):53-60.

(265) Slivka A, Pulsinelli W. Hemorrhagic complications of thrombolytic therapy in experimental stroke. Stroke 1987; 18(6):1148-1156.

(266) Hayakawa T, Waltz AG. Immediate effects of cerebral ischemia: evolution and resolution of neurological deficits after experimental occlusion of one middle cerebral artery in conscious cats. Stroke 1975; 6(3):321-327.

(267) Suzuki J, Yoshimoto T, Tnanka S, Sakamoto T. Production of various models of cerebral infarction in the dog by means of occlusion of intracranial trunk arteries. Stroke 1980; 11(4):337-341.

(268) Marshall JW, Ridley RM. Assessment of functional impairment following permanent middle cerebral artery occlusion in a non-human primate species. Neurodegeneration 1996; 5(3):275-286.

(269) Chimon GN, Wong PT. Ischemic tolerance and lipid peroxidation in the brain. Neuroreport 1998; 9(10):2269-2272.

(270) Belayev L, Alonso OF, Busto R, Zhao W, Ginsberg MD. Middle cerebral artery occlusion in the rat by intraluminal suture. Neurological and pathological evaluation of an improved model. Stroke 1996; 27(9):1616-1622.

(271) Menzies SA, Hoff JT, Betz AL. Middle cerebral artery occlusion in rats: a neurological and pathological evaluation of a reproducible model. Neurosurgery 1992; 31(1):100-106.

(272) Overgaard K, Sereghy T, Pedersen H, Boysen G. Dose-response of rt-PA and its combination with aspirin in a rat embolic stroke model. Neuroreport 1992; 3(10):925-928.

(273) Cai H, Yao H, Ibayashi S, Uchimura H, Fujishima M. Photothrombotic middle cerebral artery occlusion in spontaneously hypertensive rats: influence of substrain, gender, and distal middle cerebral artery patterns on infarct size. Stroke 1998; 29(9):1982-1986.

(274) Robinson MJ, Macrae IM, Todd M, Reid JL, McCulloch J. Reduction of local cerebral blood flow to pathological levels by endothelin-1 applied to the middle cerebral artery in the rat. Neurosci Lett 1990; 118(2):269-272.

(275) Duverger D, MacKenzie ET. The quantification of cerebral infarction following focal ischemia in the rat: influence of strain, arterial pressure, blood glucose concentration, and age. J Cereb Blood Flow Metab 1988; 8(4):449-461.

(276) Nakayama H, Ginsberg MD, Dietrich WD. (S)-emopamil, a novel calcium channel blocker and serotonin S2 antagonist, markedly reduces infarct size following middle cerebral artery occlusion in the rat. Neurology 1988; 38(11):1667-1673.

(277) Prado R, Ginsberg MD, Dietrich WD, Watson BD, Busto R. Hyperglycemia increases infarct size in collaterally perfused but not end-arterial vascular territories. J Cereb Blood Flow Metab 1988; 8(2):186-192.

(278) Bederson JB, Pitts LH, Tsuji M, Nishimura MC, Davis RL, Bartkowski H. Rat middle cerebral artery occlusion: evaluation of the model and development of a neurologic examination. Stroke 1986; 17(3):472-476.

(279) Menzies SA, Hoff JT, Betz AL. Middle cerebral artery occlusion in rats: a neurological and pathological evaluation of a reproducible model. Neurosurgery 1992; 31(1):100-106.

(280) Belayev L, Alonso OF, Busto R, Zhao W, Ginsberg MD. Middle cerebral artery occlusion in the rat by intraluminal suture. Neurological and pathological evaluation of an improved model. Stroke 1996; 27(9):1616-1622.

(281) Overgaard K, Sereghy T, Pedersen H, Boysen G. Dose-response of rt-PA and its combination with aspirin in a rat embolic stroke model. Neuroreport 1992; 3(10):925-928.

(282) Callaway JK, Knight MJ, Watkins DJ, Beart PM, Jarrott B. Delayed treatment with AM-36, a novel neuroprotective agent, reduces neuronal damage after endothelin-1-induced middle cerebral artery occlusion in conscious rats. Stroke 1999; 30(12):2704-2712.

(283) Cai H, Yao H, Ibayashi S, Uchimura H, Fujishima M. Photothrombotic middle cerebral artery occlusion in spontaneously hypertensive rats: influence of substrain, gender, and distal middle cerebral artery patterns on infarct size. Stroke 1998; 29(9):1982-1986.

(284) Lindsberg PJ, Kaste M. Thrombolysis for acute stroke. Curr Opin Neurol 2003; 16(1):73-80.

(285) Rogers DC, Campbell CA, Stretton JL, Mackay KB. Correlation between motor impairment and infarct volume after permanent and transient middle cerebral artery occlusion in the rat. Stroke 1997; 28(10):2060-2065.

(286) Longa EZ, Weinstein PR, Carlson S, Cummins R. Reversible middle cerebral artery occlusion without craniectomy in rats. Stroke 1989; 20(1):84-91.

(287) Wong PT, Qu K, Chimon GN, Seah AB, Chang HM, Wong MC et al. High plasma cyst(e)ine level may indicate poor clinical outcome in patients with acute stroke: possible involvement of hydrogen sulfide. J Neuropathol Exp Neurol 2006; 65(2):109-115.

(288) Howard-Jones N. A CIOMS ethical code for animal experimentation. WHO Chron 1985; 39(2):51-56.

(289) Wong EH, Kemp JA, Priestley T, Knight AR, Woodruff GN, Iversen LL. The anticonvulsant MK-801 is a potent N-methyl-D-aspartate antagonist. Proc Natl Acad Sci U S A 1986; 83(18):7104-7108.

(290) Isayama K, Pitts LH, Nishimura MC. Evaluation of 2,3,5-triphenyltetrazolium chloride staining to delineate rat brain infarcts. Stroke 1991; 22(11):1394-1398.

(291) Bederson JB, Pitts LH, Germano SM, Nishimura MC, Davis RL, Bartkowski HM. Evaluation of 2,3,5-triphenyltetrazolium chloride as a stain for detection and quantification of experimental cerebral infarction in rats. Stroke 1986; 17(6):1304-1308.

(292) Tureyen K, Vemuganti R, Sailor KA, Dempsey RJ. Infarct volume quantification in mouse focal cerebral ischemia: a comparison of triphenyltetrazolium chloride and cresyl violet staining techniques. J Neurosci Methods 2004; 139(2):203-207.

(293) Altman FP. The quantification of formazans in tissue sections by microdensitometry. II. The use of BPST, a new tetrazolium salt. Histochem J 1976; 8(5):501-506.

(294) Khan SH, Baziany A, Banigesh A, Hemmings SJ, Shuaib A. Evaluation of an optimal temperature for brain storage in delayed 2, 3,5-triphenyltetrazolium chloride staining. J Neurosci Methods 2000; 98(1):43-47.

(295) Menzies SA, Hoff JT, Betz AL. Middle cerebral artery occlusion in rats: a neurological and pathological evaluation of a reproducible model. Neurosurgery 1992; 31(1):100-106.

(296) Lu Q, Zhu YZ, Wong PT. Angiotensin receptor gene expression in candesartan mediated neuroprotection. Neuroreport 2004; 15(17):2643-2646.

(297) Rogers DC, Campbell CA, Stretton JL, Mackay KB. Correlation between motor impairment and infarct volume after permanent and transient middle cerebral artery occlusion in the rat. Stroke 1997; 28(10):2060-2065.

(298) Widdop RE, Li XC. A simple versatile method for measuring tail cuff systolic blood pressure in conscious rats. Clin Sci (Lond) 1997; 93(3):191-194.

(299) Wong PT, Qu K, Chimon GN, Seah AB, Chang HM, Wong MC et al. High plasma cyst(e)ine level may indicate poor clinical outcome in patients with acute stroke: possible involvement of hydrogen sulfide. J Neuropathol Exp Neurol 2006; 65(2):109-115.

(300) Goodwin LR, Francom D, Dieken FP, Taylor JD, Warenycia MW, Reiffenstein RJ et al. Determination of sulfide in brain tissue by gas dialysis/ion chromatography: postmortem studies and two case reports. J Anal Toxicol 1989; 13(2):105-109.

(301) Warenycia MW, Smith KA, Blashko CS, Kombian SB, Reiffenstein RJ. Monoamine oxidase inhibition as a sequel of hydrogen sulfide intoxication: increases in brain catecholamine and 5-hydroxytryptamine levels. Arch Toxicol 1989; 63(2):131-136.

(302) Beal MF, Swartz KJ, Hyman BT, Storey E, Finn SF, Koroshetz W. Aminooxyacetic acid results in excitotoxin lesions by a novel indirect mechanism. J Neurochem 1991; 57(3):1068-1073.

(303) Du F, Schwarcz R. Aminooxyacetic acid causes selective neuronal loss in layer III of the rat medial entorhinal cortex. Neurosci Lett 1992; 147(2):185-188.

(304) Wang R. The gasotransmitter role of hydrogen sulfide. Antioxid Redox Signal 2003; 5(4):493-501.

(305) Stipanuk MH. Sulfur amino acid metabolism: pathways for production and removal of homocysteine and cysteine. Annu Rev Nutr 2004; 24:539-577.

(306) Teague B, Asiedu S, Moore PK. The smooth muscle relaxant effect of hydrogen sulphide in vitro: evidence for a physiological role to control intestinal contractility. Br J Pharmacol 2002; 137(2):139-145.

(307) Li L, Bhatia M, Zhu YZ, Zhu YC, Ramnath RD, Wang ZJ et al. Hydrogen sulfide is a novel mediator of lipopolysaccharide-induced inflammation in the mouse. FASEB J 2005; 19(9):1196-1198.

(308) Zhao W, Wang R. H(2)S-induced vasorelaxation and underlying cellular and molecular mechanisms. Am J Physiol Heart Circ Physiol 2002; 283(2):H474-H480.

(309) Li L, Bhatia M, Zhu YZ, Zhu YC, Ramnath RD, Wang ZJ et al. Hydrogen sulfide is a novel mediator of lipopolysaccharide-induced inflammation in the mouse. FASEB J 2005; 19(9):1196-1198.

(310) Zhao W, Zhang J, Lu Y, Wang R. The vasorelaxant effect of H(2)S as a novel endogenous gaseous K(ATP) channel opener. EMBO J 2001; 20(21):6008-6016.

(311) Bian JS, Yong QC, Pan TT, Feng ZN, Ali MY, Zhou S et al. Role of hydrogen sulfide in the cardioprotection caused by ischemic preconditioning in the rat heart and cardiac myocytes. J Pharmacol Exp Ther 2006; 316(2):670-678.

(312) Li L, Bhatia M, Zhu YZ, Zhu YC, Ramnath RD, Wang ZJ et al. Hydrogen sulfide is a novel mediator of lipopolysaccharide-induced inflammation in the mouse. FASEB J 2005; 19(9):1196-1198.

(313) Collin M, Anuar FB, Murch O, Bhatia M, Moore PK, Thiemermann C. Inhibition of endogenous hydrogen sulfide formation reduces the organ injury caused by endotoxemia. Br J Pharmacol 2005; 146(4):498-505.

(314) Kimura Y, Kimura H. Hydrogen sulfide protects neurons from oxidative stress. FASEB J 2004; 18(10):1165-1167.

(315) Bhatia M, Wong FL, Fu D, Lau HY, Moochhala SM, Moore PK. Role of hydrogen sulfide in acute pancreatitis and associated lung injury. FASEB J 2005; 19(6):623-625.

(316) Dingledine R, Borges K, Bowie D, Traynelis SF. The glutamate receptor ion channels. Pharmacol Rev 1999; 51(1):7-61.

(317) Sullivan JM, Traynelis SF, Chen HS, Escobar W, Heinemann SF, Lipton SA. Identification of two cysteine residues that are required for redox modulation of the NMDA subtype of glutamate receptor. Neuron 1994; 13(4):929-936.

(318) Roberson ED, Sweatt JD. Transient activation of cyclic AMP-dependent protein kinase during hippocampal long-term potentiation. J Biol Chem 1996; 271(48):30436-30441.

(319) Abel T, Nguyen PV, Barad M, Deuel TA, Kandel ER, Bourtchouladze R. Genetic demonstration of a role for PKA in the late phase of LTP and in hippocampus-based long-term memory. Cell 1997; 88(5):615-626.

(320) Leonard AS, Hell JW. Cyclic AMP-dependent protein kinase and protein kinase C phosphorylate N-methyl-D-aspartate receptors at different sites. J Biol Chem 1997; 272(18):12107-12115.

(321) Tingley WG, Ehlers MD, Kameyama K, Doherty C, Ptak JB, Riley CT et al. Characterization of protein kinase A and protein kinase C phosphorylation of the N-methyl-D-aspartate receptor NR1 subunit using phosphorylation site-specific antibodies. J Biol Chem 1997; 272(8):5157-5166.

(322) Han Y, Qin J, Chang X, Yang Z, Bu D, Du J. Modulating effect of hydrogen sulfide on gamma-aminobutyric acid B receptor in recurrent febrile seizures in rats. Neurosci Res 2005; 53(2):216-219.

(323) Wu G, Fang YZ, Yang S, Lupton JR, Turner ND. Glutathione metabolism and its implications for health. J Nutr 2004; 134(3):489-492.

(324) Whiteman M, Cheung NS, Zhu YZ, Chu SH, Siau JL, Wong BS et al. Hydrogen sulphide: a novel inhibitor of hypochlorous acid-mediated oxidative damage in the brain? Biochem Biophys Res Commun 2005; 326(4):794-798.

(325) Whiteman M, Armstrong JS, Chu SH, Jia-Ling S, Wong BS, Cheung NS et al. The novel neuromodulator hydrogen sulfide: an endogenous peroxynitrite 'scavenger'? J Neurochem 2004; 90(3):765-768.

(326) Geng B, Chang L, Pan C, Qi Y, Zhao J, Pang Y et al. Endogenous hydrogen sulfide regulation of myocardial injury induced by isoproterenol. Biochem Biophys Res Commun 2004; 318(3):756-763.

(327) Bayir H, Kagan VE, Tyurina YY, Tyurin V, Ruppel RA, Adelson PD et al. Assessment of antioxidant reserves and oxidative stress in cerebrospinal fluid after severe traumatic brain injury in infants and children. Pediatr Res 2002; 51(5):571-578.

(328) Halliwell B. Role of free radicals in the neurodegenerative diseases: therapeutic implications for antioxidant treatment. Drugs Aging 2001; 18(9):685-716.

(329) Rice ME. Ascorbate regulation and its neuroprotective role in the brain. Trends Neurosci 2000; 23(5):209-216.

(330) Kimura Y, Kimura H. Hydrogen sulfide protects neurons from oxidative stress. FASEB J 2004; 18(10):1165-1167.

(331) Kimura Y, Kimura H. Hydrogen sulfide protects neurons from oxidative stress. FASEB J 2004; 18(10):1165-1167.

(332) Kimura Y, Dargusch R, Schubert D, Kimura H. Hydrogen sulfide protects HT22 neuronal cells from oxidative stress. Antioxid Redox Signal 2006; 8(3-4):661-670.

(333) Koehler RC, Gebremedhin D, Harder DR. Role of astrocytes in cerebrovascular regulation. J Appl Physiol 2006; 100(1):307-317.

(334) Dani JW, Chernjavsky A, Smith SJ. Neuronal activity triggers calcium waves in hippocampal astrocyte networks. Neuron 1992; 8(3):429-440.

(335) Braet K, Cabooter L, Paemeleire K, Leybaert L. Calcium signal communication in the central nervous system. Biol Cell 2004; 96(1):79-91.

(336) Nagai Y, Tsugane M, Oka J, Kimura H. Hydrogen sulfide induces calcium waves in astrocytes. FASEB J 2004; 18(3):557-559.

(337) Farber K, Kettenmann H. Physiology of microglial cells. Brain Res Brain Res Rev 2005; 48(2):133-143.

(338) Wojtera M, Sikorska B, Sobow T, Liberski PP. Microglial cells in neurodegenerative disorders. Folia Neuropathol 2005; 43(4):311-321.

(339) Kim YS, Joh TH. Microglia, major player in the brain inflammation: their roles in the pathogenesis of Parkinson's disease. Exp Mol Med 2006; 38(4):333-347.

(340) Lee SW, Hu YS, Hu LF, Lu Q, Dawe GS, Moore PK et al. Hydrogen sulphide regulates calcium homeostasis in microglial cells. Glia 2006; 54(2):116-124.

(341) Hoffmann A, Kann O, Ohlemeyer C, Hanisch UK, Kettenmann H. Elevation of basal intracellular calcium as a central element in the activation of brain macrophages (microglia): suppression of receptor-evoked calcium signaling and control of release function. J Neurosci 2003; 23(11):4410-4419.

(342) Raps SP, Lai JC, Hertz L, Cooper AJ. Glutathione is present in high concentrations in cultured astrocytes but not in cultured neurons. Brain Res 1989; 493(2):398-401.

(343) Slivka A, Mytilineou C, Cohen G. Histochemical evaluation of glutathione in brain. Brain Res 1987; 409(2):275-284.

(344) Rice ME, Russo-Menna I. Differential compartmentalization of brain ascorbate and glutathione between neurons and glia. Neuroscience 1998; 82(4):1213-1223.

(345) Eto K, Asada T, Arima K, Makifuchi T, Kimura H. Brain hydrogen sulfide is severely decreased in Alzheimer's disease. Biochem Biophys Res Commun 2002; 293(5):1485-1488.

(346) Kamoun P, Belardinelli MC, Chabli A, Lallouchi K, Chadefaux-Vekemans B. Endogenous hydrogen sulfide overproduction in Down syndrome. Am J Med Genet A 2003; 116(3):310-311.

(347) Davis SM, Lees KR, Albers GW, Diener HC, Markabi S, Karlsson G et al. Selfotel in acute ischemic stroke : possible neurotoxic effects of an NMDA antagonist. Stroke 2000; 31(2):347-354.

(348) Andersson A, Hutlberg B, Lindgren A. Redox status of plasma homocysteine and other plasma thiols in stroke patients. Atherosclerosis 2000; 151(2):535-539.

(349) Olney JW, Ho OL, Rhee V. Cytotoxic effects of acidic and sulphur containing amino acids on the infant mouse central nervous system. Exp Brain Res 1971; 14(1):61-76.

(350) Barks JD, Silverstein FS. Excitatory amino acids contribute to the pathogenesis of perinatal hypoxic-ischemic brain injury. Brain Pathol 1992; 2(3):235-243.

(351) Schurr A, West CA, Heine MF, Rigor BM. The neurotoxicity of sulfur-containing amino acids in energy-deprived rat hippocampal slices. Brain Res 1993; 601(1-2):317-320.

(352) Slivka A, Cohen G. Brain ischemia markedly elevates levels of the neurotoxic amino acid, cysteine. Brain Res 1993; 608(1):33-37.

(353) Mathisen GA, Fonnum F, Paulsen RE. Contributing mechanisms for cysteine excitotoxicity in cultured cerebellar granule cells. Neurochem Res 1996; 21(3):293-298.

(354) Dombkowski RA, Russell MJ, Olson KR. Hydrogen sulfide as an endogenous regulator of vascular smooth muscle tone in trout. Am J Physiol Regul Integr Comp Physiol 2004; 286(4):R678-R685.

(355) Fournier A, Achard JM, Boutitie F, Mazouz H, Mansour J, Oprisiu R et al. Is the angiotensin II Type 2 receptor cerebroprotective? Curr Hypertens Rep 2004; 6(3):182-189.

(356) Robert K, Vialard F, Thiery E, Toyama K, Sinet PM, Janel N et al. Expression of the cystathionine beta synthase (CBS) gene during mouse development and immunolocalization in adult brain. J Histochem Cytochem 2003; 51(3):363-371.

(357) Du F, Schwarcz R. Aminooxyacetic acid causes selective neuronal loss in layer III of the rat medial entorhinal cortex. Neurosci Lett 1992; 147(2):185-188.

(358) Beal MF, Swartz KJ, Hyman BT, Storey E, Finn SF, Koroshetz W. Aminooxyacetic acid results in excitotoxin lesions by a novel indirect mechanism. J Neurochem 1991; 57(3):1068-1073.

(359) Bredt DS, Glatt CE, Hwang PM, Fotuhi M, Dawson TM, Snyder SH. Nitric oxide synthase protein and mRNA are discretely localized in neuronal populations of the mammalian CNS together with NADPH diaphorase. Neuron 1991; 7(4):615-624.

(360) Brenman JE, Chao DS, Gee SH, McGee AW, Craven SE, Santillano DR et al. Interaction of nitric oxide synthase with the postsynaptic density protein PSD-95 and alpha1-syntrophin mediated by PDZ domains. Cell 1996; 84(5):757-767.

(361) Choi YB, Tenneti L, Le DA, Ortiz J, Bai G, Chen HS et al. Molecular basis of NMDA receptor-coupled ion channel modulation by S-nitrosylation. Nat Neurosci 2000; 3(1):15-21.