

Memantine Protects From Exacerbation of Ischemic Stroke and Blood Brain Barrier Disruption in Mild But Not Severe Hyperhomocysteinemia

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Background—Hyperhomocysteinemia is a risk factor for ischemic stroke; however, a targeted treatment strategy is lacking partly because of limited understanding of the causal role of homocysteine in cerebrovascular pathogenesis.

Methods and Results—In a genetic model of cystathionine beta synthase (CBS) deficiency, we tested the hypothesis that elevation in plasma total homocysteine exacerbates cerebrovascular injury and that memantine, a N-methyl-D-aspartate receptor antagonist, is protective. Mild or severe elevation in plasma total homocysteine was observed in *Cbs*+/- (6.1±0.3 μmol/L) or *Cbs*-/- (309±18 μmol/L) mice versus *Cbs*+/+ (3.1±0.6 μmol/L) mice. Surprisingly, *Cbs*-/- and *Cbs*+/- mice exhibited similar increases in cerebral infarct size following middle cerebral artery ischemia/reperfusion injury, despite the much higher total homocysteine levels in *Cbs*-/- mice. Likewise, disruption of the blood brain barrier was observed in both *Cbs*+/- and *Cbs*-/- mice. Administration of the N-methyl-D-aspartate receptor antagonist memantine protected *Cbs*+/- but not *Cbs*-/- mice from cerebral infarction and blood brain barrier disruption. Our data suggest that the differential effect of memantine in *Cbs*+/- versus *Cbs*-/- mice may be related to changes in expression of N-methyl-D-aspartate receptor subunits. *Cbs*-/-, but not *Cbs*+/- mice had increased expression of NR2B subunit, which is known to be relatively insensitive to homocysteine.

Conclusions—These data provide experimental evidence that even a mild increase in plasma total homocysteine can exacerbate cerebrovascular injury and suggest that N-methyl-D-aspartate receptor antagonism may represent a strategy to prevent reperfusion injury after acute ischemic stroke in patients with mild hyperhomocysteinemia. (*J Am Heart Assoc.*2020;9:e013368. DOI: 10.1161/JAHA.119.013368.)

Key Words: blood brain barrier • cystathionine beta synthase • homocysteine • stroke

Elevated plasma total homocysteine (tHcy) is an established independent risk factor for adverse vascular events, including myocardial infarction and stroke.^{1–3} The importance of tHcy as a mediator of vascular events remains controversial, however. Early interventional trials largely failed

to show a beneficial effect of homocysteine lowering therapy in reducing the risk of secondary cardiovascular events in patients with mild-to-moderate hyperhomocysteinemia,^{4–6} but subsequent prospective studies demonstrated that homocysteine lowering with vitamin therapy decreased the risk of stroke in both primary and secondary prevention settings.^{6–11} The landmark China Stroke Primary Prevention Trial reported that lowering of homocysteine with folate supplementation decreased the incidence of first stroke among adults with hypertension.¹² These randomized prospective results support the notion that homocysteine is a causal factor in patients with ischemic stroke.^{13,14} Nevertheless, the mechanistic role of homocysteine in stroke pathogenesis remains unclear and a targeted treatment strategy beyond B vitamin therapy is still lacking.

Acute ischemic stroke induces a cascade of events resulting in neuronal cell death and loss of neurologic function in focal areas of the brain. The pathophysiology of stroke is complex, involving multiple processes including excitotoxicity.¹⁵ The N-methyl-D-aspartate (NMDA) receptor is

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Clinical Perspective

What Is New?

- We report that a mild elevation in homocysteine is sufficient to produce a severe stroke phenotype in genetic murine models demonstrating a direct causal role of elevated homocysteine.
- Furthermore, we also demonstrate a novel protective role of N-methyl-D-aspartate receptor antagonism in stroke and blood brain barrier disruption in the context of hyperhomocysteinemia.
- Memantine is licensed and approved by the Food and Drug Administration for treatment of moderate-to-severe Alzheimer disease permitting the blockade of excessive N-methyl-D-aspartate receptor activity without disrupting normal synaptic transmission.

What Are the Clinical Implications?

- Findings of the current study provide a potential therapeutic strategy in preventing stroke in patients with mild-to-moderate hyperhomocysteinemia.
- Given that mild-to-moderate hyperhomocysteinemia is highly prevalent in patients with cerebrovascular disease in many parts of the world, potential clinical impact could be considerable.

an ionotropic glutamate receptor that mediates neuronal transmission at most excitatory synapses. It is well accepted that excessive activation of NMDA receptors mediates neuronal injury following ischemia.^{16,17} Homocysteine is known to directly bind and activate NMDA receptors, leading to excitotoxicity and neuronal cell death.¹⁷ NMDA receptor antagonism has thus far failed to show a beneficial effect in the secondary prevention of stroke in clinical trials,^{18,19} but 1 recent study found that NMDA receptor inhibition improved stroke outcome in a pharmacological model of hyperhomocysteinemia in rats.²⁰

Apart from the excitotoxic effects of homocysteine on neurons, studies from our group and others have also demonstrated deleterious effects of homocysteine on the cerebral vasculature,^{21–23} including blood brain barrier (BBB) disruption.^{24,25} Under physiological conditions, the BBB formed by the endothelial layer of cerebral vessels serves to limit influx of potentially neurotoxic compounds between the circulating blood and the central nervous system.²⁶ Mild increases in tHcy can directly activate NMDA receptors on cerebrovascular endothelial cells leading to increased permeability of the BBB.²⁴ However, it is not known if severe elevation in tHcy exacerbates BBB disruption or whether NMDA antagonism is protective. Using a genetic model of hyperhomocysteinemia (mice deficient in cystathionine β

synthase [CBS]), we tested the hypothesis that hyperhomocysteinemia worsens stroke injury and BBB disruption in a dose dependent manner and that inhibition of the NMDA receptor is protective. Our findings demonstrate that either mild or severe elevation in plasma tHcy leads to increased BBB disruption and a severe experimental stroke phenotype and that antagonism of NMDA receptor protects mice with mild but not severe hyperhomocysteinemia.

Methods

The data that support the findings are available from the corresponding author upon reasonable request.

Mice

All animal use was carried out in accordance with the recommendations in the *Guide for the Care and Use of Laboratory Animals of the National Institutes of Health*. The animal protocol was approved by the Institutional Animal Care and Use Committee of the University of Iowa. Mice homozygous deficient for murine *Cbs* but expressing a zinc-inducible mutant human *CBS* transgene (Tg-*I278T Cbs*-/-)²⁷ were initially bred with mice heterozygous deficient in murine *Cbs* (*Cbs*+/-) to obtain Tg-*I278T Cbs*+/- mice. Next, these mice were crossed to generate Tg-*I278T Cbs*+/, Tg-*I278T Cbs*+/- and Tg-*I278T Cbs*-/- littermates for study. These mice are referred as *Cbs*+/, *Cbs*+/- and *Cbs*-/- throughout the manuscript.

Plasma tHcy

Blood was collected from mice anesthetized with sodium pentobarbital (75–90 mg/kg IV) by cardiac puncture into EDTA (final concentration 5 mmol/L), and plasma was collected after centrifugation. Plasma tHcy, the total concentration of homocysteine after quantitative reductive cleavage of all disulfide bonds, was measured by high-performance liquid chromatography and ammonium 7-fluorobenzo-2-oxa-1,3-diazole-4-sulphonate (SBDF) fluorescence detection.²⁸

Memantine Treatment

Mice were randomly assigned to treatment with or without 30 mg/kg per day memantine (100 mg in 330 mL) in the drinking water for 14 days before the study. This dosing regimen has previously been shown to result in serum memantine concentrations of ≈ 1 $\mu\text{mol/L}$ in C57Bl/6J mice, comparable with therapeutic concentrations in humans.²⁹ To ensure adequate water intake across the groups, mice were monitored daily for water intake and signs of dehydration and weight loss.

Transient Middle Cerebral Artery Occlusion

Focal cerebral ischemia was induced by transiently occluding the right middle cerebral artery based on modification of previous protocol.³⁰ Male mice weighing 22 to 25 g were anesthetized with isoflurane and kept on a Plexiglas platform over a heating pad throughout the procedure. An incision was made in the external carotid artery and a 0.22-mm diameter silicon-covered 6-0 nylon monofilament (Doccol) was advanced 9 to 10 mm through the internal carotid artery to the proximal middle cerebral artery. Middle cerebral artery occlusion (MCAO) was documented by a decrease in laser Doppler signal to <20% of baseline values, after which the monofilament was secured in place. Following 60 minutes of ischemia, the monofilament was then removed to allow for reperfusion. After 24 hours of reperfusion, mice were evaluated for neurological deficits using a motor deficit scale and euthanized for brain histology.

Neurological Scoring

Twenty-four hours after transient MCAO, mice were evaluated in a masked manner for motor deficits using a 5-point scale: 0, no observable neurological deficit (normal); 1, failure to extend contralateral forepaw when picked up by tail (mild); 2, mild circling to the contralateral side but normal posture at rest (moderate); 3, consistent strong and immediate circling, falling to the contralateral side at rest (moderate-severe); 4, severe postural rotation at rest progressing into barreling, loss of righting reflex (severe); 5, comatose or moribund.

Measurement of Infarct Volume

Twenty-four hours after MCAO, cerebral infarct size was determined by 2,3,5 triphenyltetrazolium chloride staining. Brains were cut from the frontal pole into 1-mm-thick serial coronal sections using a mouse Brain Matrix (Roboz surgical instrument). Sections were stained with 1% triphenyltetrazolium chloride at 37°C for 15 minutes at 37°C then fixed in 10% neutral buffered formalin. Sections were scanned and infarct area was determined by a person blinded to the study and analysis was performed using National Institutes of Health Image J software. To correct for brain swelling because of edema after ischemia the corrected total infarct volume (%) was calculated as described: Corrected infarct volume (%)=[volume of contralateral hemisphere−(volume of ipsilateral hemisphere −volume of infarct)]/volume of contralateral hemisphere×100.³¹

BBB Permeability

BBB integrity was assessed by an Evans Blue (EB) perfusion method. EB solution (2% w/v) was injected (5 mL/kg) by cannulation of the common carotid artery. After 2 hours mice

were perfused with PBS and brain tissue was harvested and homogenized in 50% trichloroacetic acid (w/v) in PBS. Samples were centrifuged at 10 600g and absorbance was measured at 610 nm with a spectrophotometer. EB concentration was determined using a standard curve and normalized to wet brain weight.

Primary Neuron Cultures

Primary cortical neuronal cells were isolated according to previous protocol.³² In brief, neurons were isolated from early postnatal day 0 mice and plated in 96-well culture plates. After 3 days in culture, 5 μmol/L 5-Fluoro-2'-deoxyuridine (Sigma) was added for 24 hours to prevent glial proliferation. The neurons were subsequently maintained with serum-free NeuroBasal medium (Gibco) with B27 supplements and studied at 11 to 14 days in vitro.

Cell Death Assays

In vitro cell death was analyzed using the lactate dehydrogenase (LDH) Cytotoxicity Assay Kit (Thermo). In brief, cells grown in 96-well plates were treated with varying doses of homocysteine as indicated and incubated at 37°C for 24 hours in humidified atmosphere containing 95:5% of air/CO₂ mixture. For hypoxia-reoxygenation experiments, cultures were incubated at 37°C for 2 hours in an anaerobic chamber containing 95:5% of N₂/CO₂ mixture followed by 22 hours in humidified atmosphere containing 95:5% of air/CO₂ mixture. Memantine was added 1 hour before homocysteine treatment. LDH released into the supernatant was analyzed by measuring absorbance at 490 and 680 nm using a spectrophotometer per the manufacturer's instructions. Percent LDH release was calculated: (Compound-treated LDH activity−Spontaneous LDH activity)/(Maximum LDH activity−Spontaneous LDH activity)×100.

Real-Time Polymerase Chain Reaction

Total RNA was isolated from brain cortex using Trizol (Ambion) and reverse transcribed to obtain cDNA. The primers used for real-time polymerase chain reaction were as follows: NR1 sense: GCTGTACCTGCTGGACCGCT; NR1 antisense: GCAGTGTAGGAAGCCACGATGATC; NR2A sense: CTGACAAGGATCCGACATCCACG; NR2A antisense: CTCATCCTTGGAGCAGTAGAGC; NR2B sense: GCTACAACACCCACGAGAAGAG; NR2B antisense: CAGTCTCAGGACACATTCGAGG. Real-time polymerase chain reaction reactions were run using a SYBR green quantitative PCR kit (Invitrogen) and primers on an Applied Biosystems 7900HT Fast Real-Time PCR machine. Cycling conditions were 50°C for 2 minutes, 95°C for 10 minutes, and 30 cycles of 95°C for 15 seconds and 60°C for 1 minute. Data were analyzed using the comparative

threshold cycle ($\Delta\Delta\text{CT}$) method with values normalized to 18s rRNA and expressed relative to levels in *Cbs*^{+/+} mice.

Immunoblotting

Cells or tissues were lysed in RIPA buffer (25 mmol/L Tris pH 7.4, 150 mmol/L NaCl, 0.1% Triton X-100) containing 0.1% SDS and protease inhibitors (Roche). Total protein concentration was determined by Bradford assay (Bio-Rad). Lysates were treated with sample loading buffer and ≈ 50 μg were loaded onto a 10% SDS-polyacrylamide gel. After electrophoresis, gels were transferred to a nitrocellulose membrane. Membranes were blocked for 1 hour in blocking buffer (5% non-fat dry milk, 100 mmol/L Tris-HCl pH 7.4, 0.1% Tween-20). Membranes were incubated with the following primary antibodies overnight at 4°C: anti-NMDAR2A (1:1000, Abcam ab124913), anti-NMDAR2B (1:500, Abcam ab28373), and anti- β -Actin (1:5000, Abcam ab8227). Membranes were then incubated with respective secondary antibodies conjugated to horseradish peroxidase (Thermo Scientific). Antibody-bound protein bands are then visualized by horseradish peroxidase-dependent chemiluminescence Femto kit (Thermo Scientific). Quantification was performed by densitometry using National Institutes of Health Image J.

Statistical Analysis

Statistical analysis was performed using GraphPad Prism (Version 8 for Windows, GraphPad Software, La Jolla, California, USA). Normality was evaluated by the D'Agostino-Pearson test. Parametric data were analyzed by use of 1-way ANOVA or 2-way ANOVA with Tukey test for multiple comparisons. Kruskal-Wallis ANOVA by Ranks was used to analyze non-parametric data measured on an ordinal (rank order) scale that is based on medians rather than means (eg, neurologic score data).³³ Data are expressed as mean \pm SEM unless otherwise stated. Statistical significance was defined as $P < 0.05$.

Results

Mice With Mild or Severe Hyperhomocysteinemia Exhibit Increased Susceptibility to Cerebral Ischemia-Reperfusion Injury

We studied a cohort of *Cbs*^{+/+}, *Cbs*^{+/-} and *Cbs*^{-/-} mice containing a conditional zinc-inducible mutated human *CBS* (*I278T*) transgene that is necessary to allow the *Cbs*^{-/-} mice to survive beyond the neonatal period.³⁴ We first confirmed that plasma tHcy was mildly or severely elevated in *Cbs*^{+/-} and *Cbs*^{-/-} mice (6.1 ± 0.3 $\mu\text{mol/L}$ and 309 ± 18 $\mu\text{mol/L}$, respectively) compared with *Cbs*^{+/+} littermate

control mice (3.1 ± 0.6 $\mu\text{mol/L}$, $P < 0.05$ versus *Cbs*^{+/-} and $P < 0.0001$ versus *Cbs*^{-/-} mice, Figure 1A). These results are in agreement with previous findings.³⁴

To determine the pathologic role of hyperhomocysteinemia in ischemic stroke, we experimentally induced stroke in mice using the MCAO model. After 1 hour of ischemia and 24 hours of reperfusion, both *Cbs*^{+/-} and *Cbs*^{-/-} mice had larger cerebral infarct volumes compared with *Cbs*^{+/+} mice ($35.2 \pm 2.9\%$ in *Cbs*^{+/-} mice and $34.6 \pm 4.2\%$ in *Cbs*^{-/-} mice, versus $19.7 \pm 2.5\%$ in *Cbs*^{+/+} mice, $P < 0.01$ and $P < 0.05$, respectively, Figure 1B and 1C). Severe neurological deficits, such as head tilt and barrel rolling, were frequently observed in *Cbs*^{+/-} and *Cbs*^{-/-} mice, whereas neurological deficits were less severe in *Cbs*^{+/+} mice at 24 hours (Figure 1D). Interestingly, there were no significant differences in the degree of cerebral infarction or functional neurological outcomes between *Cbs*^{+/-} and *Cbs*^{-/-} mice following MCAO, suggesting a threshold effect of hyperhomocysteinemia.

Blood Brain-Barrier Disruption is Observed in Mouse Models of Mild or Severe Hyperhomocysteinemia

The integrity of the BBB is critical in limiting paracellular permeability, and a breakdown in the BBB can contribute to stroke pathology.²⁶ BBB integrity was assessed in using an EB dye permeation method. We found increased extravasation of EB in *Cbs*^{+/-} (3.4 ± 0.6 $\mu\text{g/g}$) and *Cbs*^{-/-} mice (4.8 ± 0.2 $\mu\text{g/g}$) compared with *Cbs*^{+/+} mice (1.6 ± 0.2 $\mu\text{g/g}$, $P < 0.05$ and 0.01 , respectively) (Figure 2).

Homocysteine-Induced Cell Death in Primary Cortical Neurons is Protected by Memantine

Previous work has suggested that homocysteine induces excitotoxicity in neurons by activating the NMDA receptor.¹⁷ Using cultured primary cortical neurons isolated from early postnatal mice, we assessed the dose-dependent effects of homocysteine on neuronal cell death using an LDH release assay. Cultures were treated under normoxic conditions or transient hypoxia for 2 hours followed by reoxygenation for 22 hours to recapitulate in vivo ischemia-reperfusion injury during stroke. We found that homocysteine induced significant cytotoxicity at concentrations of ≈ 100 $\mu\text{mol/L}$ or greater under normoxic conditions (Figure 3A) and ≈ 300 $\mu\text{mol/L}$ or greater under conditions of hypoxia reoxygenation (Figure 3C). Addition of glycine (10 $\mu\text{mol/L}$), an NMDA receptor co-agonist known to be released during cerebral ischemia,^{35,36} significantly lowered the half maximal effective concentration (EC_{50}) of homocysteine. Co-treatment with 1 $\mu\text{mol/L}$ memantine, an uncompetitive NMDA receptor antagonist, decreased the

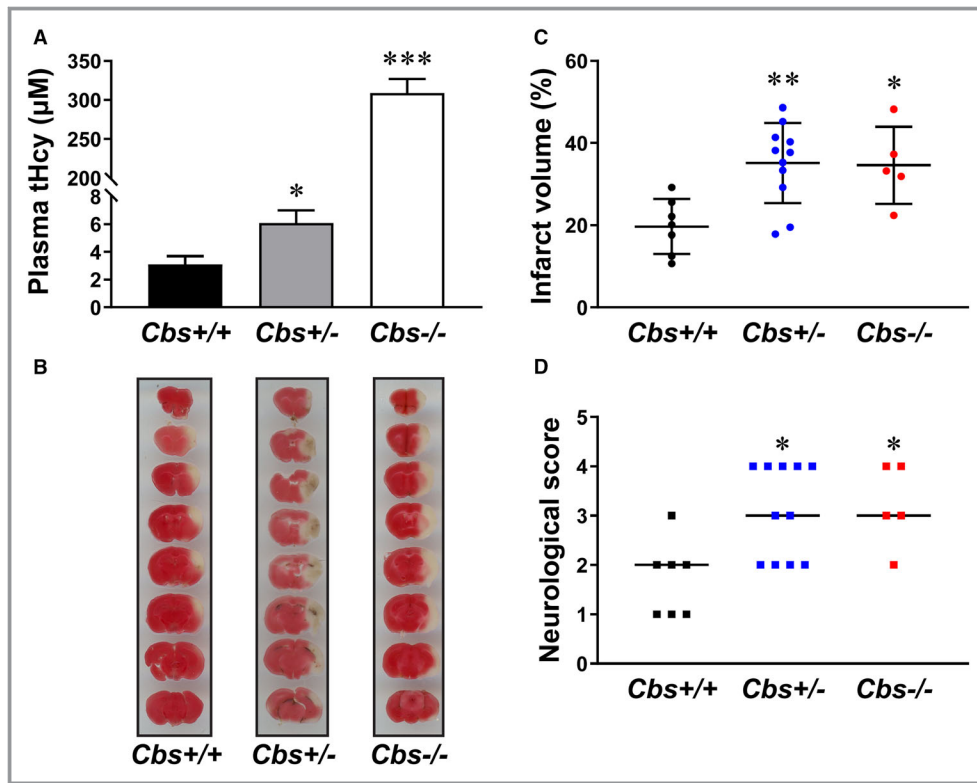


Figure 1. Murine models of mild and severe hyperhomocysteinemia because of CBS (cystathionine beta synthase) deficiency exhibit increased stroke susceptibility. **A**, Plasma total homocysteine. **B**, Representative images of 2,3,5-triphenyltetrazolium chloride staining of serial coronal sections 24 hours after experimental stroke: viable tissue was stained red, whereas the infarcted area remained unstained (white). **C**, Corrected mean infarct volume of the ipsilateral hemisphere. **D**, Neurological score expressed as scatter plots with horizontal line depicting the median. Data for **(A and C)** are expressed as mean±SEM. n=5 to 11. tHcy indicates total homocysteine. **P*<0.05, ***P*<0.01, and ****P*<0.0001 vs *Cbs*^{+/+} mice: 1-way ANOVA for **(A and C)**, Kruskal–Wallis test for **(D)**.

sensitivity of the cells to homocysteine, but did not eliminate it entirely (*P*<0.05, Figure 3B and 3D). These findings suggest

that the sensitivity of primary cortical neurons to homocysteine is mediated at least in part by the NMDA receptor.

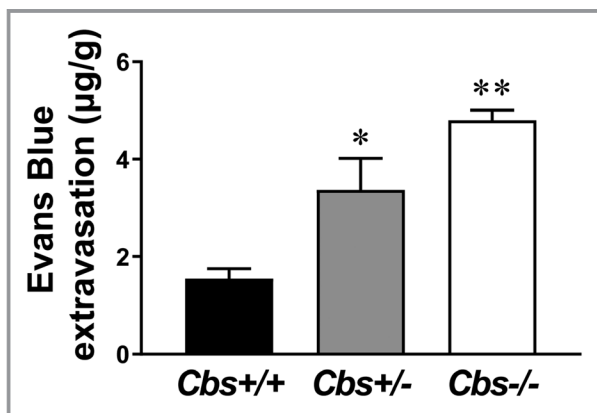


Figure 2. Increased blood brain barrier disruption in hyperhomocysteinemic mice. Evans blue extravasation was quantified in the brain parenchyma before experimental stroke. Data are normalized to total brain weight and expressed as mean±SEM (n=4–11). **P*<0.05, ***P*<0.01 vs *Cbs*^{+/+} mice, 1-way ANOVA. *Cbs*, cystathionine beta synthase.

Inhibition of the NMDA Receptor Protects *Cbs*^{+/-} But Not *Cbs*^{-/-} Mice From Blood-Brain Barrier Disruption and Experimental Stroke

To determine if the neurovascular phenotypes of hyperhomocysteinemic mice are mediated through the NMDA receptor, we administered memantine continuously in the drinking water for 2 weeks before MCAO or BBB integrity assessment. None of the mice showed signs of dehydration or >10% loss in body weight over 2 weeks of treatment. Although the severity of cerebral infarction was similar in *Cbs*^{+/-} and *Cbs*^{-/-} mice in the absence of memantine treatment, we observed that administration of memantine rescued the *Cbs*^{+/-} mice from cerebral ischemia-reperfusion injury but not *Cbs*^{-/-} mice (Figure 4A through 4C). Similarly, memantine protected *Cbs*^{+/-} but not *Cbs*^{-/-} mice from BBB disruption (Figure 5).

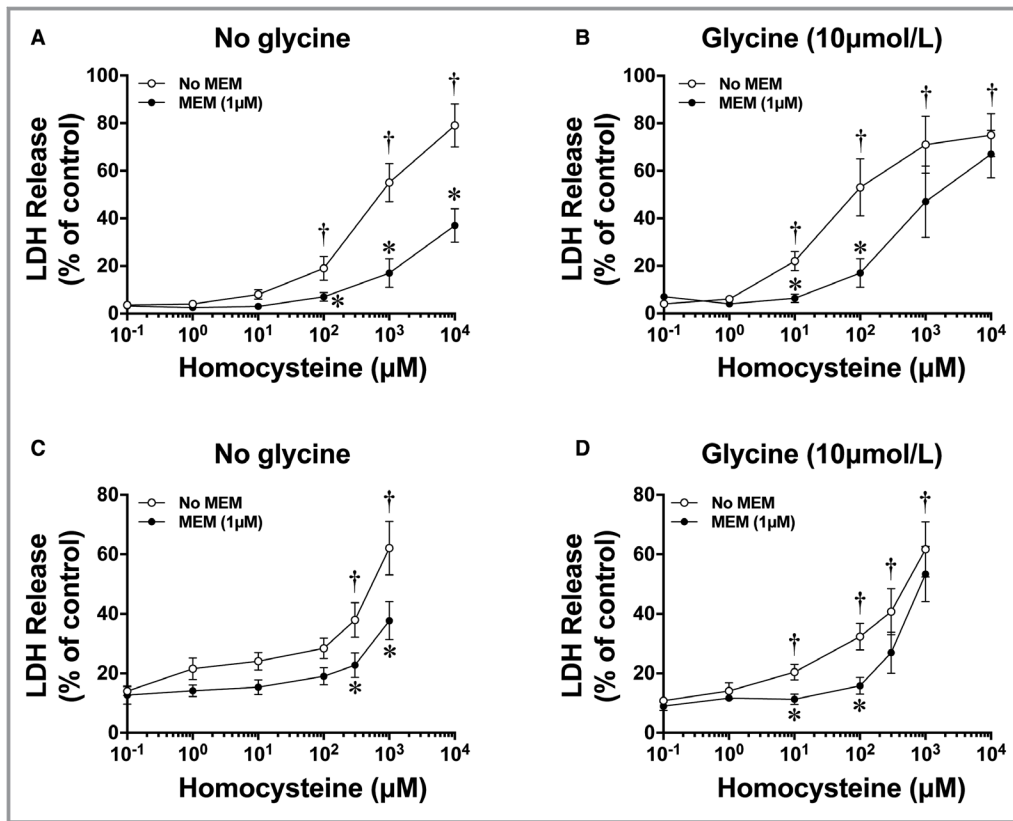


Figure 3. Memantine protects primary cortical neurons in culture from homocysteine-induced cell death. Primary murine cortical neurons isolated from early postnatal P₀ mice were grown for 11 to 14 days in culture. Cells were treated with homocysteine, memantine, and glycine as indicated (**A** and **B**) under normoxic conditions for 24 hours or (**C** and **D**) under conditions of transient hypoxia for 2 hours followed by 24 hours reoxygenation. Results are expressed as mean±SEM (n=4). **P*<0.05 vs no memantine by 2-way ANOVA and †*P*<0.05 vs 0 μmol/L homocysteine by 1-way ANOVA. LDH, lactate dehydrogenase.

NMDA Receptor Subtypes are Differentially Modulated Under Mild or Severe Hyperhomocysteinemia

Since protective effects of memantine were observed in *Cbs*^{+/-} mice with mild hyperhomocysteinemia but not in *Cbs*^{-/-} mice with severe hyperhomocysteinemia, we determined if the degree of hyperhomocysteinemia affected expression of NMDA receptor subtypes. NMDA receptor subunits NR1, NR2A, and NR2B are the predominant isoforms expressed in the mammalian forebrain and have been implicated in stroke.³⁷ We therefore measured mRNA and protein levels for these subunits in the cerebral cortex. No significant differences were observed in the mRNA expression of the NR1 subunit between the 3 groups (Figure 6A). The mRNA levels for NR2A was decreased by ≈35% in *Cbs*^{-/-} compared with *Cbs*^{+/+} mice (*P*<0.05, Figure 6B); however, NR2A protein expression was not altered between groups (Figure 6F). NR2B mRNA and protein levels were increased in *Cbs*^{-/-} compared with *Cbs*^{+/+} mice (*P*<0.05, Figure 6C

and 6G respectively). No significant differences in NR2A or NR2B expression were observed between *Cbs*^{+/+} and *Cbs*^{+/-} mice. Notably, the ratio of NR2A to NR2B mRNA (Figure 6D) and protein (Figure 6H) was significantly decreased in *Cbs*^{-/-} mice compared with *Cbs*^{+/+} mice.

Discussion

It is well recognized that hyperhomocysteinemia is an independent risk factor for adverse vascular events, especially ischemic stroke.¹ Plasma homocysteine levels are also directly associated with higher mortality rates in stroke patients,³⁸ but the mechanisms linking elevated homocysteine to stroke pathology are still poorly understood. Using genetic approaches, we examined the role of homocysteine in the pathogenesis of ischemic stroke in mice with variable degrees of hyperhomocysteinemia. We made several key observations: (1) mild or severe elevations in homocysteine produce similar degrees of cerebral injury in an experimental stroke model of focal ischemia-reperfusion, (2) mild-to-severe

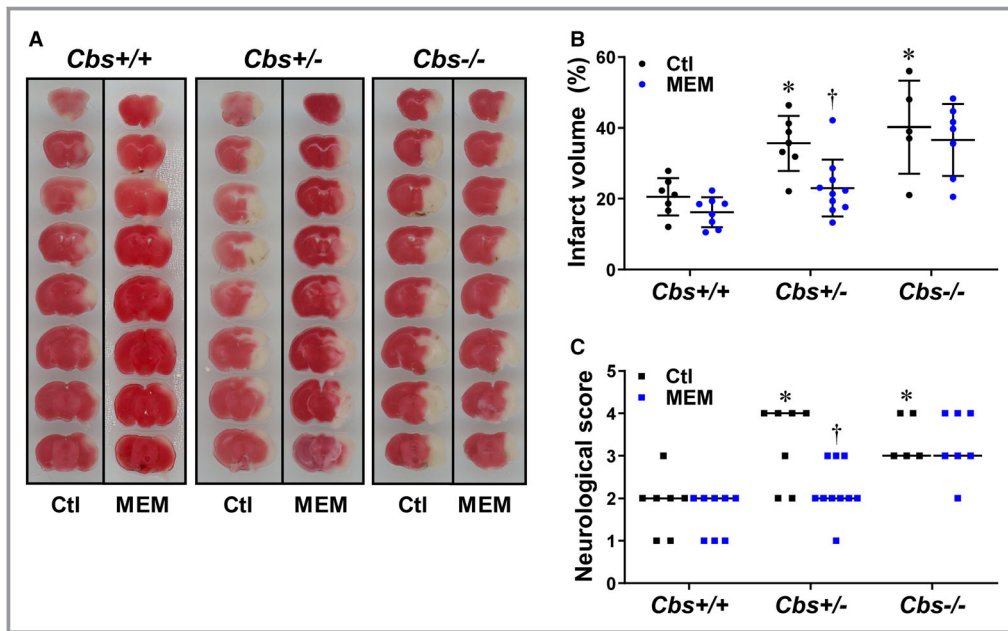


Figure 4. Memantine reverses stroke phenotype in *Cbs+/-* but not in *Cbs-/-* mice. Mice were treated with memantine or no memantine (control) in the drinking water for 2 weeks before experimental stroke. **A**, Representative images of 2,3,5-triphenyltetrazolium chloride staining of serial coronal sections 24 hours after experimental stroke. Viable tissue was stained red, whereas the infarcted area remained unstained (white). **B**, Corrected mean infarct volumes. Data are expressed as mean±SEM (n=5–11 mice/group). **P*<0.05 vs *Cbs+/+* Ctl, †*P*<0.05 vs *Cbs+/-* Ctl, 2-way ANOVA. **C**, Neurological score of each group expressed as scatter plots with horizontal line depicting the median. **P*<0.05 vs *Cbs+/+* Ctl, †*P*<0.05 vs *Cbs+/-* Ctl, 2-way ANOVA for (B), ANOVA on ranks for (C). *Cbs*, cystathionine beta synthase.

elevation in homocysteine disrupts BBB integrity, (3) selective inhibition of the NMDA receptor protects *Cbs+/-* but not *Cbs-/-* mice from experimental stroke and BBB disruption,

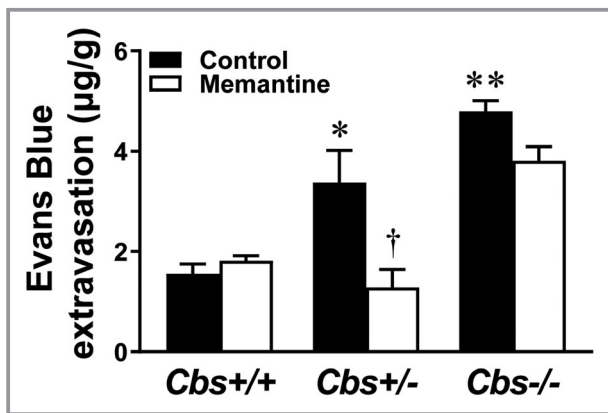


Figure 5. Memantine reverses blood brain barrier disruption in *Cbs+/-* but not in *Cbs-/-* mice. Mice were treated with memantine in the drinking water or control for 2 weeks before the study. Blood brain barrier integrity was assessed before middle cerebral artery occlusion. Evans blue extravasation was quantified in the brain parenchyma before experimental stroke. Data are normalized to total brain weight and expressed as mean±SEM (n=4–11). **P*<0.05, and ***P*<0.01 vs *Cbs+/+* control mice, and †*P*<0.05 vs *Cbs+/-* control by 2-way ANOVA. *Cbs*, cystathionine beta synthase.

and the lack of response to inhibitor in *Cbs-/-* mice may be related to the altered expression of NMDA receptor subunits. These findings provide evidence that homocysteine contributes to the severity of ischemic stroke and BBB disruption and that NMDA receptor antagonism may represent a targeted therapy in patients with mild hyperhomocysteinemia.

Severe elevation in plasma tHcy (>100 µmol/L) is rare and is primarily caused by mutations in the *CBS* gene and typically manifests as premature atherosclerosis along with various skeletal and developmental abnormalities.³⁹ On the other hand, mild-to-moderate elevations in tHcy (15–50 µmol/L) are relatively common, with a prevalence of ≈5% to 7% in the general population, and are caused most often by chronic kidney disease, nutritional deficiencies in B vitamins, or common polymorphisms in the methyltetrahydrofolate reductase (*MTHFR*) gene.⁴⁰ Although early clinical trials largely failed to show a beneficial effect of homocysteine lowering therapy on cardiovascular outcomes, accumulating clinical evidence suggests a direct causal role of dysregulated homocysteine metabolism in ischemic stroke.^{6,9} One potential explanation for this discrepancy is that the homocysteine-related pathogenesis of myocardial infarction and ischemic stroke are mechanistically distinct. Indeed, using the same murine model as in this study, we demonstrated previously that hyperhomocysteinemia did not increase thrombotic susceptibility in

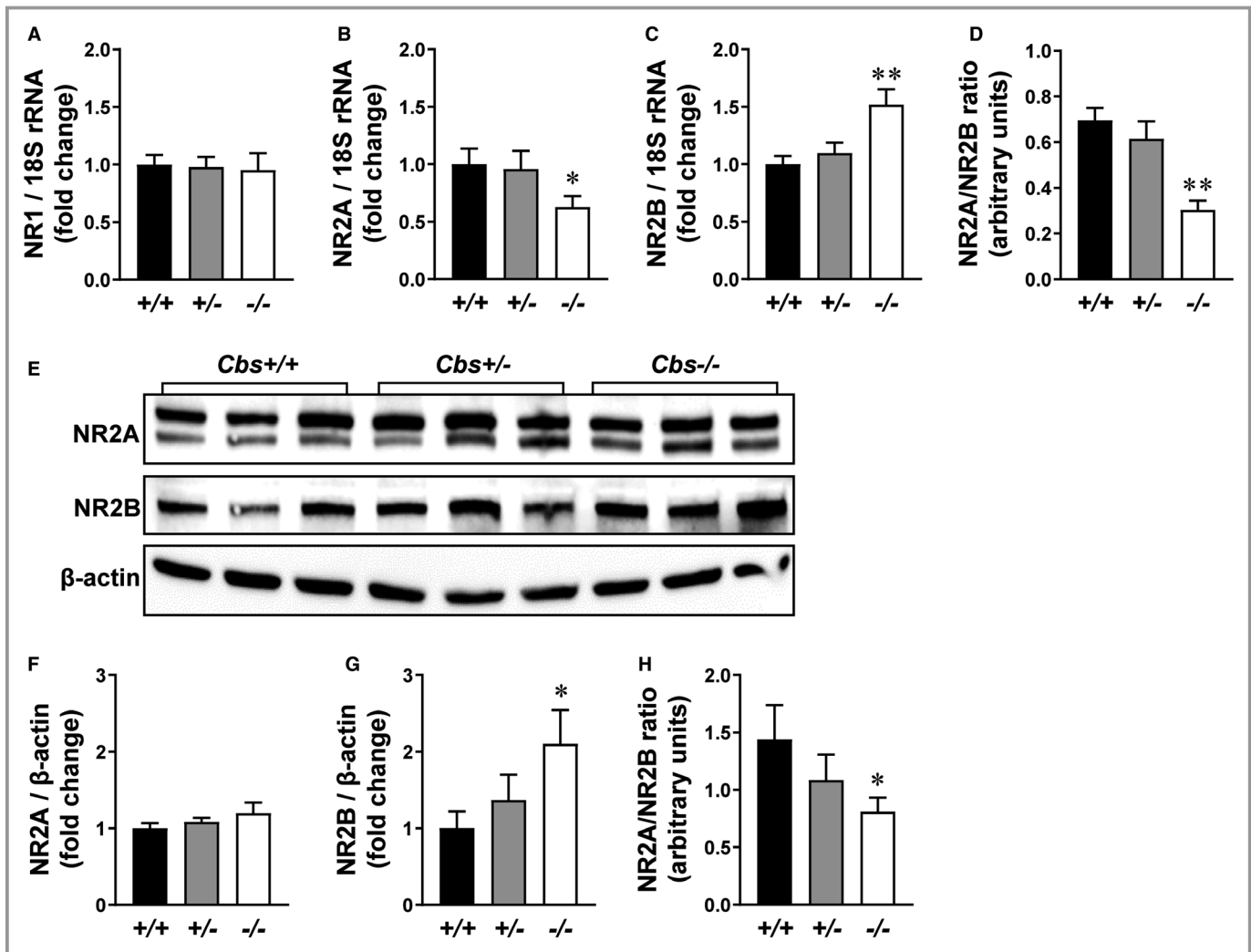


Figure 6. Differential expression of NMDA receptor subunits in hyperhomocysteinemic mice. mRNA levels for (A) NR1, (B) NR2A, and (C) NR2B in cortical tissues measured by real-time polymerase chain reaction and normalized to 18S rRNA. D, Ratio of NR2A/NR2B mRNA levels. Results are expressed as mean±SEM (n=5–7). E, Representative immunoblot of NR2A, NR2B, and β-actin protein in lysates of cortical tissues from *Cbs*+/, *Cbs*+/-, and *Cbs*-/- mice. Protein levels for (F) NR2A (top band) and (G) NR2B quantified by densitometry and normalized to β-actin. H, Ratio of NR2A/NR2B protein levels. Of technical note, 2 bands were detected using anti-NR2A with both bands showing similar levels of expression between all groups (data not shown for the bottom band which may represent a modified protein, degradation product, or non-specific band). Results are expressed as mean±SEM (n=6). **P*<0.05, ***P*<0.01 vs *Cbs*+/+ mice, 1-way ANOVA. *Cbs*, cystathionine beta synthase.

peripheral arteries or veins but did increase the sensitivity of cerebral vessels to vascular dysfunction.³⁴ Increased sensitivity to cerebral vessel vasomotor dysfunction was demonstrated in multiple genetic models of hyperhomocysteinemia.^{21,22,41} These findings imply that homocysteine may preferentially affect cerebrovascular pathophysiology versus other vascular processes outside the central nervous system.

A surprising observation in our study was that, despite the severe elevation in plasma tHcy in *Cbs*-/- mice, the degree of cerebral infarction was similar to that in *Cbs*+/- mice with mild hyperhomocysteinemia. This finding suggests a threshold effect in which even a small increase in homocysteine is

sufficient to produce a severe adverse cerebrovascular phenotype that is not exacerbated with further increases in tHcy levels. Alternatively, it is conceivable that the severe hyperhomocysteinemia in *Cbs*-/- mice is associated with elevation of a protective metabolite that counterbalances the adverse effects of homocysteine in ischemic stroke. Previous studies have suggested that some potentially protective metabolites such as thioethers accumulate in homozygous but not heterozygous CBS deficiency.^{27,42} Finally, the stroke phenotype in *Cbs*+/- and *Cbs*-/- mice may be mediated by effects of CBS deficiency that are independent, or only partially dependent on homocysteine.

It is well accepted that cerebral ischemia releases glutamate, which can stimulate neuronal NMDA receptors, induce calcium influx, and promote calcium-dependent activation of downstream signaling cascades leading to excitotoxic neuronal death. Interestingly, several prior studies have demonstrated that NMDA receptors also can be activated by homocysteine binding to the glutamate agonist site⁴³ to induce calcium-dependent neuronal excitotoxicity.^{17,44,45} Extending upon these findings, we found that in vivo administration of memantine, a specific NMDA receptor antagonist, protected *Cbs+/-* mice with mild hyperhomocysteinemia from cerebral ischemia-reperfusion injury (Figure 4). Neuroprotection by memantine was not observed in *Cbs-/-* mice with severe hyperhomocysteinemia, implying activation of additional pathologic pathways that needs to be explored in future studies.

Previous studies have suggested that glycine enhances the effects of homocysteine-induced NMDA receptor activation.¹⁷ Glycine is an NMDA receptor co-agonist^{35,36,46} that can reach micromolar levels locally in the brain during cerebral ischemia-reperfusion injury.⁴⁷ Our findings in primary cortical neurons demonstrate that in the absence of glycine, homocysteine induced significant neuronal cell death at concentrations of 100 $\mu\text{mol/L}$ or higher under normoxic conditions (Figure 3A) and 300 $\mu\text{mol/L}$ or higher under conditions of hypoxia-reoxygenation (Figure 3C). In the presence of glycine (10 $\mu\text{mol/L}$), however, concentrations of homocysteine as low as 10 $\mu\text{mol/L}$ were sufficient to induce neuronal cell death (Figure 3B and 3D). In concordance with our in vivo observations (Figure 4), protection from cell death by memantine was observed at low (10–100 $\mu\text{mol/L}$) but not higher homocysteine concentrations in the presence of glycine (10 $\mu\text{mol/L}$) (Figure 3B). These findings may explain the differential neuroprotection by memantine between the *Cbs+/-* and *Cbs-/-* mice (Figures 4 and 5).

Alternatively, the differential effects of memantine in *Cbs+/-* and *Cbs-/-* mice may be related to changes in expression of NMDA receptor subunits. In mammals, functional NMDA receptors are multimeric complexes.⁴⁸ The well-characterized neuronal glutamate/glycine responsive NMDA receptor contains 2 NR1 and 2 NR2 subunits. The NR1 subunit is an essential component found ubiquitously in all NMDA receptors whereas NR2 members are differentially incorporated based on developmental stage and synaptic or extrasynaptic location.⁴⁹ NR2A and NR2B are the predominant NR2 subunits in the adult forebrain where ischemic stroke occurs most frequently.^{37,50} We found that *Cbs-/-* mice have increased NR2B subunit expression and a decreased NR2A/NR2B ratio (Figure 6). A recent study using pharmacologic administration of free L-homocysteine via osmotic pumps in rats showed that mild increases in plasma homocysteine (analogous to plasma levels in

Cbs+/- mice) significantly increased cerebral ischemia-reperfusion injury.²⁰ It was further demonstrated that the NR2A and not the NR2B subunit of the NMDA receptor mediated the exacerbation in stroke phenotype in the setting of mild hyperhomocysteinemia. These prior observations are consistent with our findings in *Cbs+/-* mice, which have mild hyperhomocysteinemia and preserved expression of NR2A. In vitro studies have demonstrated that the NR2A and NR2B subunits display differential sensitivity to homocysteine, with a 6-fold higher EC50 for activation of NMDA receptors containing NR2B compared with NR2A.^{51,52} The relative insensitivity of NR2B to homocysteine suggests that the cerebrovascular phenotype in *Cbs-/-* mice is less likely to be mediated by direct effects of homocysteine on the NMDA receptor and might explain why *Cbs-/-* mice expressing predominantly NR2B with a decreased NR2A/NR2B ratio are not protected by memantine. In contrast, *Cbs+/-* mice expressing physiological levels of NR2A and a normal NR2A/NR2B ratio may be more susceptible to increases in homocysteine and thus display protection by memantine. However, further experiments are necessary to substantiate this hypothesis.

While neuronal NMDA receptors have been well characterized, NMDA receptors are also expressed on a variety of non-neuronal cells, including cerebrovascular endothelial cells.⁵³ Previous studies have demonstrated that endothelial NMDA receptors can be activated by homocysteine resulting in altered expression of tight junction and adherent proteins that are critical in maintaining the integrity of the BBB.²⁴ Consistent with prior observations,^{24,25,54} we observed that elevation in plasma homocysteine compromises the BBB in our genetic model of hyperhomocysteinemia (Figure 2). Given the well-defined link between the BBB and stroke pathogenesis,²⁶ it is likely that BBB disruption is a prerequisite to neuronal injury in this experimental stroke model. Consistent with our experimental stroke studies, BBB disruption was reversed only in *Cbs+/-* but not *Cbs-/-* mice with administration of memantine (Figure 5). Nevertheless, it remains unclear whether activation of NMDA receptors on vascular endothelial cells or neurons, or both, contribute to the stroke phenotypes of hyperhomocysteinemic mice. Future studies using tissue-specific NMDA receptor targeted mice can address this question.

We acknowledge that other homocysteine-related metabolites or pathways in *Cbs-/-* mice may contribute to neurovascular injury in cerebral ischemia-reperfusion injury through effects on oxidative stress, inflammation, mitochondria, or apoptosis. Homocysteine is metabolically linked to several sulfur metabolites, and alterations in homocysteine metabolism can modulate levels of related metabolites such as methionine, cysteine, and methylfolates and affect methyltransferase reactions that use S-adenosylmethionine as a

methyl donor. We reported previously that plasma levels of folate are reduced in *Cbs*^{-/-} mice,³⁴ and 1 study of mice fed a low folate diet found an increase in cerebral infarction after MCAO.⁵⁵ Additional homocysteine-related metabolites may also be important; high levels of the cyclic homocysteine analog homocysteine thiolactone, which can modify proteins via N-Hcy (N-homocysteinylation), have been reported in some animal models of hyperhomocysteinemia. In 1 study, hepatic levels of N-Hcy-protein were found to be increased >10-fold in *Cbs*^{-/-} mice versus *Cbs*^{+/-} or *Cbs*^{+/+} mice.⁵⁶ Similarly, urinary homocysteine thiolactone and N-Hcy-protein were reported to be elevated in *Cbs*^{-/-} mice relative to *Cbs*^{+/-} mice.^{57,58} Because of the parallel changes in the concentrations of homocysteine with several other metabolites in most clinical and experimental studies, the individual contributions of these metabolites to pathological states are difficult to establish. Future study design should consider these potential mechanisms and limitations.

Conclusions

We report that a mild elevation in hyperhomocysteinemia is sufficient to produce a severe stroke phenotype in genetic murine models demonstrating a direct causal role of elevated homocysteine. Furthermore, we also demonstrate a novel protective role of NMDA receptor antagonism in stroke and BBB disruption in the context of mild hyperhomocysteinemia.

Clinical Perspective

Memantine is licensed and approved for treatment of moderate-to-severe Alzheimer disease at low systemic concentrations^{59,60} permitting the blockade of excessive NMDA receptor activity without disrupting normal synaptic transmission which produce few side effects.⁶¹ Our findings suggest a potential role for memantine or other NMDA receptor antagonists as a strategy for preventing stroke in patients with mild or moderate hyperhomocysteinemia. Given that mild-to-moderate hyperhomocysteinemia is highly prevalent in patients with cerebrovascular disease in many parts of the world,^{12,38,62,63} the potential clinical impact could be considerable.

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Dr. Gu designed the research, conducted the experiments, acquired data, analyzed data, and wrote the manuscript. Dr. Sonkar conducted experiments, analyzed data, and revised the manuscript. Dr. Katara conducted the experiment, analyzed data, and revised the manuscript. Dr. Kumar conducted experiments, acquired data, analyzed data, and revised the manuscript. Dr. Arning conducted experiments, analyzed data, and revised the manuscript. Dr. Bottiglieri analyzed data and revised the manuscript. Dr. Kruger provided mouse model

and revised the manuscript. Dr. Lentz assisted with the interpretation of the results and revised the manuscript. Dr. Dayal directed the project, designed studies, analyzed data, and co-wrote the manuscript.

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Disclosures

None.

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