Published in final edited form as:

Brain Res. 2008 July 24; 1221: 147–153. doi:10.1016/j.brainres.2008.04.021.

GABA_A receptor agonist mitigates homocysteine-induced cerebrovascular remodeling in knockout mice

Munish Kumar, Neetu Tyagi, Karni S. Moshal, Utpal Sen, S.B. Pushpakumar, Thomas Vacek, David Lominadze, and Suresh C. Tyagi*

Department of Physiology and Biophysics, University of Louisville School of Medicine, Louisville, Kentucky 40202, USA

Abstract

Individuals with homozygous deficiency in cystathionine-β-synthase (CBS) develop high levels of homocysteine in plasma, a condition known as homocysteinuria. Mental retardation ensues with death in teens; the heterozygous live normally but develop vascular dementia and Alzheimer's disease (AD) in later part of life. The treatment with muscimol, a gamma amino butyric acid receptor-A (GABA_A) agonist, mitigates the AD syndrome and vascular dementia. We tested the hypothesis that homocysteine (Hcy) antagonizes the GABA_A receptor and behaves as an excitotoxic neurotransmitter that causes blood brain barrier (BBB) permeability and vascular dementia. The BBB permeability was measured by infusing Evan's blue dye (2% in saline 5 ml/kg concentration) in CBS^{-/+}, GABA_A^{-/-}, CBS^{-/+}/GABA_A^{-/-} double knockout, CBS^{-/+} mice treated with muscimol and wild type (WT) mice. Matrix Metalloproteinase (MMP-2, MMP-9), Tissue Inhibitor of Matrix Metalloproteinase (TIMP-3, TIMP-4), collagen-III and elastin levels were measured in whole brain by Western blot. These results suggested an increase in Evan's blue permeability: CBS^{-/+} + <GABA_A^{-/-} < CBS^{-/+}/GABA_A^{-/-} compared to WT mice. Interestingly, in CBS^{-/+} mice treated with muscimol, BBB permeability was significantly decreased compared with the CBS^{-/+} group. There was a decrease in the TIMP-4 protein expression level, whereas the TIMP-3 level increased in CBS^{-/+}, GABA_A^{-/-}, and CBS^{-/+}/GABA_A^{-/-} mice compared to the WT. MMP-2 and MMP-9 expression significantly increased in all the groups compared to the wild type. The results suggested that Hcy caused cerebral interstitial remodeling in brain by distorting the extracellular matrix, thus increasing the blood brain permeability; treatment with muscimol mitigated BBB permeability.

Keywords

Blood brain barrier permeability; MMP; TIMP; Double knockout

1. Introduction

Homocysteine is a thiol amino acid. An increase of homocysteine level in plasma leads to various pathophysiological conditions and cardiovascular diseases (Refsum et al., 1998; Seshadri, 2002). Various genetic and epigenetic factors, food habits, and changes in metabolic condition lead to increase in homocysteine (Siri et al., 1998; Frosst et al., 1995). In fact, the most common cause of hyperhomocysteinemia is the folate deficiency and lack of CBS gene that leads to kidney diseases and vascular dysfunction (Lentz, 1997). CBS gene is involved in

homocysteine clearance via transsulfuration pathway. Heterozygous CBS^{+/-} are predisposed to elevated homocysteinemic condition (Bar-Or et al., 2004).

TIMPs have a wide range of important functions such as inhibition of MMPs and other growth regulatory roles in developments. TIMPs play a pivotal role in the regulation of metabolism of extracellular matrix (ECM) (Lou, 2005). The MMPs constitute a large family of proteolytic enzymes responsible for ECM degradation and remodeling under normal and pathological conditions (Tyagi et al., 2005a,b; Shastry et al., 2005, 2006). In general, studies have demonstrated that an imbalance between MMPs and TIMPs occurs in various pathophysiological conditions. In addition to these functions, TIMPs also regulate cell growth. In fact, decreased production of TIMPs resulted in greater activity of MMPs, leading to cancer growth (Malemud, 2006). Previous studies from our lab have demonstrated that during cardiovascular diseases, the level of TIMP-4 decreased, whereas the level of TIMP-3 increased (Moshal et al., 2005, 2006; Shastry et al., 2006). Activation of MMPs and alternation of basement membrane (BM) associated with BBB injury is documented in stroke and BBB permeability (Haorah et al., 2007; Sellner and Leib, 2006; Cunningham et al., 2005).

GABA, an endogenous amino acid is present in central nervous system and function as an inhibitory neurotransmitter (Erdo, 1985). Hcy competes with GABA_A receptor and acts as an excitotoxic neurotransmitter (Griffiths et al., 1983; Tyagi et al., 2007a,b). The GABA receptors play a significant role in brain microvascular permeability; its inhibition leads to ECM degradation and increases in brain permeability (Tyagi et al., 2007a, b; Shastry et al., 2005; 2006; Lazzarini et al., 2001). Increased Hcy level may attenuate the function of GABA_A receptor, leading to disruption in BBB layer. Therefore, we hypothesized the role of GABA_A receptor in hyperhomocysteinemia, TIMPs and MMPs in blood brain barrier since it has a role in regulating the metabolism of ECM and permeability.

2. Results

Brain cortex Hcy phenotypic data suggested that there were significantly higher Hcy in CBS^{-/+} and CBS^{-/+}/GABA_A^{-/-} as compared to WT and GABA_A^{-/-} (Fig. 1). These data suggested mild hyperhomocysteinemia in CBS^{-/+} and CBS^{-/+}/GABA_A^{-/-}. Although we did not report the levels of homocysteine in CBS^{-/+} mice treated with muscimol, we expected no change in the homocysteine level in these mice.

To determine whether increased Hcy in CBS^{-/+} and CBS^{-/+}/GABA_A^{-/-} mice caused an increase in BBB permeability, we measured interstitial diffusion of Evans blue in WT, GABA_A^{-/-}, CBS^{-/+}, CBS^{-/+}/GABA_A^{-/-} and CBS^{-/+} mice treated with muscimol. We observed that Evans blue diffusion was significantly higher in all other group compared to the WT. Permeability was significantly higher in CBS^{-/+}/GABA_A^{-/-} mice compared to GABA_A^{-/-} and CBS^{-/+}. Interestingly, muscimol treatment mitigated BBB permeability in CBS^{-/+} mice (Fig. 2).

Brain sections were stained from each group with trichrome and Van Gieson to see the pattern of collagen and elastin. There was a significant difference in CBS^{-/+} mice compared to WT. In other groups, we could not find a clear difference. Blue color signifies collagen whereas the dark blue signifies elastin straining (Fig. 3). Western blot showed that elastin level was significantly decreased in CBS^{-/+}, CBS^{-/+}/GABA_A^{-/-}, and GABA_A^{-/-} mice compared to the WT, whereas collagen-III level was significantly higher in these groups compared with WT. CBS^{-/+} mice treated with muscimol showed significant decrease in collagen-III and increase in elastin level compared to the CBS^{-/+} mice (Fig. 4A). These are associated with an increase in the expression of MMPs.

Protein levels and activity of MMPs in WT, CBS^{-/+}, CBS^{-/+}/GABA_A^{-/-}, GABA_A^{-/-} and CBS^{-/+} mice treated with muscimol were measured using Western blot and in-gel gelatin zymography (Figs. 5A and 6). There were significant increases in MMP-2 and MMP-9 expression in CBS^{-/+}, CBS^{-/+}/GABA_A^{-/-}, and GABA_A^{-/-} mice compared to WT. Interestingly, in CBS^{-/+} mice treated with muscimol, results showed a significant decrease in MMP-2 and -9 protein expression. In addition, we observed an increase in MMP-9 activity in CBS^{-/+} and CBS^{-/+}/GABA_A^{-/-} mice measured by gelatin zymography (Fig. 6). The result suggested that during homocysteinemia the levels of MMPs increased several fold.

The activities of MMPs are controlled by TIMPs; therefore, we measured the protein level of TIMPs using Western blot (Fig. 7 A). A significant increase in TIMP-3 expression was observed in CBS^{-/+}, CBS^{-/+}/GABA_A^{-/-} and GABA_A^{-/-} compared to the WT. In CBS^{-/+} mice treated with muscimol, TIMP-3 expression was significantly decreased compared with CBS^{-/+} mice. TIMP-4 protein levels were significantly decreased in CBS^{-/+}, CBS^{-/+}/GABA_A^{-/-} and GABA_A^{-/-} compared to the WT. Interestingly, CBS^{-/+} mice treated with muscimol showed a significant increase in TIMP-4 expression. The data suggested that Hcy increases TIMP-3 and decreases TIMP-4 expression in CBS^{-/+}, GABA_A^{-/-} and CBS^{-/+}/GABA_A^{-/-} knockout mice and muscimol treatment mitigated these changes.

3. Discussion

GABA is the primary inhibitory neurotransmitter in the brain (Tyagi et al., 2007a,b). Muscimol competes with Hcy, a GABA_A receptor agonist and displaces Hcy interaction to GABA_A. Therefore, muscimol attenuates cerebral dysfunction by inhibiting the binding of Hcy to the GABA_A receptor. MMPs and TIMPs are involved in ECM remodeling that is essential in development and morphogenesis (Malemud, 2006). MMPs degrade proteins and polysaccharides that compose the neurovascular matrix (collagen, laminin, elastin and fibronectin) (Lo et al., 2003; Shastry et al., 2006). BBB is formed by microvascular endothelial cells (MVEC) and regulates flow of molecules in and out of the brain (Haorah et al., 2007). Microcirculation in the brain may be more susceptible to Hcy thus effecting the permeability in the brain. It is well documented that Hcy causes cell detachment and brain cell death by creating oxidative stress (Tyagi et al., 2005a,b, 2007a,b). Thus, the free radicals generated under oxidative stress induced by Hcy is the underlying cause of neurodegenerative and neuroinflammatory disorders and BBB permeability. Reactive oxygen species activate MMPs and decrease TIMPs in a protein tyrosine kinase-dependent manner (Moshal et al, 2006). Increasing evidence has indicated that MMP-2 and MMP-9 level increases in pathophysiological and brain damage conditions (Zhao et al., 2007; Wang and Tsirka, 2005; Mun-Bryce and Rosenberg, 1998). In HHcy, early appearance of MMP-2, MMP-9 has been reported in cardiovascular diseases and neurovascular diseases (Sen et al., 2007a,b; Lominadze et al., 2006).

In the present study, Hcy mice showed a significant increase in the MMP-2, MMP-9 protein expression in brain cortex. Matrix-in activities are regulated by activation of the precursor zymogens and inhibition by endogenous inhibitors TIMPs. Thus, the balance between MMPs and TIMPs is critical for the eventual ECM remodeling in the tissue (Shastry et al., 2005, 2006). The level of TIMP-4 was significantly decreased in Hcy mice. On the other hand, TIMP-3 increased in the Hcy mice. TIMP-3 is the specific inhibitor of MMP-2 and -9 (Hashimoto et al., 2003). In the present study we observed that in CBS^{-/+}/GABA_A^{-/-} double KO there was an increase in TIMP-3 levels which may have downregulated the expression for MMP-2 and -9. The second reason for an increase in TIMP-3 is that the whole brain is taken and is abundantly present throughout the brain. TIMP-4 is abundant only in cerebellum (Fager and Jaworski, 2000; Zhao et al., 2004). TIMP-3 is known to promote apoptosis in normal cells through increased death receptor signaling (Bond et al., 2002; Wetzel et al., 2008); this may

be the another reason for the increase in its expression. An increase in MMPs and a decrease in TIMPs degrade the ECM, thus the collagen level is increased, and the elastin level is decreased (Hashimoto et al., 2003). Brain section stained for collagen and elastin showed that the collagen level increases in CBS^{-/+} mice compared to the wild type, whereas the elastin decreases. The present study showed that in CBS^{-/+}, GABA_A^{-/-} and CBS^{-/+}/GABA_A^{-/-}, there were significant increases in Evans blue permeability. These results coincided with the earlier reports that Hcy increased albumin leakage through EC monolayer in pial vessels (Tyagi et al., 2007a,b; Lominadze et al., 2006). Hcy-induced albumin leakage drastically decreased with treatment of GABA or its agonist, muscimol, suggesting that Hcy may directly affect EC properties through binding the GABA_A receptor (Tyagi et al., 2007a,b).

Hcy specifically competes with muscimol (GABA_A receptor agonist) for binding to the GABA_A receptor (Griffiths et al., 1983) and behaves as an excitatory neurotransmitter. GABA_A receptors play a significant role in brain microvascular permeability; alteration in functions results in matrix degradation and edema (Lazzarini et al., 2001). A previous report from our lab showed that antagonist of GABA_{A/B} receptor (muscimol and baclofen) inhibits Hcy-mediated collagen constriction to MVEC (Shastri et al., 2005).

The result of the present study suggested that the activation of GABA_A receptor in brain cortex may prevent Hcy-induced BBB leakage and, therefore, highlighted the functional role of GABA_A receptor in brain cortex and its possible involvement in Hcy-induced permeability.

Taken together, the present study supported that Hcy caused injurious effects on the central nervous system and may contribute to neurodegenerative diseases and vascular dementia. Hcy caused brain injury via imbalance in MMPs and TIMPs level and collagen/elastin ratio. Understanding the MMP pathways, role of TIMPs, underlying BBB dysfunction, GABA_A receptor and its agonist may lead to the development of therapeutic intervention for Hcy-induced cerebral complications.

4. Experimental procedures

Mice (wild type C57BL/6J, GABA_A^{-/-} and CBS^{-/+}) were obtained from Jackson Laboratories (Bar Harbor, ME) and housed in the animal care facility at University of Louisville. The CBS^{-/+} and GABA_A^{-/-} mice were crossbred for 6 generations to create CBS^{-/+}/GABA_A^{-/-} double knockout. CBS^{-/+} male mice were administered with muscimol (0.8 g/kg) in drinking water for 4 weeks. Mice were genotyped for each group with specific set of primers. All animal procedures were in accordance with the National Institute of Health Guidelines for animal research. The Institutional Animal Care and Use Committee of the University of Louisville School Of Medicine approved this study.

4.1. Homocysteine measurement

High-pressure liquid chromatography (HPLC) analyses were performed in a Shimadzu Class-VP 5.0 chromatograph (Shimadzu Corp, Japan) equipped with LC-10ADvp pump, SIL-10ADvp autoinjector, CTO-10Avp column oven and SPD-10Avp detector.

Chromatography: The chromatographic conditions were maintained as described elsewhere (Malinow et al., 1989). Briefly, 0.1 M monochloroacetic acid with 1.8 mM octylsulfate at pH 3.2 was used as mobile phase which was filtered through a Millipore filter (0.45 µm) and degassed under vacuum. The isocratic solvent was pumped and circulated through the column at a constant flow of 0.8 ml/min. Samples were injected through the autoinjector, and an injection volume of 20 µl was used. During HPLC analysis, Hcy level in brain homogenate was identified according to their retention times and co-chromatography with standards.

Sample preparation: Fresh brain supernatants were collected and centrifuged to remove debris. To determine Hcy in the supernatants, 200 µl supernatant was diluted with 100 µl of water and

then 300 μ l of 9 M urea (pH 9.0) was added. The 50 μ l of *n*-amyl alcohol was added to the solution as an antifoaming agent. Reduction of disulfides and cleavage of the protein-bound sulfur-containing amino acids was performed by the addition of 50 μ l solution of NaBH₄ (10%, wt/vol) in 0.1 N NaOH. To perform reaction, samples were incubated in a water bath at 50 °C for 30 min. Samples were cooled down at room temperature and reaction was stopped by the addition of 500 μ l of 20% trichloroacetic acid.

4.2. Blood brain permeability

BBB permeability was carried out by Evans blue dye as reported earlier with some modification (Kamath et al., 2006). Mice from each group were injected with 50 μ g/g Evans blue dye. After 3 h, excess dye was removed from circulation by infusing saline and cutting the inferior vena cava. Brain was taken out by opening the skull, weighed and homogenized in 50% trichloroacetic acid. The samples were centrifuged at 10,000 rpm for 20 min. The supernatant was diluted with 100% alcohol (1:4). Evans blue fluorescence was read at 610 nm and 595 nm. The readings were divided by the weight of the brain and data were analyzed.

4.3. Histological studies

A part of fresh brain was sectioned on cryostat and was stained with trichrome and elastic staining kit (Van Gieson).

Mouse polyclonal antibodies to MMPs (MMP-2, MMP-9), TIMPs (TIMP-3, TIMP-4), anti- β -actin antibodies, collagen and Elastin antibodies were from Santa Cruz (Santa Cruz, CA). Evans blue and other analytical reagents were from Sigma-Aldrich (St. Louis, MO). Horseradish Peroxidase (HRP)-linked anti-rabbit IgG antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA). Polyvinylidene difluoride (PVDF) membrane was from Bio-Rad (Hercules, CA). Brain homogenates were prepared in protein extraction buffer (0.01 M cacodylic acid pH 5.0, 0.15 M NaCl, 1 μ M ZnCl₂, 0.02 M CaCl₂, 0.0015 M NaN₃ and 0.01% v/v Triton X-100) and kept for overnight digestion in cold room. Next day protein was extracted by centrifugation at 5000 rpm for 10 min. Western blot: 50 μ g of proteins were analyzed on 10%–12% SDS-PAGE as described earlier (Sen et al., 2007a,b) for MMP-2, MMP-9, TIMP-3, TIMP-4, Collagen-III and Elastin protein levels. Further, the blots were stripped and reprobed for β -actin expression for all above protein to standardize the data. The developed X-ray images were analyzed using UMAX Power Lock II (Taiwan).

MMP-9 activity in brain tissue was measured using gelatin gel zymography as described previously (Tyagi et al., 1993). Briefly, samples were electrophoretically resolved on 7.5% SDS-PAGE containing 1.5 mg/ml gelatin as a substrate. At the end, gel was incubated in renaturation buffer (2.5% Triton X-100) for 30 min to remove SDS, rinsed in distilled water, and then incubated for 36 h at 37 °C in water bath in activation buffer (50 mM Tris-HCl, pH 7.4, and 5 mM CaCl₂). Gel was stained using 0.5% Coomassie blue R-250 for 1 h. MMP activity was detected as a white band on a dark blue background and quantitated densitometrically using Un-Scan-It software (Silk Scientific, Orem, UT).

4.4. Statistical analysis

The means and standard errors of mean (SEM) in five experimental groups of mice (WT, CBS^{-/+}, CBS^{-/+} treated with muscimol, CBS^{-/+}/GABA^{-/-} and GABA^{-/-}) were determined. There were nine responses observed (Homocysteine level, Evans blue permeability, Western Blot for Elastin, Collagen-III, MMP-2, MMP-9, TIMP-3, TIMP-4 and MMP-9 activity). For each of these nine responses, we checked normality assumptions using Shapiro–Wilk test (Shapiro and Wilk, 1965) and found the data to be valid. The ANOVA procedure analyzes the data to be compared between the groups, and declared results significant at alpha level of 0.05 were applied, ($p < 0.05$).

Acknowledgments

The authors wish to thank Dr. Shesh N. Rai, Director, Biostatistics stand Facility, JG Brown Cancer Center, University of Louisville for helping in statistical analysis.

The study is supported by National Heart, Lung, and Blood Institute Grants HL-71010, HL-74185, and HL-88012.

Abbreviations

CBS	cystathionine- β -synthase
GABA	gamma amino butyric acid

REFERENCES

- Bar-Or D, Curtis CG, Sullivan A, Rael LT, Thomas GW, Craun M, Bar-Or R, Maclean KN, Kraus JP. Plasma albumin cysteinylolation is regulated by cystathionine beta-synthase. *Biochem. Biophys. Res. Commun* 2004;325:1449–1453. [PubMed: 15555590]
- Bond M, Murphy G, Bennett MR, Newby AC, Baker AH. Tissue inhibitor of metalloproteinase-3 induces a Fas-associated death domain-dependent type II apoptotic pathway. *J. Biol. Chem* 2002;277:13787–13795. [PubMed: 11827969]
- Cunningham LA, Wetzel M, Rosenberg GA. Multiple roles for MMPs and TIMPs in cerebral ischemia. *Glia* 2005;50:329–339. [PubMed: 15846802]
- Erdo SL. Peripheral GABAergic mechanism. *Trends Pharmacol. Sci* 1985;5:141–158.
- Fager N, Jaworski DM. Differential spatial distribution and temporal regulation of tissue inhibitor of metalloproteinase mRNA expression during rat central nervous system development. *Mechanisms Dev* 2000;98:105–109. [PubMed: 11044612]
- Frosst P, et al. A candidate genetic risk factor for cardiovascular disease: a common mutation in methylenetetrahydrofolate reductase {letter}. *Nat. Genet* 1995;10:111–113.
- Griffiths R, Williams DC, O'Neill C, Dewhurst IC, Ekuwem CE, Sinclair CD. Synergistic inhibition of (3H) muscimol binding to calf brain synaptic membranes in the presence of L-homocysteine and pyridoxal 5-phosphate: a possible mechanism for homocysteine-induced seizures. *Eur. J. Biochem* 1983;137:467–478. [PubMed: 6319125]
- Haorah J, Ramirez SH, Schall K, Smith D, Pandya R, Persidsky Y. Oxidative stress activates protein tyrosine kinase and matrix metalloproteinases leading to blood-brain barrier dysfunction. *J. Neurochem* 2007;101:566–576. [PubMed: 17250680]
- Hashimoto T, Wen G, Lawton MT, Boudreau NJ, Bollen AW, Yang GY, Barbaro NM, Higashida RT, Dowd CF, Halbach VV, Young WL. Abnormal expression of matrix metalloproteinases and tissue inhibitors of metalloproteinases in brain arteriovenous malformations. *Stroke* 2003;34:925–931. [PubMed: 12649522]
- Kamath AF, Chauhan AK, Kisucka J, Dole VS, Loscalzo J, Handy DE, Wagner DD. Elevated levels of homocysteine compromise blood-brain barrier integrity in mice. *Blood* 2006;107:591–593. [PubMed: 16189268]
- Lazzarini R, Malucelli BE, Palermo-Neto J. Reduction of acute inflammation in rats by diazepam: role of peripheral benzodiazepine receptors and corticosterone. *Immunopharmacol. Immunotoxicol* 2001;23:253–265. [PubMed: 11417852]
- Lentz SR. Homocysteine and vascular dysfunction. *Life Sci* 1997;61:1205–1215. [PubMed: 9324062]
- Lo EH, Dalkara T, Moskowitz MA. Mechanisms, mechanism and challenges and opportunities in stroke. *Nat. Rev. Neurosci* 2003;4:399–415. [PubMed: 12728267]
- Lominadze D, Roberts AM, Tyagi N, Moshal KS, Tyagi SC. Homocysteine causes cerebrovascular leakage in mice. *Am. J. Physiol. Heart Circ. Physiol* 2006;290:H1206–H1213. [PubMed: 16258031]
- Lou J. The role of matrix metalloproteinases in the morphogenesis of the cerebellar cortex. *Cerebellum* 2005;4:239–245. [PubMed: 16321879]
- Malemud CJ. Matrix metalloproteinases (MMPs) in health and disease: an overview. *Front Biosci* 2006;11:1696–1701. [PubMed: 16368548]

- Malinow MR, Kang SS, Taylor LM, Wong PW, Coull B, Inahara T, Mukerjee D, Sexton G, Upson B. Prevalence of hyperhomocyst(e)inemia in patients with peripheral arterial occlusive disease. *Circulation* 1989;79:1180–1188. [PubMed: 2785871]
- Moshal KS, Tyagi N, Moss V, Henderson B, Steed M, Ovechkin A, Aru GM, Tyagi SC. Early induction of matrix metalloproteinase-9 transduces signaling in human heart end stage failure. *J. Cell. Mol. Med* 2005;9:704–707. [PubMed: 16202218]
- Moshal KS, Sen U, Tyagi N, Henderson B, Steed M, Ovechkin AV, Tyagi SC. Regulation of homocysteine-induced MMP-9 by ERK1/2 pathway. *Am. J. Physiol. Cell Physiol* 2006;290:C883–C891. [PubMed: 16251475]
- Mun-Bryce S, Rosenberg GA. Matrix metalloproteinase in cerebrovascular diseases. *J. Cereb. Blood flow Metab* 1998;18:1163–1172. [PubMed: 9809504]
- Refsum H, Ueland PM, Nygard O, Vollset SE. Homocysteine and cardiovascular disease. *Annu. Rev. Med* 1998;49:31–62. [PubMed: 9509248]
- Sellner J, Leib SL. In bacterial meningitis cortical brain damage is associated with changes in parenchymal MMP-9/TIMP-1 ratio and increased collagen type IV degradation. *Neurobiol. Dis* 2006;21:647–656. [PubMed: 16257222]
- Sen U, Herrmann M, Herrmann W, Tyagi SC. Synergism between AT1 receptor and hyperhomocysteinemia during vascular remodeling. *Clin. Chem. Lab. Med* 2007a;45:1771–1776. [PubMed: 17990952]
- Sen U, Tyagi N, Kumar M, Moshal KS, Rodriguez WE, Tyagi SC. Cystathionine-beta-synthase gene transfer and 3-deazaadenosine ameliorate inflammatory response in endothelial cells. *Am. J. Physiol. Cell Physiol* 2007b;293:C1779–C1787. [PubMed: 17855772]
- Seshadri S. Plasma homocysteine as a risk factor for dementia and Alzheimer's disease. *N. Eng. J. Med* 2002;346:476–483.
- Shapiro SS, Wilk MB. An analysis of variance test for normality complete samples. *Biometrika* 1965;52:591–611.
- Shastry S, Moning L, Tyagi N, Steed M, Tyagi SC. GABA receptors and nitric oxide ameliorate constrictive collagen remodeling in hyperhomocysteinemia. *J. Cell Physiol* 2005;205:422–427. [PubMed: 15895389]
- Shastry S, Tyagi N, Moshal KS, Lominadze D, Hayden MR, Tyagi SC. GABA receptors ameliorate Hcy-mediated integrin shedding and constrictive collagen remodeling in microvascular endothelial cells. *Cell Biochem. Biophys* 2006;45:157–165. [PubMed: 16757816]
- Siri PW, Verhoef P, Kok FJ. Vitamins B6, B12, and folate: association with plasma total homocysteine and risk of coronary atherosclerosis. *J. Am. Coll. Nutr* 1998;17:435–441. [PubMed: 9791839]
- Tyagi SC, Ratajska A, Weber KT. Myocardial matrix metalloproteinase(s): localization and activation. *Mol. Cell Biochem* 1993;126:49–59. [PubMed: 8107689]
- Tyagi N, Sedoris KC, Steed M, Ovechkin AV, Moshal KS, Tyagi SC. Mechanisms of homocysteine-induced oxidative stress. *Am. J. Physiol. Heart Circ. Physiol* 2005a;289:H2649–H2656. [PubMed: 16085680]
- Tyagi SC, Lominadze D, Roberts AM. Homocysteine in microvascular endothelial cell barrier permeability. *Cell Biochem. Biophys* 2005b;43:37–44. [PubMed: 16043881]
- Tyagi N, Lominadze D, Gillespie W, Moshal KS, Sen U, Rosenberger DS, Steed M, Tyagi SC. Differential expression of gamma-aminobutyric acid receptor A (GABA(A)) and effects of homocysteine. *Clin. Chem. Lab. Med* 2007a;45:1777–1784. [PubMed: 17990949]
- Tyagi N, Moshal KS, Tyagi SC, Lominadze D. gamma-Aminobutyric acid A receptor mitigates homocysteine-induced endothelial cell permeability. *Endothelium* 2007b;14:315–323. [PubMed: 18080868]
- Wang J, Tsirka SE. Neuroprotection by inhibition of matrix metalloproteinases in a mouse model of intracerebral haemorrhage. *Brain* 2005;128:1622–1633. [PubMed: 15800021]
- Wetzel M, Li L, Harms KM, Roitbak T, Ventura PB, Rosenberg GA, Khokha R, Cunningham LA. Tissue inhibitor of metalloproteinases-3 facilitates Fas-mediated neuronal cell death following mild ischemia. *Cell Death and Diff* 2008;15:143–151.
- Zhao H, Bernardo MM, Osenkowski P, Sohail A, Pei D, Nagase H, Kashiwagi M, Soloway PD, DeClerck YA, Fridman R. Differential inhibition of membrane type 3 (MT3)-Matrix Metalloproteinase (MMP)

and MT1-MMP by Tissue Inhibitor of Metalloproteinase (TIMP)-2 and TIMP-3 regulates Pro-MMP-2 activation. *J. Biol. Chem* 2004;279:8592–8601. [PubMed: 14681236]

Zhao BQ, Tejima E, Lo EH. Neurovascular proteases in brain injury, hemorrhage and remodeling after stroke. *Stroke* 2007;38:748–752. [PubMed: 17261731]

Fig. 1.

Brain cortical levels of homocysteine in WT, CBS^{-/+}, CBS^{-/+}/GABA_A^{-/-} and GABA_A^{-/-}.

The values represented mean $\pm$ SEM ($n=4$). GroupWise statistical comparison was performed.

* $p<0.05$ compared to WT. # $p<0.05$ compared to GABA_A^{-/-}.

Fig. 2.

Evans blue permeability in brain interstitium. Brain cortical leakage of Evans blue dye in WT, CBS-/+ , CBS-/+ /GABA_A^{-/-}, GABA_A^{-/-} and CBS-/+ mice treated with muscimol. The values represented mean±SEM ($n=4$). GroupWise statistical comparison was performed.

* $p<0.05$ compared to WT. # $p<0.05$ compared to CBS-/+ /GABA_A^{-/-}. ≠ $p<0.05$ compared to CBS-/+ mice.

Fig. 3. Histological analysis of brain cortex in wild type and CBS^{-/+} mice. Tissue section was labeled with elastic stain (Van Gieson) for elastin (A, B) and trichrome for collagen (C, D). Note: the arrow indicates increased blue stain for collagen and decreased dark blue stain for elastin in CBS^{-/+}. Magnification $\times 40$.

Fig. 4.

Representative Western blot analysis of elastin and collagen protein (A) levels in WT, CBS^{-/-}, CBS^{-/-} +, CBS^{-/-}/GABA_A^{-/-}, GABA_A^{-/-} and CBS^{-/-} mice treated with muscimol in lanes 1, 2, 3, 4 and 5. The bar graph (B and C) values represent mean $\pm$ SEM ($n=4$) and are normalized with β -actin. * $p<0.05$ compared to WT. $\neq p<0.05$ compared to CBS^{-/-} mice.

Fig. 5. Representative Western blot analysis of MMP-2 protein (A) levels in WT, CBS^{-/+}, CBS^{-/+}/GABA_A^{-/-}, GABA_A^{-/-} and CBS^{-/+} mice treated with muscimol in lanes 1, 2, 3, 4 and 5. The bar graphs (B and C) represent mean±SEM (*n*=4) and are normalized with β-actin. **p*<0.05 compared to WT. †*p*<0.05 compared to CBS^{-/+} mice.

Fig. 6. Representative MMP-9 activity in gelatin zymography for WT, CBS-/-, CBS-/-/GABA_A^{-/-}, GABA_A^{-/-} and CBS-/- mice treated with muscimol in lanes 1, 2, 3, 4 and 5. The bar graph represents mean±SEM ($n=4$). * $p<0.05$ compared to WT. ≠ $p<0.05$ compared to CBS-/- mice.

Fig. 7.

Representative Western blot analysis of TIMP-3 and TIMP-4 protein levels (A) in WT, CBS^{-/+}, CBS^{-/+}/GABA_A^{-/-}, GABA_A^{-/-} and CBS^{-/+} mice treated with muscimol in lanes 1, 2, 3, 4 and 5. The bar graphs (B and C) represent mean±SEM (*n*=4) and are normalized with β-actin. **p*<0.05 compared to WT. #*p*<0.05 compared to CBS^{-/+}/GABA_A^{-/-}. *p*<0.05 compared to CBS^{-/+} mice.