Analysis of MTHFR, CBS, Glutathione, Taurine, and Hydrogen Sulfide Levels in Retinas of Hyperhomocysteinemic Mice

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Submitted: December 8, 2016
Accepted: March 2, 2017
Citation: Cui X, Navneet S, Wang J, et al. Analysis of MTHFR, CBS, glutathione, taurine, and hydrogen sulfide levels in retinas of hyperhomocysteinemic mice. Invest Ophthalmol Vis Sci. 2017;58:1954–1963. DOI: 10.1167/iovs.16-21247

PURPOSE. Hyperhomocysteinemia (Hhcy) is implicated in certain retinal neurovascular diseases, although whether it is causative remains uncertain. In isolated ganglion cells (GCs), mild Hhcy induces profound death, whereas retinal phenotypes in Hhcy mice caused by mutations in remethylation (methylene tetrahydrofolate reductase [Mthfr+/−]) or transulfuration pathways (cystathionine β-synthase [Cbs+/−]) demonstrate mild GC loss and mild vasculopathy. The current work investigated compensation in vivo of one pathway for the other, and, because the transsulfuration pathway yields cysteine necessary for formation of glutathione (GSH), taurine, and hydrogen sulfide (H2S), they were analyzed also.

METHODS. Retinas isolated from wild-type (WT), Mthfr+/−, and Cbs+/− mice (12 and 22 weeks) were analyzed for methylene tetrahydrofolate reductase (MTHFR), cystathionine-β-synthase (CBS), and cystathionase (CTH) RNA/protein levels. Retinas were evaluated for levels of reduced:oxidized GSH (GSH:GSSG), Slc7a11 (xCT), taurine, taurine transporter (TAUT), and H2S.

RESULTS. Aside from decreased CBS RNA/protein levels in Cbs+/− retinas, there were minimal alterations in remethylation/transsulfuration pathways in the two mutant mice strains. Glutathione and taurine levels in Mthfr+/− and Cbs+/− retinas were similar to WT, which may be due to robust levels of xCT and TAUT in mutant retinas. Interestingly, levels of H2S were markedly increased in retinas of Mthfr+/− and Cbs+/− mice compared with WT.

CONCLUSIONS. Ganglion cell loss and vasculopathy observed in Mthfr+/− and Cbs+/− mouse retinas may be milder than expected, not because of compensatory increases of enzymes in remethylation/transsulfuration pathways, but because downstream transsulfuration pathway products GSH, taurine, and H2S are maintained at robust levels. Elevation of H2S is particularly intriguing owing to neuroprotective properties reported for this gasotransmitter.

Keywords: remethylation pathway, transsulfuration pathway, retina, ganglion cell, homocysteine, one carbon metabolism

Homocysteine (Hcy) is a sulfur-containing nonproteinogenic amino acid, which sits at the intersection of the remethylation and transsulfuration metabolic pathways important for synthesis of methionine (Fig. 1). When the diet is replete with methionine, Hcy is catabolized to cystathionine by cystathionine-β-synthase (CBS) via the transsulfuration pathway. Cystathionine is converted to cysteine via cystathionase (CTH), also known as cystathionine γ-lyase (CSE). Cysteine is used in synthesis of downstream products such as glutathione (GSH), taurine, and hydrogen sulfide (H2S). When the diet is deficient in methionine, Hcy is remethylated back to methionine via the remethylation pathway involving the enzymes methylene tetrahydrofolate reductase (MTHFR) and methionine synthase. The remethylation pathway requires folate and cobalamin (vitamin B12) as cofactors. Perturbation in these pathways can lead to elevated plasma Hcy levels. Homozygous mutations in Cbs and Mthfr lead to homocystinuria characterized by exceedingly high levels of plasma Hcy, severe mental and skeletal abnormalities, premature thromboembolism, and lens dislocation.1 Heterozygous mutations in these enzymes or nutritional deficiency of cofactors lead to moderate increase in plasma Hcy known as hyperhomocysteinemia (Hhcy).2

Hyperhomocysteinemia, an independent risk factor for cardiovascular diseases,3 is implicated in certain neurodegenerative diseases.4 Given that retina is a neurovascular tissue, there has been interest in the role of excess Hcy in retinal disease.5 Some studies suggest that Hhcy plays a role in AMD,6,7 diabetic retinopathy,8–10 and retinal vein occlusion11; however, the mechanisms are not certain. Earlier studies suggested a link between Hhcy and glaucoma,12,13 although recent reports raise doubt about this association.14–20

It is feasible to study effects of severely elevated Hhcy on retina using mice with global knockout of the Cbs or Mthfr genes.21,22 It is perhaps more relevant to the general population to understand the effects of milder Hhcy on retina because of its greater prevalence. To understand the effects of chronic, but
milder elevation of Hcy on retina structure and function, our laboratory characterized the retinal phenotype in CBS- and MTHFR-deficient mice. CBS<sup>−/−</sup> mice have an approximate 2-fold increase in retinal Hcy. The retinal morphology is comparable to that of age-matched wild-type (WT) mice initially, but by 15 to 30 weeks, morphologic changes are observed, including loss of cells in the ganglion cell (GC) layer and decreased thickness of inner plexiform and nuclear layers. Functional studies reveal a gradual decrease in electroretinogram (ERG) amplitudes by 10 weeks of age. There is mild vasculopathy observed in CBS<sup>−/−</sup> mice that worsens significantly with age and includes neovascularization, vessel tortuosity, vascular leakage, and areas of ischemia/hypoxia. Retinas of these mice demonstrate elevated glial fibrillary acidic protein (GFAP) consistent with retinal gliosis. CBS<sup>−/−</sup> mice also have a slight elevation (approximately 2-fold) of retinal Hcy. The mice have no significant differences in scotopic or photopic a- or b-wave amplitudes of the ERG; however, they demonstrate a decrease in the positive scotopic threshold responses (STRs) at wave amplitudes of the ERG; however, they demonstrate a decrease in the positive scotopic threshold responses (STRs) at 12 weeks, which becomes more pronounced at 24 weeks, consistent with modest attenuation of ganglion cell function. Fluorescein angiographic analysis of Mthfr<sup>−/−</sup> mutants showed focal leakage and vascular tortuosity by 24 weeks. Optical coherence tomography (OCT) imaging of Mthfr<sup>−/−</sup> mice revealed a statistically significant decrease in the nerve fiber layer (NFL) thickness at 24 weeks. The loss of GCs and decreased NFL thickness is not associated with increased intraocular pressure.

The data from studies of CBS<sup>−/−</sup> and Mthfr<sup>−/−</sup> mice support the idea that the mild retinal neurovasculopathy is due to chronic exposure to slightly elevated Hcy levels. It is important to note, however, that the retinopathy develops over many weeks and does not overwhelm the visual system to the point of blindness. This observation contrasts with data obtained in isolated primary GCs harvested from WT mice exposed to Hcy. Even fairly low level exposure to Hcy (50 μM for 18 hours) can induce death in ~50% of cells. Clearly, the intact retinal milieu has the capacity to withstand exposure to mild Hcy. We do not know whether in the in vivo models there is any compensatory effect, such that CBS expression is altered in Mthfr mutants or vice versa. We addressed this in the current study by evaluating CBS and MTHFR at the RNA and protein level in retinas of CBS<sup>−/−</sup> and Mthfr<sup>−/−</sup> mice. In addition, we had not previously assessed levels of potentially beneficial downstream products of the transsulfuration pathway (GSH, taurine, and H<sub>2</sub>S; Fig. 1) in retinas of these mutant mice, but do so in the current study. Glutathione is an abundant antioxidant in the retina that affords protection to the highly oxidative retinal environment. A naturally occurring tripeptide formed of glutamate, glycine, and cysteine, GSH acts as a reductant of peroxides. Taurine is the most abundant amino acid in retina and is essential for normal retinal architecture and function. Hydrogen sulfide is a gasotransmitter that has neuroprotective properties. The current study analyzed levels of GSH, taurine, and H<sub>2</sub>S in retinas of both mutant mouse strains. We found that GSH and taurine levels were similar between the mutant mouse strains and WT. Most interesting was the observation that levels of H<sub>2</sub>S were actually elevated in retinas of both mutant mouse strains, which may account for the milder-than-expected phenotypes in the mice. The elevated H<sub>2</sub>S also raises intriguing questions about the role of Hcy in regulating levels of this gasotransmitter. For the current work, two ages were selected for analysis (12 and 22 weeks) because they reflect time points in which only modest GC loss is observed, mild retinal function alteration is detected, and vasculopathy is modest in both mutant strains.

**Methods**

**Animals**

Eighty-two mice were used in the study (Table 1). Breeding pairs of Mthfr<sup>−/−</sup> mice (Rozen Lab, McGill University, Montreal, Canada) and CBS<sup>−/−</sup> mice (B6.129P2-Cbs<sup>−/−</sup>/J; Jackson Laboratories, Bar Harbor, ME, USA) were used to establish colonies of these strains. Confirmation of the genotype was performed as described. Briefly, DNA was prepared from a small tissue sample from the mouse ear by NaOH (HotSHOT) extraction followed by PCR. Primers used for genotyping Mthfr were as follows: mmrEx3S (5'-GAA GCA GAG GAG AGG CCT CAG-3'); nE0s5 (5'-AGG CTG AAG AAC GAC ATC AGC AGC-3'); and mIn3A1 (5'-AGC TAG CTG GTG ATC TTC TCA TG-3'). Polymerase chain reaction yielded a single band of ~145 bp for WT mice and two bands for the heterozygous mice (~216 bp; Fig. 2A). Primers used for genotyping CBS were as follows: P10 (5'-GAA GTG GAC TCA GCA CA-3'); Pneo (5'-GAG GTC GAC GGT ATC GAT A-3'); and P13 (5'-GGG ATG ACC TGC ATT CAT CT-3'). The PCR yielded a single band of ~510 bp for WT mice and two bands for the heterozygous mice (~450 bp; Fig. 2B). Mice were also screened for the Crb1<sup>DBR/DNB</sup> mutation, which causes focal disruption of the retina, has
been reported in various mouse strain stocks, and it is important to exclude these mutant alleles before studying new retinal models.30 Breeder C57BL/6J (WT) mice were from Jackson Labs. Mice were fed Teklad Irradiated Rodent Diet 8904 for breeding or Diet 2918 for maintenance (Teklad, Jackson Labs. Mice were fed Teklad Irradiated Rodent Diet (Table 2). Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and analyzed using the comparative CQ method31,32 to obtain fold-changes in gene expression. The analysis was performed in triplicate.

**Table 1.** Animals Used in This Study

| Mouse Genotype  | n   | Age, wk |
|-----------------|-----|---------|
| Mthfr+/+ & Cbs+/+ (wild-type) | 3  | 12      |
| Mthfr+/+ & Cbs+/+ (heterozygous) | 3  | 12      |
| Cbs+/+ (heterozygous) | 3  | 12      |
| Mthfr+/+ & Cbs+/+ (wild-type) | 3  | 22      |
| Mthfr+/+ & Cbs+/+ (heterozygous) | 3  | 22      |
| Cbs+/+ (heterozygous) | 3  | 22      |
| Western blot analysis to examine MTHFR, CBS, CTH, xCT, TAUT, and 3MST protein expression in the mouse retina |
| Mthfr+/+ & Cbs+/+ (wild-type) | 5  | 12      |
| Mthfr+/+ & Cbs+/+ (heterozygous) | 5  | 12      |
| Cbs+/+ (heterozygous) | 5  | 12      |
| Mthfr+/+ & Cbs+/+ (wild-type) | 5  | 22      |
| Mthfr+/+ & Cbs+/+ (heterozygous) | 5  | 22      |
| Cbs+/+ (heterozygous) | 5  | 22      |
| H2S and reduced GSH detection in the mouse retina |
| Mthfr+/+ & Cbs+/+ (wild-type) | 4  | 12      |
| Mthfr+/+ & Cbs+/+ (heterozygous) | 4  | 12      |
| Cbs+/+ (heterozygous) | 4  | 12      |
| Mthfr+/+ & Cbs+/+ (wild-type) | 4  | 22      |
| Mthfr+/+ & Cbs+/+ (heterozygous) | 4  | 22      |
| Cbs+/+ (heterozygous) | 4  | 22      |
| Amino acid profile for taurine levels in the mouse retina |
| Mthfr+/+ & Cbs+/+ (wild-type) | 2  | 12      |
| Mthfr+/+ & Cbs+/+ (heterozygous) | 1  | 12      |
| Cbs+/+ (heterozygous) | 2  | 12      |
| Mthfr+/+ & Cbs+/+ (wild-type) | 2  | 22      |
| Mthfr+/+ & Cbs+/+ (heterozygous) | 1  | 22      |
| Cbs+/+ (heterozygous) | 2  | 22      |

**Immunodetection of Proteins in Mouse Retina**

Protein was extracted from neural retinas of WT, Mthfr+/−, and Cbs+/− mice per our method.21,24,31,32 Forty to 100 μg protein was subjected to SDS-PAGE at 100 V and transferred to nitrocellulose membranes at 90 V for 1.5 hours. Membranes were blocked in 5% milk in TBST (1 hour at room temperature), followed by incubation with primary antibodies: goat anti-MTHFR (Abcam, Cambridge, MA, USA; 1:500), rabbit anti-CBS (Cell Signaling, Danvers, MA, USA; 1:250), rabbit anti-CTH (Thermo-Fisher Scientific, Waltham, MA, USA; 1:500), rabbit anti-3MST (Aviva Systems Biology, San Diego, CA, USA; 1:500), rabbit anti-xCT35 (1:800), and rabbit anti–taurine transporter (TAUT) (Alomone Labs, Jerusalem, Israel; 1:200) in 5% milk in TBST buffer at 4°C overnight. Membranes were washed three times for 5 minutes in TBST and incubated with horseradish peroxidase-conjugated goat anti-rabbit IgG antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA; 1:2000) for 1 hour at room temperature. The ECL Western blot detection system (Thermo Scientific) was used to visualize protein bands. Membranes were reprobed with GAPDH (Millipore, Temecula, CA, USA), which served as the loading control. All immunoblot images are representative of three or more independent experiments. The bands from Western blotting were quantified using Image J 1.48v software (http://imagej.nih.gov/ij/; National Institutes of Health, Bethesda, MD, USA).

**Assessment of GSH, Taurine, and H2S Levels in Mouse Retina**

Retinas were harvested from euthanized WT, Mthfr+/−, and Cbs+/− mice by propoting the eye using Dumont #5 forceps and slititng the cornea using a #15 scalpel blade. After the lens was genty ejected, the retina was lifted way from the eyecup using the forceps. Retinas were homogenized in ice-cold PBS.
and centrifuged at 10,000g for 10 minutes at 4°C; supernatant was used for GSH and H₂S detection. Protein concentration was determined using the Pierce BCA protein assay kit (Thermo-Fisher Scientific).

To estimate levels of GSH, cellular GSH levels per protein and glutathione redox state (GSH/GSSG) were determined using the Glutathione Detection Kit (ADI-900-160; Enzo Life Sciences, Farmingdale, NY, USA) according to the manufacturer’s instructions. The supernatant was treated with metaphosphoric acid (MPA; final concentration 5%; Sigma-Aldrich Corp., St. Louis, MO, USA) for 5 minutes. A diluted known volume of MPA extract was treated without (for total GSH) or with 4-vinylpyridine (Sigma-Aldrich Corp.) (only for GSSG analysis), and appropriate GSSG standards were treated similarly to prepare a standard curve. After appropriate volumes of freshly prepared reaction mix (glutathione reductase with reaction mix buffer) were added, a kinetic GSH-reductase recycling assay was performed following the manufacturer’s instructions using a Synergy Multi-Mode Reader (BioTek, Winooski, VT, USA) set at excitation:emission of 485:525 nm. The zero point was determined using the Pierce BCA protein assay kit (ThermoFisher). Fluorescence was measured using the Synergy 2 plate reader (BioTek).

### RESULTS

#### Assessment of MTHFR and CBS in Mthfr<sup>−/−</sup> and Cbs<sup>−/−</sup> Retinas

We evaluated Mthfr, Cbs, and Cth mRNA levels and MTHFR, CBS, and CTH protein levels in retinas of Mthfr<sup>−/−</sup> mice at 12 and 22 weeks. We used both retinas from a single mouse for RNA expression studies with at least three repetitions of the analysis. Similarly, we used both retinas from a single mouse to evaluate protein levels, again with at least three repetitions. Retinal expression of Mthfr in Mthfr<sup>−/−</sup> mice was noticeably decreased by 22 weeks (Fig. 3A); protein levels of MTHFR were only mildly (and not significantly) altered at this age (Fig. 3B). Quantitative data from three separate immunoblotting experiments are presented in Figure 3C. Interestingly, expression of CBS increased significantly in Mthfr<sup>−/−</sup> mice at 12 weeks, although it decreased significantly by 22 weeks (Fig. 3D). Protein levels of CBS showed a similar trend in levels, but did not reach statistical significance (Figs. 3E, 3F). Evaluation of Cbs in Mthfr<sup>−/−</sup> mice showed a significant increase in expression at 12 weeks but no difference at 22 weeks (Fig. 3G). The CTH protein levels evaluated in WT and Mthfr<sup>−/−</sup> mice showed no difference at either 12 or 22 weeks (Figs. 3H, 3I). The data suggest that, in the Mthfr<sup>−/−</sup> mouse retina, there is a detectable difference in expression of Cbs as well as Mthfr, but no significant change in levels of these proteins in mice hyperhomocysteinemic due to Mthfr mutation.

We next evaluated mRNA and protein levels in retinas of Cbs<sup>−/−</sup> mice at 12 and 22 weeks. Following the same sequence as described above, we first examined expression of Mthfr in Cbs<sup>−/−</sup> mice: there was no change at 12 weeks; there was, however, a significant decrease in Mthfr expression at 22 weeks (Fig. 4A). Analysis of MTHFR protein levels in three independent samples showed a trend toward decreasing protein levels at 22 weeks in Cbs<sup>−/−</sup> mutant mice compared with WT (Figs. 4B, 4C); however, the data did not reach statistical significance. Examination of Cbs expression in Cbs<sup>−/−</sup> retinas revealed significant decreases at 12 and 22 weeks compared with WT (Fig. 4D). Levels of the CBS protein were slightly lower in retinas of Cbs<sup>−/−</sup> mice at 12 weeks compared with WT but were markedly decreased by 22 weeks in the mutant compared with age-matched WT mice (Figs. 4E, 4F). Regarding RNA and protein levels of CTH, there were no differences between Cbs<sup>−/−</sup> and WT at either 12 or 22 weeks (Figs. 4G–I).

Taken collectively, data shown in Figures 3 and 4 suggest that there is not a compensatory increase in levels of key enzymes of the transsulfuration pathway (CBS, CTH) in Mthfr<sup>−/−</sup> mice nor a compensatory increase in levels of

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**Table 2.** PCR Primers Used for Gene Expression Studies

| Gene   | NCBI Accession Number | Forward Sequence | Reverse Sequence |
|--------|-----------------------|------------------|------------------|
| Gapdh  | NM_000804             | 5'-AGGTCCGTGTTAGCAAGCGATTG-3' | 5'-TGTAGACCAATGTAGTGAGTGTA-3' |
| Mthfr  | NM_010840             | 5'-ACAGCCCATAGCGAGAGCGCAA-3' | 5'-CACTTTAGCAAGCTGCGGAAGGA-3' |
| Cbs    | NM_144955             | 5'-TCTTTGGCCATTGTTCCAGCAT-3' | 5'-GGGACCTGCTGTTAAATGCTGGA-3' |
| Ct7a11 (xCT) | NM_011990         | 5'-GGCACCGCTACGCAGGATCAG-3' | 5'-CTCCACAGCAGCAGCAGA-3' |

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**Statistical Analysis**

Two-way ANOVA was used to test for significant differences between the three mouse groups (WT, Mthfr<sup>−/−</sup>, and Cbs<sup>−/−</sup>) at the two ages (12 and 22 weeks) studied. Tukey’s paired comparison test was the post hoc statistical test. Statistical analysis used the GraphPad Prism software (version 6; GraphPad Software, Inc., La Jolla, CA, USA). P < 0.05 was considered significant.
MTHFR in retinas of Cbs+/−/C0 mice. There is a substantial decrease in CBS protein levels in retinas of the Cbs heterozygous mice, whereas levels of MTHFR protein in the Mthfr+/−/C0 mouse retina are similar to WT. Protein levels of CTH were not altered in either mutant strain.

Given that there was a significant decrease in CBS protein level in Cbs+/−/C0 mouse retinas, we were interested in determining levels of downstream products in the transsulfuration pathway secondary to synthesis of cysteine, including GSH, taurine, and H2S.

Assessment of GSH, Taurine, and H2S in Retinal Hyperhomocysteinemia

Reduced GSH is one of the most important scavengers of reactive oxygen species (ROS), and its ratio with GSSG may be used as a marker of oxidative stress. We used a commercially available kit to analyze levels of GSH and determine the GSH/GSSG ratio in retinal samples. Our data show that GSH levels in retinas of Mthfr+/−/C0 and Cbs+/−/C0 retinas did not differ significantly from WT retinas (Fig. 5A). Similarly, the ratio of reduced to oxidized glutathione was similar between WT and mutant retinas (Fig. 5B). Glutathione is a tripeptide formed by linkage of glutamate-cysteine-glycine. Cysteine is the rate-limiting substrate for de novo GSH synthesis. Although the transsulfuration pathway is a source of cysteine, there are other mechanisms by which cells can acquire cysteine for GSH synthesis including the Na+/independent cystine-glutamate exchanger, system xc−.

System xc− is composed of two subunits: the transport specific xCT subunit and the ubiquitous 4F2hc subunit. Under physiologic conditions, system xc− transports cysteine into cells coupled to efflux of glutamate out of cells. Once inside the cell, cysteine is converted to cysteine and used in GSH synthesis. We examined xCT at the RNA and protein level in Mthfr+/−/C0 retinas (Figs. 5C–E) and Cbs+/−/C0 retinas (Figs. 5F–I).
5-F-H) and found that expression levels were similar to WT. Our data suggest that levels of GSH are maintained in the Hhcy retinas, due at least in part, to robust expression of system xc$^-$/C0, and are therefore not decreased by disruption of the transulfuration pathway.

We then evaluated the level of taurine in retinas of $\text{Mthfr}^{+/+}/\text{C0}$ and $\text{Cbs}^{+/+}/\text{C0}$ mice. Taurine is an enigmatic molecule. It is the most abundant amino acid in the retina, and it is essential for maintenance of photoreceptor cells, yet its physiologic role is unclear. Because taurine is downstream in the transulfuration pathway, we were interested in determining whether its normally high levels would be altered in retinas of mice with disruption of this pathway. Our analysis used retinal samples from mice at 12 and 22 weeks. The levels of taurine were quite high at 12 weeks in WT and the $\text{Mthfr}^{+/+}/\text{C0}$ and $\text{Cbs}^{+/+}/\text{C0}$ mice (≈800 nmol/mg protein) and slightly less at 22 weeks (Fig. 6A). Two-way ANOVA showed no difference in taurine levels for either age or mouse group analyzed. It addition to its synthesis in the transsulfuration pathway, taurine can be supplied also to tissues via TAUT, which is present in a number of cells within the retina.38 We examined the level of TAUT in WT and mutant mouse retinas at 12 and 22 weeks. In all cases, TAUT was detected abundantly, suggesting its active role in retinas of these mice (Fig. 6B). It appears that even though the transulfuration pathway is decreased in $\text{Cbs}^{+/+}/\text{C0}$ mice, taurine levels and levels of TAUT are maintained in retinas of these mice and $\text{Mthfr}^{+/+}/\text{C0}$ mice.

We next evaluated levels of H2S in retinas of $\text{Mthfr}^{+/+}/\text{C0}$ and $\text{Cbs}^{+/+}/\text{C0}$ mice using a probe that is specific for this gasotransmitter.35 The basis of detection is the dual nucleophilicity of
H₂S and a H₂S-mediated tandem nucleophilic substitution-cyclization reaction, which is used to release fluorophores and turn on fluorescence. The probe (WSP-5) shows high sensitivity and selectivity for H₂S over other reactive sulfur species (e.g., cysteine and GSH).35 Interestingly, H₂S levels were elevated significantly in mutant retinas compared to WT (Fig. 7A). Hydrogen sulfide measured at 12 weeks in retinas of WT animals averaged $\sim$1.35 μM/g protein, whereas age-

![Figure 5](image1.png)

**FIGURE 5.** Detection of GSH and xCT (Slc7a11). Retinas were harvested from 12- and 22-wk WT, Mthfr⁺⁻⁻, and Cbs⁺⁻⁻ mice and were used to detect (A) reduced GSH (pmol/mg protein) and (B) glutathione redox ratio (GSH/GSSG) using a commercially available kit according to the manufacturer’s instructions. There were no statistically significant differences between the groups. Each experiment was performed in triplicate. Additional retinas were harvested from WT, Mthfr⁺⁻⁻, and Cbs⁺⁻⁻ mice to evaluate expression of xCT. (C, F) Quantitative real-time RT-PCR analysis of xCT mRNA extracted from 12- and 22-wk WT, Mthfr⁺⁻⁻, and Cbs⁺⁻⁻ mice. Primers are provided in Table 2. There were no significant differences in gene expression. (D, G) Representative immunoblots to detect xCT in proteins extracted from 12- and 22-wk WT, Mthfr⁺⁻⁻, and Cbs⁺⁻⁻ mice. (E, H) Quantification of immunoblotting data. Error bars represent mean ± SEM from three separate experiments (n = 3). There were no significant differences in levels of xCT.

![Figure 6](image2.png)

**FIGURE 6.** Analysis of retinal taurine levels and taurine transporter (TAUT). Retinas were harvested from 12- and 22-wk WT, Mthfr⁺⁻⁻, and Cbs⁺⁻⁻ mice and were used to detect taurine levels using an amino acid analyzer and levels of TAUT by immunoblotting. (A) Quantitative data of retinal taurine levels analyzed as described in the text. (B) Representative immunoblot showing detection of TAUT. GAPDH was used as the internal control. There were no statistically significant differences between the groups from triplicate experiments.
retinas as has been reported.39 Quantification of five separate immunoblotting experiments showed a slight increase in MST in retinas of Mthfr+/− compared with WT and Cbs+/− mice at 12 weeks and a slight decrease in this protein in Mthfr+/− versus Cbs+/− and WT mice at 22 weeks (Fig. 7C); however, quantitative analysis revealed no statistically significant difference in MST protein levels between the three groups at either age.

**DISCUSSION**

Elevated Hcy levels are implicated in retinal neurovascular diseases including central retinal vein occlusion (CRVO), pseudoxefoliation glaucoma, and diabetic retinopathy.6 Controversy exists as to whether Hhcy is a biomarker or pathogenic in retinopathies. Efforts to resolve this controversy have used in vitro models and subsequently in vivo models. In vitro studies performed using GCS isolated from mouse retina showed marked vulnerability to micromolar concentrations of Hcy such that incubation of the cells 18 hours with 50 μM n.l. hcy thiolactone led to 50% cell death.40 In vivo studies demonstrated profound GC loss and inner retinal disruption within 5 days of intravitreal injection of high dosage (200 μM) of n.l. hcy thiolactone.41 Marked photoreceptor cell loss within 15 days and complete ablation of the outer nuclear layer within 90 days after hcy-thiolactone intravitreal injection (25 and 200 μM) was reported by Chang et al.12

Effects of more physiologically relevant concentrations of Hhcy can be studied using mice that harbor mutations of genes, such as Cbs and Mthfr, which cause Hhcy in the human population. We reported previously that the retinal phenotype is similar in mice with heterozygous mutations of either Cbs21,23,24 or Mthfr.25 For example, Cbs+/− and Mthfr+/− mice demonstrate loss of GCs yet little involvement of inner or outer nuclear retinal layers. Both mutant mice demonstrate electrophysiologic deficits that reflect compromised GC function, although intact photoreceptor-bipolar responses. There are alterations in the thickness in Mthfr+/− mice25 and decreased mitochondrial size in the NFL in Cbs+/− mice.43 Both mutant models have elevated glial acidic fibrillary protein (GFAP) levels, suggestive of active gliosis ongoing within the retina. Finally, the mutant mice show vascular changes, although the extent of the vasculopathy appears worse in Cbs+/− compared with Mthfr+/− mice.23 The data suggest that chronic endogenous Hhcy is mildly deleterious to retinal neurons and vasculature. These observations in the mouse models are relevant to humans. For examples, higher serum Hcy levels and altered one-carbon metabolism have been reported in a subset of astronauts on the International Space Station who developed ophthalmic changes including altered NFL thickness, optic disc edema, globe flattening, choroidal folds, hyperopic shifts, and cotton wool spots.44 There was no evidence of glaucoma or central retinal vein occlusion in the astronauts.

The functional and structural findings in the two mutant mouse models of Hhcy prompted the present study to analyze levels of key enzymes at ages that reflect time points in which only modest GC loss is observed, mild retinal function alteration is detected, and vasculopathy is modest (e.g., 12 and 22 weeks). We were interested in whether there was a compensatory effect of one pathway for the other, for example, that Cbs levels are increased in Mthfr+/− mutants or MTHFR levels are increased in Cbs+/− mutants. We did not observe marked compensatory changes. In Mthfr+/− mice, there were no significant changes in MTHFR protein levels despite decreased expression of Mthfr RNA at 22 weeks. There was a slight increase in Cbs RNA at 12 weeks, which decreased significantly by 22 weeks; there was no change in protein levels. There was an increase in Cth
expression initially, but this did not persist, and no changes were observed in CTH protein levels.

In Cbs−/− mutant mice, there was no compensatory increase in MTHFR or CTH at the RNA or protein level. There was a marked decrease in Cbs RNA expression at 12 and 22 weeks, with a dramatic decrease in Cbs protein by 22 weeks. This finding was intriguing because, in the transulfuration pathway, there are several factors that are beneficial to retina downstream of CBS including GSH, taurein, and potentially H2S. We predicted that Cbs−/− mutant retinas would have diminished levels of GSH or taurein; however, this was not the case. Total GSH levels and levels of reduced oxidized GSH in Cbs−/− retinas were similar to Mthfr−/− and WT mice. A major source of cysteine, the rate-limiting substrate for GSH production, is the cystine-glutamate exchanger, system xc−. As reviewed by McBean, the exchanger is considered the major mechanism for provision of cysteine for GSH, at least in astrocytes, although the transsulfuration pathway can also participate. In the case of the Hhcy models we investigated in this study, it appears that the exchanger is fully functional. Our finding that expression of xCT, the unique component of the exchanger, is robust in Cbs−/− and Mthfr−/− mice supports this notion. It is not feasible to study the activity of the transporter in the neural retina tissue; however, in future studies we will examine its function in isolated retinal Müller cells under Hhcy conditions. The data suggest that at least through 22 weeks, a decrease in GSH levels does not account for the GC loss and vascular changes observed in the Hhcy models.

We predicted also that because taurein is a downstream product of the transsulfuration pathway that its levels might be altered in Cbs−/− retinas. However, our analysis showed very high taurein levels in Cbs−/− retinas that were similar to Mthfr−/− and WT mice. The transsulfuration pathway is not the sole source of this amino acid in the retina, emphasizing the importance of the Na−/dependent TAUT in delivery of taurein to retina. Taurein has long been known to be essential for photoreceptor cell survival; however, new studies demonstrate a key role in protecting against retinal GC loss as well.28 We detected an abundance of TAUT in neural retinas of Hhcy and WT retinas. It may be that taurein in Cbs−/− and Mthfr−/− retinas is neuroprotective in Hhcy mice, attenuating GC loss. Future studies examining the Hhcy models that lack TAUT would permit this speculation to be tested.

A third important downstream product of the transsulfuration pathway that its levels might be altered in Cbs−/− retinas. Hydrogen sulfide levels were markedly elevated in both Cbs−/− and Mthfr−/− retinas compared with WT. The major enzymes involved in endogenous production of H2S include CBS and CTH; however, a third enzyme MST can also generate H2S.46 We examined levels of MST in Cbs−/− and Mthfr−/− retinas, but did not observe a statistical difference in protein levels in either mutants and a dramatic decrease in MST protein by 22 weeks. This MST does not account for the increased level of H2S in retinas of these mice. The prevailing thought for many years had been that H2S was a toxic and noxious gas; later, it was considered a metabolic byproduct or a marker of enzymatic activity.47 Recent studies have ascribed a much more important role to this gaseous molecule. Kimmura et al.48 showed that H2S facilitates induction of hippocampal long-term potentiation by enhancing activity of N-methyl-D-aspartate receptors and might act as a neuromodulator. Indeed, H2S is now proposed as a novel neurotransmitter, or more accurately a gasotransmitter. Work from Snyder’s group suggests that H2S signals by sulfhydration, which involves modification of cysteine residues on target proteins. There are number of proteins, whose functions are regulated by sulfhydration.29 It is noteworthy that rather than having a deleterious effect on neurons, H2S can protect primary cultures of these cells from oxidative toxicity and excitotoxicity. This observation is particularly relevant to retina, in which oxidative stress and excitotoxicity are implicated in disease.

The current findings may help in understanding whether Hhcy is pathogenic or merely a biomarker in certain retinal diseases. Using two mutant models with excess levels of Hcy due to mutations of two arms of the Hcy-methionine metabolic pathway, we were able to interrogate comprehensively levels of several key enzymes and important downstream factors directly relevant to retinal health. We did not observe altered enzyme levels nor marked decreases in GSH or taurein. These data suggest that the neurovascular phenotype may be due to directly to elevated retinal Hcy, which characterizes both models, providing some support for the notion that Hcy is pathogenic and not merely a biomarker for retinal disease. We made an additional observation, however, that was surprising regarding elevated retinal levels of H2S in the two models. We do not know whether H2S represents a beneficial phenomenon or whether it is toxic. In other words, would the GC loss and vascular phenotype in the Hhcy mutant mice be worse or better if H2S was not elevated? There has been one report that elevated H2S contributes to retinal neovascularization in an ischemia-induced retinopathy; however, the levels of CBS were actually elevated in that model.39 Future studies will explore the role of elevated H2S in the Hhcy models, as well various proteins regulated by sulfhydration that may help explain the retinal phenotype observed under Hhcy conditions.

Acknowledgments

The authors thank Rima Rozen, PhD, (McGill University, Montreal, Canada) for providing the founder Mthfr−/− mouse breeding pairs to establish our colony. Supported by National Institutes of Health Grants R01 EY012830 and R01 HG116571.

Disclosure: X. Cui, None; S. Navneet, None; J. Wang, None; P. Roon, None; W. Chen, None; M. Xian, None; S.B. Smith, None

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