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ORIGINAL RESEARCH

Diabetes Accelerates Retinal Neuronal Cell Death In A Mouse Model of Endogenous Hyperhomocysteinemia

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Abstract: Hyperhomocysteinemia has been implicated in visual dysfunction. We reported recently that mice with endogenous hyperhomocysteinemia, due to mutation of the cystathionine-β-synthase (*cbs*) gene, demonstrate loss of neurons in the retinal ganglion cell (RGC) layer and other retinal layers as homocysteine levels increase. Some clinical studies implicate hyperhomocysteinemia in the pathogenesis of diabetic retinopathy, which is also characterized by RGC loss. The present study used $cbs^{+/-}$ mice to determine whether modest elevation of plasma homocysteine, in the presence of diabetes, accelerates neuronal cell loss. Diabetes (DB) was induced in 3 wk old $cbs^{+/-}$ and wildtype mice using streptozotocin; four groups of mice were studied: DB $cbs^{+/-}$; non-DB $cbs^{+/-}$; DB $cbs^{+/+}$; non-DB $cbs^{+/+}$. One group of diabetic $cbs^{+/-}$ mice was maintained on a high methionine diet (HMD, 0.5% methionine drinking water) to increase plasma homocysteine slightly. Eyes were harvested at 5, 10 and 15 weeks post-onset of diabetes; retinal cryosections were examined by light microscopy and subjected to systematic morphometric analysis. Diabetic $cbs^{+/-}$ had significantly fewer RGCs at 5 weeks compared to age-matched, non-diabetic $cbs^{+/-}$ and wildtype controls (10.0 ± 0.5 versus 14.9 ± 0.5 and 15.8 ± 0.6 cells/100 μm retina length, respectively). Significant differences in retinas of DB/high homocysteine versus controls were obtained 15 wks post-onset of diabetes including fewer RGCS and decreased thickness of inner nuclear and plexiform layers. Moderate increases in plasma homocysteine coupled with diabetes cause a more dramatic alteration of retinal phenotype than elevated homocysteine or diabetes alone and suggest that diabetes accelerates the retinal neuronal death in hyperhomocysteinemic mice.

Keywords: mouse, homocysteine, diabetes, morphometric analysis

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Introduction

Homocysteine, a sulfur-containing, non-proteinogenic amino acid, is an intermediate in methionine metabolism. Elevation of homocysteine, termed hyperhomocysteinemia, has been implicated in the pathogenesis of a variety of diseases including cardiovascular disorders[1,2] and neurodegenerative disorders.[3,4] There is evidence that hyperhomocysteinemia may play a role in diseases of the visual system including maculopathy, open-angle glaucoma, and optic atrophy.[5–12] Severe hyperhomocysteinemia due to methionine synthase deficiency appears to decrease rod photoreceptor responses and induce retinal ganglion cell loss based on electrophysiological findings.[13] Given the potential relationship between hyperhomocysteinemia, vascular disease and neurodegeneration it is not surprising that clinical studies have examined also the relationship of hyperhomocysteinemia and diabetic retinopathy. Diabetic retinopathy is a complex disease characterized by vascular dysfunction and neuronal cell loss.[14,15] There are reports in the clinical literature suggesting a link between excess homocysteine and diabetic retinopathy.[16–21]

Over the past several years, our laboratory has used various model systems to investigate the effects of homocysteine on retinal neuronal viability. Initially we used a retinal neuronal cell line (RGC-5)[22] and subsequently primary retinal ganglion cells isolated from mouse retina[23] and showed that homocysteine induced apoptotic death of these cells, although the homocysteine levels required in the cell line were much greater than in the primary neurons. We then demonstrated that injection of micromolar concentrations of homocysteine into mouse vitreous induced apoptotic death of ganglion cells providing the first *in vivo* experimental evidence of homocysteine-induced ganglion cell loss.[24] In these studies homocysteine was applied exogenously. Recently, however, we examined the retinas of mice with a deletion of the gene coding for cystathionine-β-synthase *(cbs)*, a model for endogenous elevation of plasma homocysteine.[25] The availability of this model offered an opportunity to examine *in vivo* the effects of long term endogenous exposure to elevated levels of homocysteine in the retina. Homozygous mice ($cbs^{-/-}$) have a ~30-fold increase in plasma homocysteine levels (~200 μm compared to ~6 μM in wildtype mice) and a shortened lifespan of only 3–5 weeks.[26] They mice are a useful model of extreme elevations of homocysteine. Heterozygous mice ($cbs^{+/-}$) have a ~4-fold increase in plasma homocysteine, with a lifespan comparable to that of wildtype mice. They are a valuable model for evaluations of the effects of mild elevation of endogenous homocysteine on a variety of organs and tissues.[27] Plasma homocysteine levels had been reported previously for the *cbs* mutant mouse;[26] we examined retinal homocysteine levels by HPLC and detected ~7-fold elevation of homocysteine in retinas of $cbs^{-/-}$ mice and ~2-fold elevation of homocysteine in retinas of $cbs^{+/-}$ mice compared to age-matched wildtype mice.[25] The retinal architecture of $cbs^{+/-}$ mice was similar to that of wildtype mice during the first six months postnatally, whereas a much more profound retinal phenotype was observed in older $cbs^{+/-}$ animals (1–2 years) and in the homozygous $cbs^{-/-}$ mice involving more retinal layers including hypertrophy of the retinal pigment epithelium and decreased thickness of inner retinal layers. Dietary supplementation of drinking water with methionine increases plasma homocysteine levels in $cbs^{+/-}$ mice to ~7-fold compared to wildtype.[28] Systematic morphometric examination at various ages of retinas of heterozygous *cbs* mice ($cbs^{+/-}$) maintained on this diet demonstrated a significant loss (~20%) of cells in the retinal ganglion cell layer during the first 30 weeks postnatally.[25] The remaining nuclear and plexiform layers of the $cbs^{+/-}$ mice showed minimal disruption under these modest hyperhomocysteinemic conditions.

In the present study, we used the *cbs* mutant mouse to determine whether the retinal phenotype would be altered if the mice were also diabetic. We used a standard protocol (streptozotocin injection) to induce diabetes and systematically examined the retinas of diabetic, hyperhomocysteinemic mice. We observed that the loss of cells in the ganglion cell layer found in diabetes accelerated the cell loss observed in hyperhomocysteinemic mice.

Methods

Animals

A total of 56 mice were used in this study (Table 1). Breeding pairs of $cbs^{+/-}$ mice (B6.129P2-Cbs^{tm1Unc}/J) were purchased from the Jackson Laboratories

Table 1. Average mouse weights and blood glucose levels of hyperhomocysteinemia/diabetes study.

Treatment group	n	Mean weight ± S.D. (grams)	Blood glucose ± S.D. (mg/dL)	Age at analysis/duration of diabetes
cbs$^{+/+}$	5	19.76 ± 2.62	117 ± 31.3	8 wks/non-diabetic
	5	21.50 ± 3.06	112 ± 18.8	13 wks/non-diabetic
cbs$^{+/-}$	5	20.00 ± 3.25	109 ± 23.2	8 wks/non-diabetic
	5	21.26 ± 2.78	98 ± 17.8	13 wks/non-diabetic
Diabetic *cbs*$^{+/+}$	4	18.75 ± 2.84	419 ± 109.5	8 wks/5 wks
	4	21.25 ± 1.80	491 ± 92.5	13 wks/10 wks
Diabetic *cbs*$^{+/-}$	5	19.72 ± 2.87	450 ± 82.8	8 wks/5 wks
	5	19.18 ± 0.74	362 ± 59.3	13 wks/10 wks
Mice maintained on high methionine (HM) diet				
				Age at analysis/duration of diabetes/ duration of HM diet
Diabetic *cbs*$^{+/-}$ HM	5	21.36 ± 2.41	479 ± 61.7	18 wks/15 wks/15 wks
Non-diabetic *cbs*$^{+/-}$ HM	7	24.42 ± 1.39	116 ± 14.9	18 wks/non-diabetic/15 weeks HM
Non-diabetic *cbs*$^{+/+}$	6	23.25 ± 2.73	112 ± 18.8	18 wks/non-diabetic/normal diet

(Bar Harbor, ME) and the colony established in our laboratory as described.[25] Mice were maintained in clear plastic cages and exposed to 12 h alternating light/dark cycles (light levels 6.0–10.0 lux). Room temperature was 23 ± 1 °C. Animals were fed Harlan's Teklad rodent diet no. 8604 and administered tap water ad libitum. Diabetes was induced by injecting 3-week-old *cbs*$^{+/-}$ mice intraperitoneally with 75 mg/kg streptozotocin (Sigma, St. Louis, MO) dissolved in sodium-citrate buffer (0.01 M, pH 4.5) on 3 consecutive days following the method of Martin et al.[29] Diabetic mice were not administered insulin. Mice were screened for diabetes beginning three days after the first dose of streptozotocin by testing for the presence of glucose in urine using the Urine Strip Test. At the time of sacrifice (5 or 10 weeks post-onset of diabetes), the diabetic state of the animal was confirmed by measuring blood glucose levels via a glucometer. Fasting blood glucose levels >250 mg/dl were considered to be diabetic. To increase plasma homocysteine levels slightly, a group of *cbs*$^{+/-}$ mice was administered drinking water containing methionine (final concentration, 0.5%) at the time of weaning[28] and some of these were made diabetic as described above. Three groups of mice were studied: diabetic/high methionine *cbs*$^{+/-}$ mice, non-diabetic/high methionine *cbs*$^{+/-}$ mice and wildtype controls (non-diabetic, normal drinking water, *cbs*$^{+/+}$). At 15 wks post onset of diabetes, eyes were obtained from these mice and processed as described below. Maintenance and treatment of animals adhered to the institutional guidelines for the humane treatment of animals and to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

Microscopic evaluation and measurement procedures

Mice were euthanized by carbon dioxide asphyxiation, followed by cervical dislocation. Eyes were enucleated and flash frozen in Tissue-Tek OCT (Miles Laboratories, Elkhart, IN) by immersion in liquid nitrogen. Cryostat sections (10 µm) were obtained and stained with hematoxylin and eosin. Microscopic evaluation of retinas included scanning tissue sections for evidence of gross pathology followed by systematic morphometric analysis, which included measurements of cell height of the retinal pigment epithelium, the number of cell rows in the inner nuclear layer and outer nuclear layer, the thickness of these layers, the thickness of the inner and outer plexiform layers, the thickness of the inner and outer segments of photoreceptor cells, and the number of cells in the ganglion cell layer per 100 µm. Both the left and right eyes were measured for each of the mice used in the study; at least 3 separate sections

were examined for data collection. Measurements were made using 3 adjacent fields on the nasal and temporal sides for a total of 6 measurement points; the initial image on each side was taken ~200 μm from the optic nerve. Two independent observers performed the measurements in a masked fashion. All measurements were made using a Zeiss Axioplan-2 microscope and an HRM camera (Carl Zeiss, Inc., West Germany) and quantified using the AxioVision v. 4.5.0. program. The average of measurements for these six images in each eye was determined for each animal and an overall average was calculated for each parameter in each test group.

Statistical analysis

One-way analysis of variance was used to determine whether there were significant differences between measurements of total retinal thickness, the thicknesses of the individual retinal layers and the number of cells in the ganglion cell layer among the four groups of animals examined. Tukey's paired comparison test was the post-hoc statistical test. Data were analyzed using the NCSS 2007 program (Kaysville, UT). A p value < 0.05 was considered significant.

Results

Mouse weights and blood glucose levels

At the time animals were euthanized for study, they were weighed and blood glucose levels determined. As shown in Table 1, the non-diabetic *cbs*$^{+/-}$ mice demonstrated blood glucose levels and average weights that were comparable non-diabetic *cbs*$^{+/+}$ mice. Wildtype mice, in which diabetes was induced using streptozotocin had blood glucose levels that averaged >400 mg/dL; diabetic mice with a mutation of the *cbs* gene (*cbs*$^{+/-}$) mice had similar blood glucose levels and weights to the diabetic wildtype mice.

Histologic and morphometric analysis of mouse retinas

Histologic sections were prepared from mice in each of the four groups and subjected to systematic morphometric examination as described above. Diabetes was induced in the mice at 3 weeks postnatally and animals were examined either 5 or 10 weeks post-onset of diabetes; thus mice in the 5 week post-diabetes onset group were actually 8 weeks of age and the mice in the 10 week post-diabetes group were 13 weeks. Data were compared to age-matched, non-diabetic mice (wild-type and *cbs*$^{+/-}$). These morphometric data are provided in Figure 1. As shown, the mice that had been diabetic 5 weeks had retinal measurements similar to non-diabetic mice, wildtype and *cbs*$^{+/-}$ mice. When the total retinal thickness was measured (from inner limiting membrane to Bruch's membrane), there was no statistically significant difference between diabetic and non-diabetic nor between heterozygous *cbs* and wildtype mice. The inner and outer nuclear layers of 5 week diabetic mice were not different in

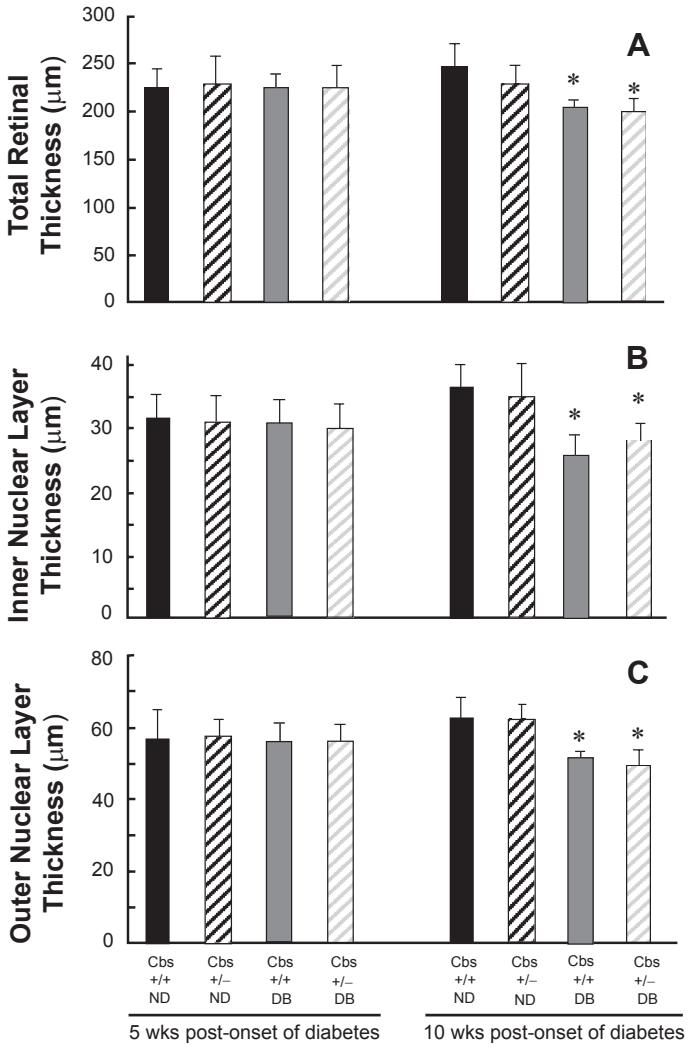

Figure 1. Morphometric analysis of retinas. Retinal cryosections from diabetic and age-matched control *cbs*$^{+/-}$ and *cbs*$^{+/+}$ mice were subjected to morphometric analysis at 5 and 10 weeks post-onset of diabetes as described in the text. (**A**) Total retinal thickness. (**B**) Thickness of inner nuclear layer (INL). (**C**) Thickness of outer nuclear layer (ONL). Data are the means ± S.E. *Significantly different from wildtype, non-diabetic control ($p < 0.05$).

thickness compared to the non-diabetic mice. When retinas of mice that had been diabetic for 10 weeks were examined, however, significant differences were observed in the total retinal thickness of mice that were diabetic. The average retinal thickness determined for non-diabetic mice (wildtype (*cbs*$^{+/+}$) and mutant (*cbs*$^{+/-}$)) was about 225–250 µm, whereas the thickness of the retinas of diabetic *cbs*$^{+/-}$ mice was ~200 µm thick. The layers that were most affected were inner and outer nuclear layers. The inner nuclear layer was typically 35–40 µm in thickness in the non-diabetic *cbs*$^{+/+}$ as well as in the *cbs*$^{+/-}$ mice. It was reduced in thickness to ~25–28 µm thick in the diabetic *cbs*$^{+/-}$ mice. The outer nuclear layer was ~60 µm thick in non-diabetic wildtype and *cbs*$^{+/-}$ mice, however in diabetic *cbs*$^{+/-}$ mice the outer nuclear layer was ~50 µm thick. The decreased thickness of the retinal layers was similar between the two diabetic mouse groups *(cbs*$^{+/-}$ and *cbs*$^{+/-}$). It appeared that diabetes was associated with the thinning of the two retinal layers, and the hyperhomocysteinemia did not accelerate this reduction in layer thickness.

Representative photomicrographs of mice in the 10 weeks post-onset diabetes group (and non-diabetic controls) are shown in Figure 2. The retinal morphology of the wildtype mouse (Fig. 2A) is well-preserved. The inner and outer nuclear layers are of uniform thickness; no disruption is noted in the inner/outer segments of the photoreceptor cells. The retinal pigment epithelial layer is intact with no visible disruption. Non-diabetic *cbs*$^{+/-}$ mice at this age have retinas that are similar to the age-matched wildtype mice, with minimal alterations notable (Fig. 2B). The observations in these mice are consistent with our earlier findings.[25] Representative retinas of diabetic *cbs*$^{+/+}$ and *cbs*$^{+/-}$ mice are shown in Figure 2C and 2D, respectively. There is noticeable thinning of the inner retinal layers as reflected in the morphometric analyses (Fig. 1).

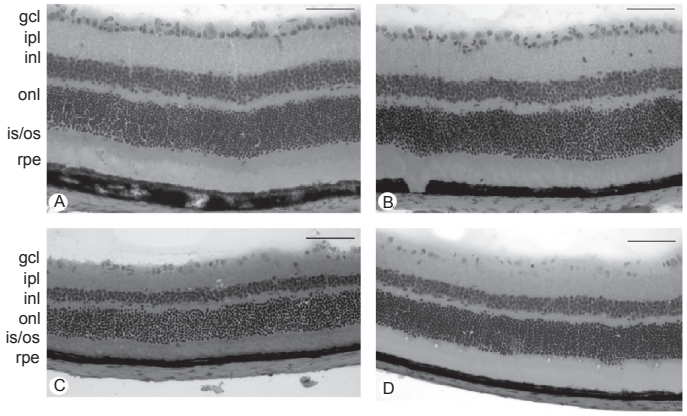

Figure 2. Photomicrographs of H&E-stained retinal cryosections (10 weeks post-onset of diabetes). Representative hematoxylin and eosin-stained cryosections of retinas harvested from (**A**) *cbs*$^{+/+}$ mouse (non-diabetic), (**B**) *cbs*$^{+/-}$ mouse (non-diabetic), (**C**) *cbs*$^{+/+}$ (diabetic) and (**D**) *cbs*$^{+/-}$ (diabetic). There are fewer cells in the gcl than in the non-diabetic, wildtype mouse (shown in A) or in the non-diabetic, mutant *cbs*$^{+/-}$ mouse. Magnification scale bar = 50 µm.
Abbreviations: gcl, ganglion; cell layer, inl, inner nuclear layer; onl, outer nuclear layer; is/os, inner segments and outer segments; rpe, retinal pigment epithelium.

In addition to measuring the thickness of the various retinal layers, we examined also the number of cells in the ganglion cell layer (Fig. 3). In our previous study reporting the retinal phenotype of the *cbs* mutant mice,[25] we found that there were fewer cells in the ganglion cell layer of hyperhomocysteinemic mice compared to wildtype mice. In the current work, retinal sections were examined from temporal to nasal ora serrata, cells of the ganglion cell layer were counted and data expressed as number of cells per 100 µm length of retina. Typically, the wildtype mouse has 14–15 cells per 100 µm length and this was observed in non-diabetic wildtype mice (Fig. 3C). The data obtained at 5 weeks post-onset diabetes the data were particularly interesting. The non-diabetic wildtype mice had ~14 cells/100 µm length, the non-diabetic *cbs*$^{+/-}$ mice had ~12.5 cells/100 µm which was similar to the number of cells detected in the diabetic, normal homocysteine (*cbs*$^{+/+}$) mice. The diabetic, hyperhomocysteinemic *cbs*$^{+/-}$ mice had significantly fewer cells than all other groups with only 11 cells/100 µm length. Thus, the combination of insults (hyperglycemia and hyperhomocysteinemia) at this early age induced greater cell death than either of the two insults individually. Interestingly, by the time the mice had been diabetic for 10 weeks, the number of cells counted in the ganglion cell layer of the diabetic mice was equivalent (~10 cells per 100 µm length) regardless of the homocysteine status of the mice. These data suggest that the death of cells in the ganglion cell layer is accelerated when the two insults (hyperhomocysteinemia and diabetes) are both present. The photomicrographs in Figure 3 (A and B) show the ganglion cell layer of a wildtype, non-diabetic mouse compared to a diabetic *cbs*$^{+/-}$ mouse. At early ages it appears that hyperglycemia accelerates the ganglion cell death associated with

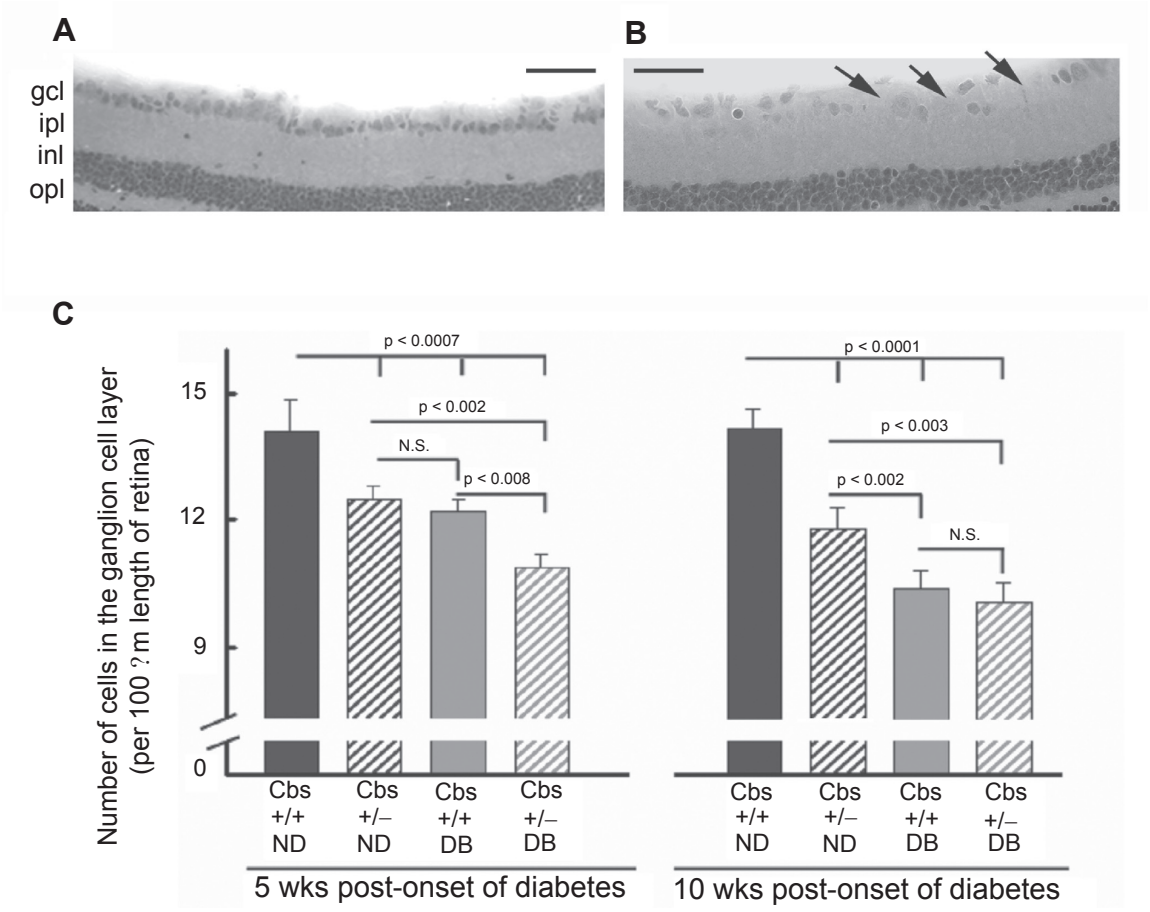

Figure 3. Analysis of cells in the ganglion cell layer of diabetic and non-diabetic cbs mice. Photomicrographs of the retinal ganglion cell layer of non-diabetic *cbs*$^{+/+}$ (wildtype) mouse (**A**) and diabetic *cbs*$^{+/+}$ mouse (**B**) at 10 weeks post-onset diabetes. Arrows point to regions of significant cell loss compared to wildtype retinas in which cells are uniformly distributed. (**C**) Retinas were harvested from *cbs*$^{+/+}$ (wildtype) or *cbs*$^{+/+}$ mutant mice that were either non-diabetic (ND) or diabetic (DB) for either 5 or 10 weeks, cryosections were prepared and analyzed morphometrically. The number of cells in the ganglion cell layer was counted and data were expressed as number of cells/100 µm length. Data were analyzed by ANOVA. In the 5 week group $F = 11.29$, $p < 0.007$ and in the 10 week group $F = 15.37$, $p < 0.001$. Post hoc analysis revealed in which groups there were statistically significant differences and these are indicated on the graph as p-values. Magnification scale bar = 50 µm.

hyperhomocysteinemia caused by a mutation in the cystathionine-β-synthase gene; as the mice age this effect is eclipsed by the effects of the diabetes alone.

It has been reported that homocysteine levels can be increased in *cbs*$^{+/-}$ mice by maintaining them on a high methionine (HM) diet.[28] We used this method to increase the endogenous homocysteine in one group of mice and concomitantly induced diabetes by streptozotocin injection. In this group of mice, there was also an effect of hyperhomocysteinemia/hyperglycemia on retinal integrity. At 15 weeks, total retinal thickness was significantly decreased in the diabetic *cbs*$^{+/-}$ HM mice compared to wildtype, non-diabetic controls (188.0 µm ± 7.0 versus 243.2 µm ± 12.9 (Fig. 4C). Measurements of the inner plexiform and nuclear layers revealed a marked decrease in diabetic *cbs*$^{+/-}$ HM mice compared to controls (34.1 µm ± 1.3 versus 43.5 µm ± 2.1 (Fig. 4D) and 27.1 µm ± 1.1 versus 36.2 µm ± 1.7 (Fig. 4E), respectively. In addition, diabetic *cbs*$^{+/-}$ HM mice had significantly fewer retinal ganglion cells than controls (9.3 ± 0.1 cells/100 µm versus 14.1 ± 0.4 cells/100 µm, respectively, Fig. 4F). Data are provided also for comparison of the diabetic *cbs*$^{+/-}$ HM mice to non-diabetic *cbs*$^{+/-}$ HM mice. In this case, the alterations observed in the diabetic retina were more pronounced when there was an even greater elevation of homocysteine. The data provide further evidence that the effect of hyperhomocysteinemia on the retina is compounded in the presence of diabetes (Fig. 4C–F).

Discussion

The current study was designed to examine *in vivo* effects of diabetes and hyperhomocysteinemia on the

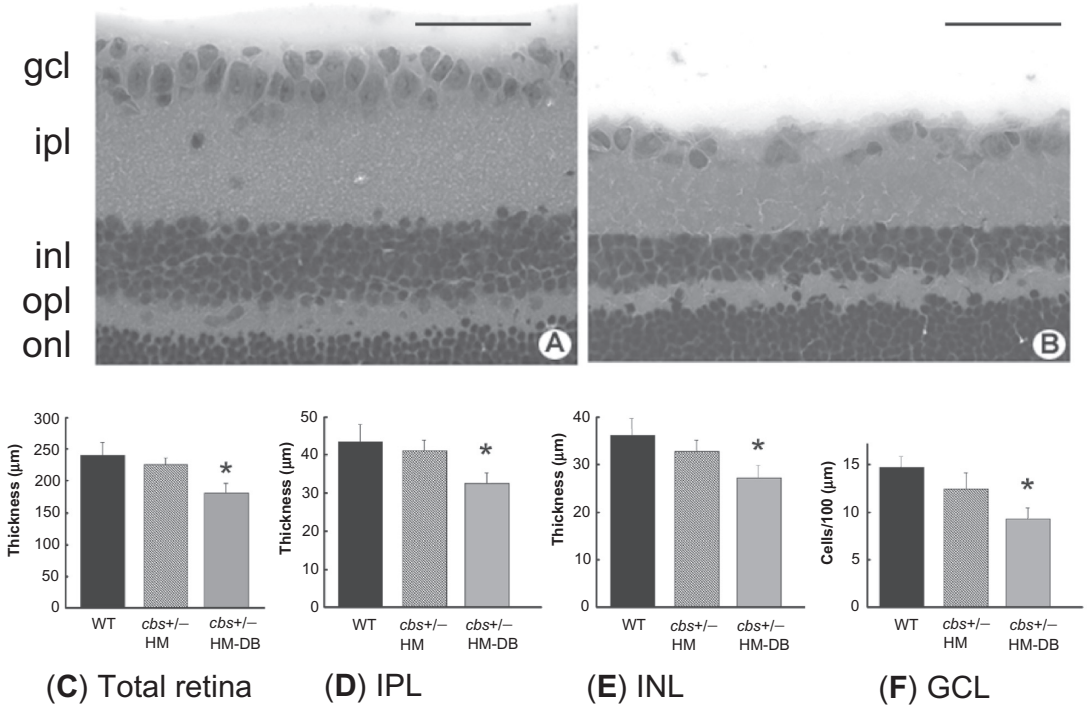

Figure 4. Analysis of retinas of diabetic cbs$^{+/-}$ mice maintained on a high-methionine diet. At the time of weaning, *cbs*$^{+/-}$ mice were placed on a diet containing 0.5% methionine (HM) in drinking water to increase endogenous homocysteine; some of these were made diabetic using streptozotocin as described in the text. Representative photomicrographs of the retina of a non-diabetic *cbs*$^{+/+}$ mouse (**A**) and a diabetic *cbs*$^{+/-}$ HM mouse (**B**) (15 weeks post-onset of diabetes). Retinas were subjected to systematic morphometric examination as described in the text. Significant differences were obtained in measurements of the total retinal thickness (**C**), the inner plexiform layer, IPL (**D**), the inner nuclear layer, INL (**E**) and in the number of ganglion cells, GCL (**F**). The number of cells in the ganglion cell layer was counted and data were expressed as number of cells/100 µm length. (*Significantly different from wildtype mice and from non-diabetic *cbs*$^{+/-}$HM mice, $p < 0.05$.)

Abbreviations: gcl, ganglion cell layer; ipl, inner plexiform layer; inl, inner nuclear layer; opl, outer plexiform layer; onl, outer nuclear layer; wt, non-diabetic; wildtype (*cbs*$^{+/+}$) mouse; *cbs*$^{+/-}$HM, non-diabetic; heterozygous *cbs* mouse on high methionine diet; *cbs*$^{+/-}$HM-DB, diabetic heterozygous *cbs* mouse on high methionine diet.

mammalian retina. We sought to extend our earlier findings regarding the retinal degeneration that occurs in the *cbs*$^{+/-}$ mouse, a model of endogenous hyperhomocysteinemia.[25] In that study, the most significant alteration in the retina of *cbs*$^{+/-}$ mice was a decrease in the number of cells in the retinal ganglion cell layer.[25] When homocysteine was elevated further in these mice, either by maintenance on a high methionine diet or in mice homozygous for the *cbs* mutation (*cbs*$^{-/-}$), the phenotype was even more severe and thinning of retinal layers was noted. In the present study, we were interested in determining whether hyperglycemia would alter the severity or temporal expression of retinopathy observed in these hyperhomocysteinemic mice. Most notably, we were interested in determining whether the loss of ganglion cells would be accelerated if the *cbs*$^{+/-}$ mice were also diabetic.

The data obtained from the current morphometric analysis suggest that ganglion cell death is accelerated when diabetes is induced in the hyperhomocysteinemic mice. That is, the retinas of the hyperhomocysteinemic mice that had been diabetic 5 weeks had fewer ganglion cells than retinas of mice in the other age-matched groups. In the group of mice in which the homocysteine was elevated slightly by dietary manipulation (methionine drinking water), the phenotype in the presence of diabetes was even more profound with greater loss of ganglion cells and marked thinning of inner nuclear and plexiform layers of the retina. The data suggest that the combined effects of homocysteine and hyperglycemia are injurious to retinal neurons and provide the first evidence of this relationship in an endogenous animal model of hyperhomocysteinemia. Owing to reports that insulin confers neuroprotection,[30] the mice used in this study were not maintained on insulin; as a consequence the diabetic mice did not live beyond ~20 weeks.[29]

Our interest in the relationship between hyperhomocysteinemia and diabetes is based

on clinical studies that report an elevation of homocysteine in patients with diabetes mellitus.[16–21] Most of these studies analyze patients with diabetes or diabetic retinopathy for homocysteine levels in their plasma and/or vitreous. In general, the purpose of these clinical studies is to examine whether there is a correlation between hyperhomocysteinemia and various stages of progression of diabetic retinopathy; the data suggest that there is. Many of the reports however center on homocysteine and its effects on vascular changes in the retina of diabetic patients. For example, Aydemir et al[16] report an elevation of homocysteine in vitreous of diabetic patients and speculate that the homocysteine elevation is due to a breakdown of the blood retinal barrier.[16] Aydin and co-workers examined the association of plasma homocysteine and macular edema in type 2 diabetes and suggested that hyperhomocysteinemia may play a role in vascular dysregulation and endothelial dysfunction in patients with diabetic retinopathy, particularly in development of macular edema.[31] In a separate clinical study Brazionis and colleagues suggest that plasma total homocysteine concentration may be a useful biomarker and/or a novel factor for increased risk of diabetic retinopathy in people with type 2 diabetes.[32] Others have evaluated the role of homocysteine and extra-cellular matrix changes in vitreous associated with diabetic retinopathy. Coral and colleagues evaluated homocysteine levels and the activity of lysyl oxidase, an enzyme that participates in collagen-elastin cross-linking, in vitreous samples of patients with diabetic retinopathy and found that increased homocysteine was associated with decreased lysyl oxidase activity.[31]

Thus, the literature is replete with evidence of a link between hyperhomocysteinemia and vasculopathy associated with diabetic retinopathy. There have been fewer clinical analyses of retinal neuronal changes associated with elevated homocysteine; however Poloschek provided a detailed investigation of the visual system in a patient with isolated methionine synthase deficiency.[13] Severe hyperhomocysteinemia is a consequence of methionine synthase deficiency. The investigators observed decreased photoreceptor function and ganglion cell loss as indicated by pathological flash visual evoked potentials (VEPs) suggesting the cytotoxic impact of homocysteine on neurons of the visual pathway. The investigators also report reduced oscillatory potentials suggesting microvascular damage to the retina through homocysteine. The relationship of hyperhomocysteinemia and visual function warrants further investigation particularly given that excess homocysteine is implicated in other neuronal degenerations.[3,4]

The present study utilized an animal model to examine the effects of excess homocysteine on retinal neuronal health in the presence of hyperglycemia. While long-acknowledged as a vascular disease, diabetic retinopathy also has a neuronal component that diminishes visual function.[14,15] The plethora of data from clinical studies demonstrating elevated homocysteine in the vitreous of patients with diabetes may be relevant not only to the vascular aspects of diabetic retinopathy, but also may play a role in death of retinal neurons characteristic of this disease. While the current studies induced diabetes in the $cbs^{+/-}$ mutant mouse using streptozotocin, there are endogenously occurring models of diabetes that have been described in mice. Indeed the retinal neuronal death is not as severe in the streptozotocin-induced model[29] as it is for example in $Ins2^{Akita/+}$ mouse, an endogenous model of diabetes.[34] In this model ganglion cells die by apoptosis and inner retinal layers are significantly thinner.[35–38] It will be informative in future studies to analyze retinas of mice harboring mutations of both of these genes (*cbs* and $Ins2^{Akita}$) for the synergistic consequences on retina of hyperglycemia and homocysteine. Additionally, it will be important to perform electrophysiological studies on the *cbs* mutant mice to determine visual function in the presence and absence of diabetes.

Disclosure

The authors report no conflicts of interest.

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