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Age-related changes in visual function in *cystathionine-beta-synthase* mutant mice, a model of hyperhomocysteinemia

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ABSTRACT

Homocysteine is an amino acid required for the metabolism of methionine. Excess homocysteine is implicated in cardiovascular and neurological disease and new data suggest a role in various retinopathies. Mice lacking *cystathionine-beta-synthase* ($cbs^{-/-}$) have an excess of retinal homocysteine and develop anatomical abnormalities in multiple retinal layers, including photoreceptors and ganglion cells; heterozygous ($cbs^{+/-}$) mice demonstrate ganglion cell loss and mitochondrial abnormalities in the optic nerve. The purpose of the present study was to determine whether elevated homocysteine, due to absent or diminished *cbs*, alters visual function. We examined $cbs^{-/-}$ (3 weeks) and $cbs^{+/-}$ mice (5, 10, 15, 30 weeks) and results were compared to those obtained from wild type (WT) littermates. Conventional dark- and light-adapted ERGs were recorded, along with dc-ERG to assess retinal pigment epithelial (RPE) function. The visual evoked potential (VEP) was used to assess transmission to the visual cortex. The amplitudes of the major ERG components were reduced in $cbs^{-/-}$ mice at age 3 weeks and VEPs were delayed markedly. These findings are consistent with the early retinal disruption observed anatomically in these mice. In comparison, at 3 weeks of age, responses of $cbs^{+/-}$ mice did not differ significantly from those of WT mice. Functional abnormalities were not observed in $cbs^{+/-}$ mice until 15 weeks of age, at which time amplitude reductions were noted for the ERG a- and b-wave and the light peak component, but not for other components generated by the RPE. VEP implicit times were delayed in $cbs^{+/-}$ mice at 15 and 30 weeks, while VEP amplitudes were unaffected. The later onset of functional defects in $cbs^{+/-}$ mice is consistent with a slow loss of ganglion cells reported previously in the heterozygous mutant. Light peak abnormalities indicate that RPE function is also compromised in older $cbs^{+/-}$ mice. The data suggest that severe elevations of homocysteine are associated with marked alterations of retinal function while modest homocysteine elevation is reflected in milder and delayed alterations of retinal function. The work lays the foundation to explore the role of homocysteine in retinal diseases such as glaucoma and optic neuropathy.

1. Introduction

Homocysteine, a nonproteinogenic and sulfur-containing amino acid, is an intermediate compound in methionine metabolism. Excess homocysteine is detrimental to normal physiological function; hence homocysteine is typically remethylated to methionine or converted via the transsulfuration pathway to cysteine under the control of the enzyme *cystathionine-beta-synthase* (*CBS*). *CBS* activity plays a critical role in regulating homocysteine concentrations, and *CBS* dysfunction is associated with elevated homocysteine levels. In human, *CBS* mutations underlie a complex phenotype with ocular, skeletal, neurological and cardiovascular components (Mudd, 2011).

In patient-based studies, elevated homocysteine has been detected in a number of clinical conditions affecting the eye, including AMD (Axer-Siegel et al., 2004; Seddon et al., 2006); maculopathy and retinal degeneration (Tsina et al., 2005); primary

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and secondary open-angle glaucoma (Bleich et al., 2002); exfoliative glaucoma (Vessani et al., 2003), pigmentary glaucoma (Jaksic et al., 2010), proliferative vitreoretinopathy (Aydemir et al., 2008), type 2 diabetes mellitus (Yang et al., 2002), central retinal vein occlusion (Lahey et al., 2003). Poloschek et al. (2005) reported abnormal electroretinograms (ERGs) and visual evoked potentials (VEPs) in a child with severe hyperhomocysteinemia due to methionine synthase deficiency.

To understand mechanisms of homocysteine-induced disruption of retina, *in vitro* and *in vivo* model systems have been used. Homocysteine upregulates VEGF expression and induces ER stress in cultured ARPE-19 cells (Roybal et al., 2004). It induces apoptotic death in the RGC-5 cell line (Ganapathy et al., 2010) and very modest levels of homocysteine induce apoptotic death of primary ganglion cells harvested from neonatal mice (Dun et al., 2007). Analysis of retinas of mice in which high concentrations of homocysteine were administered via an intravitreal route revealed marked ganglion cell loss and disruption of the inner retina within a few days of injection (Moore et al., 2001) and significant loss of photoreceptor cells when the retinas were exposed to homocysteine for several months (Chang et al., 2011). The availability of a mouse in which the *cbs* gene is deleted ($cbs^{-/-}$) (Watanabe et al., 1995) has permitted analysis of the effects of endogenous elevation of homocysteine on retinal neurons. Depending upon whether the mouse has one *cbs* allele ($cbs^{+/-}$) or no copies of *cbs* ($cbs^{-/-}$), the plasma homocysteine ranges from moderate to severe, respectively. Comprehensive morphological analysis of retinas of homozygous mutant mice ($cbs^{-/-}$) revealed alterations in the RPE, loss of cells in the ganglion cell layer, and marked disruption of the inner/outer nuclear retinal layers (Ganapathy et al., 2009). These mice have ~30-fold increase in plasma homocysteine, ~7-fold increase in retinal homocysteine and a lifespan of ~3–5 weeks; they are a model of severe hyperhomocysteinemia (Watanabe et al., 1995). Analysis of retinas of heterozygous mice ($cbs^{+/-}$) reveals a much milder phenotype characterized by modest cell loss in the ganglion cell layer and decreased thickness of the inner plexiform and nuclear layers. Gross disruption of the retina has not been observed in heterozygous mice ($cbs^{+/-}$), which have ~4–7-fold increase in plasma homocysteine and reflect moderate hyperhomocysteinemia. Interestingly, the retinal ganglion cell loss is accompanied by alterations of the mitochondria of the nerve fiber layer detectable at the ultrastructural level and by increased expression of two mitochondrial proteins, Opa1 and Fis1 (Ganapathy et al., 2011). Thus, the histological appearance of retinas of the *cbs* mutant mice has been analyzed comprehensively; however it is not known what functional alterations accompany these retinal abnormalities. The present study addressed this by using visual electrophysiological protocols to study *cbs* mutant mice at different ages.

2. Methods

2.1. Mice

Generation of mice deficient in *cbs* was reported by Watanabe et al. (1995) and a colony of mice has been established at the Jackson Laboratories (Bar Harbor, ME). Breeding pairs of $cbs^{+/-}$ mice (B6.129P2-Cbs^{tm1Unc}/J) were used to generate $cbs^{-/-}$, $cbs^{+/-}$ and $cbs^{+/+}$ (wild type; WT) mice. Genotyping, husbandry and housing conditions for the mice were as described (Ganapathy et al., 2009). All experiments involving mice were approved by the Institutional Animal Care and Use Committee of the Cleveland Clinic Foundation and adhered to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

2.2. Visual electrophysiology

Three recording protocols were used. All were conducted following overnight dark adaptation, after which mice were anesthetized with ketamine (80 mg/kg) and xylazine (16 mg/kg), and placed on a temperature-regulated heating pad. The pupils were dilated with eyedrops (2.5% phenylephrine HCl, 1% cyclopentolate, 1% tropicamide); for ERG studies, the corneal surface was anesthetized with 1% proparacaine HCL eyedrops.

2.2.1. Dark- and light-adapted ERG

To evaluate responses of the outer retina, we used a conventional strobe-flash ERG protocol. The ERG was recorded using a stainless-steel wire active electrode that contacted the corneal surface through 1% methylcellulose. Needle electrodes placed in the cheek and tail served as reference and ground leads, respectively. Responses were differentially amplified (0.3–1500 Hz), averaged, and stored using a UTAS E-3000 signal averaging system (LKC Technologies, Gaithersburg, MD). Strobe flash stimuli were initially presented in darkness within a ganzfeld bowl; across-trials flash luminance ranged from −3.6 to 2.1 log cd s/m^2. Stimuli were presented in order of increasing luminance and the interstimulus interval (ISI) was progressively increased from 4 to 90 s. A steady adapting field (20 cd/m^2) was presented in the ganzfeld. After a 7 min light adaptation period cone ERGs were evoked by strobe flash stimuli superimposed upon the adapting field. Flash luminance ranged from −0.8 to 1.9 log cd s/m^2. Stimuli were presented at 2 Hz in order of increasing luminance and 25 successive responses were averaged for each stimulus condition. The amplitude of the a-wave was measured 7 ms after flash onset from the prestimulus baseline. The amplitude of the b-wave was measured from the a-wave trough to the peak of the b-wave or, if no a-wave was present, from the prestimulus baseline.

2.2.2. Direct current (dc)-ERG

To measure ERG components generated by the RPE, responses were obtained from the corneal surface of the left eye using a 1-mm-diameter borosilicate capillary tube with filament (BF100-50-10; Sutter Instrument, Novato, CA). The capillary was filled with Hank's buffered salt solution to make contact with an Ag/Ag Cl wire electrode that was shielded from light stimulation. A similar electrode placed in contact with the right eye served as the reference. Responses were differentially amplified at dc-100 Hz (gain = ×1000; DP-301, Warner Instruments, Hamden, CT), digitized at 20 Hz, and stored using LabScribe Data Recording Software (iWorx; Dover, NH). After the initial setup for each mouse was complete, the recorded baseline was monitored until the baseline became stable. White light stimuli were then derived from an optical channel using a Leica microscope illuminator as the light source and delivered to the test eye with a 1-cm-diameter fiber-optic bundle. The stimulus luminance was 2.4 log cd/m^2. A Uniblitz shutter system was used to control stimulus duration at 7 min.

The mouse dc-ERG has four major components (Wu et al., 2004). The amplitude of the c-wave was measured from the prestimulus baseline to the peak of the c-wave. The amplitude of the fast oscillation (FO) was measured from the c-wave peak to the trough of the FO. The amplitude of the light peak (LP) was measured from the FO trough to the asymptotic value. The off-response amplitude was measured from the LP value just prior to stimulus light offset to the peak of the initial component.

2.2.3. Visual evoked potential

To measure responses of the visual cortex, VEPs were recorded using an active electrode positioned along the midline of the visual cortex. The positions of the reference and ground leads were the