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***Mthfr* as a modifier of the retinal phenotype of *Crb1*^{rd8/rd8} mice**

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Abstract

Mutations in crumb homologue 1 (*CRB1*) in humans are associated with Leber's congenital amaurosis (LCA) and retinitis pigmentosa (RP). There is no clear genotype–phenotype correlation for human *CRB1* mutations in RP and LCA. The high variability in clinical features observed in *CRB1* mutations suggests that environmental factors or genetic modifiers influence severity of *CRB1* related retinopathies. Retinal degeneration 8 (*rd8*) is a spontaneous mutation in the *Crb1* gene (*Crb1*^{rd8/rd8}). *Crb1*^{rd8/rd8} mice present with focal disruption in the outer retina manifesting as white spots on fundus examination. Mild retinal dysfunction with decreased b-wave amplitude has been reported in *Crb1*^{rd8/rd8} mice at 18 months. Methylene tetrahydrofolate reductase (*MTHFR*) is a crucial enzyme of homocysteine metabolism. *MTHFR* mutations are prevalent in humans and are linked to a broad spectrum of disorders including cardiovascular and neurodegenerative diseases. We recently reported the retinal phenotype in *Mthfr*-deficient (*Mthfr*^{+/-}) heterozygous mice. At 24 weeks the mice showed decreased RGC function, thinner nerve fiber layer, focal areas of vascular leakage and 20% fewer cells in the ganglion cell layer (GCL). Considering the variability in *CRB1*-related retinopathies and the high occurrence of human *MTHFR* mutations we evaluated whether *Mthfr* deficiency influences rd8 retinal phenotype. *Mthfr* heterozygous mice with rd8 mutations (*Mthfr*^{+/-} *rd8/rd8*) and *Crb1*^{rd8/rd8} mice (*Mthfr*^{+/+} *rd8/rd8*) mice were subjected to comprehensive retinal evaluation using ERG, funduscopy, fluorescein angiography (FA), morphometric and retinal flat mount immunostaining analyses of isolectin-B4 at 8–54 wks. Assessment of retinal function revealed a significant decrease in the a-, b- and c- wave amplitudes in *Mthfr*^{+/-} *rd8/rd8* mice at 52 wks. Fundoscopic evaluation demonstrated the presence of signature rd8 spots in *Mthfr*^{+/+} *rd8/rd8* mice and an increase in the extent of these rd8 spots in *Mthfr*^{+/-} *rd8/rd8* mice at 24 weeks and beyond. FA revealed marked vascular leakage, ischemia and vascular

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tortuosity in *Mthfr*^{+/-}*rd8/rd8* mice at 24 and 52 weeks. Retinal dysplasia was observed in ~14–33% *Mthfr*^{+/-}*rd8/rd8* mice by morphometric analysis. This was accompanied by a ~20% reduction in cells of the GCL of *Mthfr*^{+/-}*rd8/rd8* mice at 24 and 52 weeks. Retinal flat mount immunostaining with isolectin-B4 showed neovascularization and loss of blood vessel integrity in *Mthfr*^{+/-}*rd8/rd8* mice in contrast to mild vasculopathy in *Mthfr*^{+/+}*rd8/rd8* mice. Taken together, our data support an earlier onset and worsened retinal phenotype when *Mthfr* and *rd8* mutations coexist. Our study sets the stage for future studies to investigate the role of *MTHFR* deficiency in human *CRB1* retinopathies.

Keywords

CRB1; homocysteine; retina; rd8; MTHFR; mouse; LCA; RP

Introduction

In polarized cells such as retinal pigment epithelial (RPE) cells and photoreceptor cells, maintenance of apical and basal compartment polarity is imperative for cell to cell connections, communication and directional transport (Gosens et al, 2008). Crumbs proteins are highly conserved between species (humans, mice, fish, and drosophila) and play a crucial role in the maintenance of apico-basal cell polarity (Tepass et al, 1990). Homozygous *crb* mutations in drosophila result in loss of the cuticle of the embryos with a few remaining grains resembling crumbs, hence the name “crumbs” was derived (Tepass et al, 1990; Grawe et al, 1996). Crumbs homologue (*CRB*) is found in humans and mice with crumbs family members (*CRB1/Crb1*, *CRB2/Crb2* and *CRB3/Crb3*). *Crb1* is limited in expression to brain and retina (Hollander et al, 2001). *Crb2* is expressed in RPE, heart, lung, kidney and placenta (Vanden Hurk et al, 2005) and *Crb3* is expressed in retina and various other tissues (Makarova et al, 2003). The *Crb1* is expressed in the subapical region (SAR) above the outer or external limiting membrane (ELM). The ELM is formed by adherens junctions between Müller glia and photoreceptors. SAR is located above adherens junctions and consists of a group of adaptor proteins including CRB1 (Richard et al, 2006). Mutations in the *CRB1* gene in humans are implicated in various human retinal degenerative diseases such as Leber’s congenital amaurosis (LCA), autosomal recessive retinitis pigmentosa (ArRP), and retinitis pigmentosa with coats-like exudative vasculopathy (Hollander et al, 2004; McKay et al, 2005; Mehalow, et al 2003). LCA is one of the most severe forms of retinal dystrophies leading to blindness or severe visual damage in humans (Hollander et al, 2004). ArRP is characterized by a progressive loss of photoreceptor cells. A rare complication of ArRP is the development of coats-like exudative vasculopathy, characterized by vascular defects, extravascular lipid depositions and retinal detachment (Richard et al, 2006). Interestingly, there is no clear genotype–phenotype correlation for human *CRB1* mutations in RP and LCA. The high variability in clinical features observed in *CRB1* mutations suggests that environmental factors or genetic modifiers influence the severity of the *CRB1* related retinopathies (Bujakowska et al, 2012; Gosens et al, 2006; Henderson et al, 2011; Hollander et al, 2004; Richard et al, 2006; Luhmann et al, 2015). Identification of modifiers that impact the severity of *CRB1*-related diseases is therefore timely.

Three murine models are available to investigate the consequences of *CRB1* mutation, *Crb1*^{-/-}, *CrbC*^{249W} knock-in mouse and *Crb1*^{rd8/rd8} mouse. The *Crb1*^{-/-} (knock-out) mouse model has no functional CRB1. The retinal phenotype includes disrupted ELM, half or pseudo rosettes and cell death in the INL and ONL after 3–9 months (Van de Pavert et al, 2004). The *CrbC*^{249W} knock-in mouse manifests late onset loss of photoreceptor cells (Van de Pavert et al, 2007). The *Crb1*^{rd8/rd8} mouse is homozygous for a single base pair deletion in exon 9 of the mouse *Crb1* gene (nt3481) (Gosens et al, 2008; Hollander et al, 1999). The retinal abnormalities include focal disruption of the ELM resulting in loss of connection between the Müller and photoreceptors cells, shortened inner and outer segments, retinal folds and half-rosettes observed ~5 weeks after birth (Mehalow et al, 2003). The morphological changes of the *Crb1*^{rd8/rd8} retina are not accompanied by ERG dysfunction. ERG in rd8 mice is comparable to that of wild type (WT) mice through 18 months of age, although a decrease in the b-wave amplitude has been reported after this age (Aleman et al, 2011).

Our laboratory has been investigating the effects of elevated levels of homocysteine in the retina. The most common genetic cause for excess homocysteine is mutation of methylene tetrahydrofolate reductase (*MTHFR*), a key enzyme in the remethylation pathway converting homocysteine to methionine (Perla-Kajan et al, 2007; Selhub, 1991). Methionine is used further in synthesis of S-adenosylmethionine (SAM). SAM is a crucial methyl donor for several methylation reactions in the body including DNA methylation (Perla-Kajan et al, 2007; Selhub, 1991). Recently, we reported the retinal phenotype of *Mthfr* heterozygous mice, a murine model of elevated homocysteine (Markand et al, 2015). By 24 weeks of age, *MTHFR* heterozygous mice exhibit reduced ganglion cell function by ERG, thinner nerve fiber layer, ~20% reduction in the number of cells in the ganglion cell layer and mild retinal vasculopathy.

Various *MTHFR* polymorphisms have been identified and investigated; one of the most common single nucleotide polymorphisms (SNP) is 677C>T mutation observed in the heterozygous state in 44% of Americans. The 677C>T mutation predisposes an individual to mild or moderate increases in plasma homocysteine levels and is termed as hyperhomocysteinemia (Hhcy) (Frosst et al, 1995). The 677C>T mutation is linked with cardiovascular disorders, neurodegenerative diseases (Föding et al, 1991), infertility (Chen et al, 2001), inflammatory bowel disease (Mahmud et al, 1999) and genomic instability (Sohn et al, 2009; Ueland et al 2001). Considering the high occurrence of *MTHFR* mutations and association with various disorders coupled with the need for identification of genetic modifiers in *CRB1* related retinal diseases, it is possible that these two mutations coexist in humans and influence the severity of *CRB1* related retinal disease. Indeed, the identification of genes that modify *CRB1* was the topic of discussion at a recent ARVO special interest group meeting (Tsang, 2015). The *Mthfr*^{+/-} mice bred on rd8 background afford a valuable tool to investigate this possibility. We hypothesize that *Mthfr* deficiency is a modifier in rd8-related retinal pathologies.

Methods

Animals

Eighty one (81) mice were used in the study (Table I). *Mthfr*^{+/-} mice generated on the C57BL/6 background in the Rozen laboratory (McGill University Montreal, Canada) were shipped to the animal facility of Georgia Regents University. The mice harbored the rd8 mutation. The mice were bred to establish colonies of *Mthfr*^{+/+ rd8/rd8} and *Mthfr*^{+/- rd8/rd8} mice. Our nomenclature throughout this report is: *Mthfr*^{+/+ rd8/rd8} to represent the rd8 (homozygous) mutant and *Mthfr*^{+/- rd8/rd8} to represent the 'double mutant'. Body weight measurements and genotyping were performed (Chen et al, 2001; Mattapallil et al, 2012). Experiments were approved by the Institutional Animal Care and Use Committee of Georgia Regents University and adhered to the institutional guidelines for humane treatment of animals and to the ARVO statement for Use of Animals in Ophthalmic and Vision Research.

Electroretinogram (ERG)

Visual function was assessed at 32 and 52 weeks in *Mthfr*^{+/+ rd8/rd8} and *Mthfr*^{+/- rd8/rd8} mice as described (Markand et al, 2015). Briefly, mice were dark adapted overnight. Mice were anesthetized using isoflurane anesthesia. Proparacaine HCl (0.5%, Akorn, Lake Forest IL), Tropicamide (0.5%, Akorn), and Phenylephrine HCl (2.5%, Paragon, Portland OR) eye drops were applied for topical anesthesia and mydriasis. Stimuli were generated by a white LED. Light from the LED was presented to the eyes by 1 mm diameter optic fibers that were placed just in front of the pupils. Signals were acquired by silver-impregnated threads placed gently on the corneas, with a drop of hypromellose to keep eyes moist and enhance electrical contact. These signals were transferred to a Psylab amplifier (Contact Precision Instruments, Boston MA), with a gain of 10000, filtered between 0.3 and 400 Hz, with a notch filter at 60 Hz. The amplified signals were digitized by a National Instruments (Austin TX) 6323 data acquisition module, and read into custom software written in Igor Pro (WaveMetrics Inc, Lake Oswego OR). Stimulus intensity was calibrated in scotopic lumens. A series of increasing intensities of 5 ms flashes was presented while dark adapted. Amplitude and timing were measured for a-, b-, and c-waves, and a frequency domain analysis of the kernels derived by correlating the flash stimuli with the responses provided more global measures of amplitude and timing (Saul and Humphrey, 1990).

In vivo retinal imaging (fundoscopy and fluorescein angiography)

Retinal morphology and vasculature were examined *in vivo* in *Mthfr*^{+/+ rd8/rd8} and *Mthfr*^{+/- rd8/rd8} mice (8–52 weeks) by fundoscopy and fluorescein angiography (FA) using the Micron III camera (Phoenix Research Laboratories, Pleasanton, CA). Mice were anesthetized using ketamine/xylazine rodent anesthesia cocktail. Pupils were dilated with 1% tropicamide (Bausch & Lomb, Tampa, FL). For maintenance of corneal moisture, Systane lubricant eye drops (Alcon, Ft. Worth, TX) were applied. The mouse was positioned on the imaging stage for fundoscopy. For FA, the mouse was injected i/p with 10 to 20 µL fluorescein sodium (10% Lite; Apollo Ophthalmic, Newport Beach, CA). Fluorescent images were acquired rapidly at 30 sec intervals for ~ 5 minutes.

Microscopic evaluation and measurement procedures

Retinal morphology was evaluated in hematoxylin and eosin-stained retinal cryosections of *Mthfr*^{+/+} *rd8/rd8* and *Mthfr*^{+/-} *rd8/rd8* mice at 8–52 weeks. Morphometric evaluation of retinas adhered to our published methods (Ganapathy et al, 2009; Markand et al, 2015) and included scanning tissue sections for evidence of gross pathology, which included quantifying the number of retinas that had rosettes, half-rosettes, excessive numbers of photoreceptor cell nuclei. This was followed by systematic morphometric analysis, which included measurements of cell height of the retinal pigment epithelium (RPE), the number of cell rows in the inner nuclear layer (INL) and outer nuclear layer (ONL), the thickness of these layers, the thickness of the inner and outer plexiform layers (IPL and OPL), and the thickness of the inner and outer segments of photoreceptor cells (IS and OS). The number of cells in the ganglion cell layer (GCL) was expressed as cell count per 100 μ m length of retina. Measurements were made in 3 adjacent fields (peripheral, mid-peripheral, central) on the nasal and 3 on the temporal side of the optic nerve for a total of 6 fields; the initial measurement was made approximately ~200 μ m from the optic nerve. Layer thicknesses were measured at a single point in each field. 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.

Isolectin-B4 Staining of Flat-Mounted Retinal Preparations

To assess retinal vasculature, *Mthfr*^{+/+} *rd8/rd8* and *Mthfr*^{+/-} *rd8/rd8* mice (56 weeks) were subjected to retinal flat mount immunostaining with the endothelial cell marker, biotinylated griffonia simplicifolia isolectin-B4. Eyes were enucleated and fixed in 4% paraformaldehyde (4°C), subsequently retinas were dissected and incubated with the isolectin-B4 (7.5 μ l in 1 ml, Vector Labs, Burlingame, CA) overnight at 4°C. Retinas were washed in PBS-TritonX100, incubated with avidin-conjugated Texas Red (7.5 μ l in 1 ml, Vector Labs, Burlingame, CA) at 37°C for 1 h and washed again. Retinas were incised partially at four places along the rim to allow the tissue to be flattened on Superfrost microscope slides (Fisher Scientific, Pittsburgh, PA). Retinal flat mount preparations were viewed by epifluorescence using an Axioplan-2 microscope equipped with the Axiovision Program (Version 4.8) and a high-resolution camera (Carl Zeiss, Oberkochen, Germany).

Statistical analysis

For ERG and morphometric studies, two-way ANOVA was used to determine whether there were significant differences between mouse groups (factors: mouse group and stimulus intensity/age). Bonferroni post-hoc test was used to compare means. Data were analyzed using the GraphPad Prism software (version 6; GraphPad Software Inc., La Jolla, CA). A *p* value < 0.05 was considered significant.

Results

Body weight

The *Mthfr*^{+/-} *rd8/rd8* mice had a normal life span with normal reproductive performance in both males and females. The average body weight of *Mthfr*^{+/-} *rd8/rd8* and *Mthfr*^{+/+} *rd8/rd8*

mice is provided in Fig. 1. The average body weight in both groups was similar from 8–24 weeks. Interestingly, by 52 weeks, a significant increase in the body weight in *Mthfr*^{+/-rd8/rd8} mice (~42 gm) compared to *Mthfr*^{+/+rd8/rd8} mice (~32 gm) was observed.

ERG

ERG dysfunction has been reported in rd8 mice by 18 months as a subtle decrease in b-wave amplitude (Aleman et al, 2011). To evaluate whether *Mthfr* deficiency in combination with the rd8 mutation alters retinal function, we performed light- and dark- adapted ERGs at 32–52 weeks in *Mthfr*^{+/+rd8/rd8} and *Mthfr*^{+/-rd8/rd8} mice. Fig 2 represents the summary of scotopic flash response ERG results in both groups of mice at 32 wk. No difference in the a-wave (Fig. 2A), the b-wave (Fig. 2B) or the c-wave (Fig. 2C) amplitudes were observed in mouse groups. At 52 weeks, a significant decline in the amplitude of the a-wave (Fig. 3A), the b-wave (Fig. 3B) and the c-wave (Fig. 3C) were observed reflecting dysfunction in photoreceptor cells, bipolar cells and RPE cells, respectively in retinas of *Mthfr*^{+/-rd8/rd8} mice. Latencies to the peak of the c-wave were shortened (Fig. 3F), but significant differences were not seen for a- or b-wave latencies (Fig. 3D, Fig. 3E). In addition, significant declines in amplitude (Fig. 3G) and timing (Fig. 3H) of these scotopic responses from *Mthfr*^{+/-rd8/rd8} mice were observed when using frequency domain measures, kernel amplitude (Fig. 3G) and absolute phase (Fig. 3H), that integrated the a-, b-, and c-wave components of the flash ERG. Global latencies (Fig. 3I) did not differ significantly.

Fundus and Fluorescein angiography (FA)

Additional *in vivo* retinal assessments were performed in *Mthfr*^{+/+rd8/rd8} and *Mthfr*^{+/-rd8/rd8} mice including funduscopy and FA (aged 8–52 weeks). Typically, the rd8 mutation manifests as focal white spots limited to the inferior temporal quadrant of the fundus (Gosens et al, 2008; Aleman et al, 2011). Our fundus data from *Mthfr*^{+/+rd8/rd8} were consistent with these reports (Fig. 4A, 4E, 4I and 4M). Interestingly, in the rd8 mice with *Mthfr* deficiency, the number and the distribution of white spots increased over the ages studied (8–52 weeks) (Fig. 4G, 4K and 4O). By 24 weeks, marked retinal disruption including geographic atrophy (GA) was observed (Fig 4K, arrow) which occurred in 60–70% of *Mthfr*^{+/-rd8/rd8} mice. The incidence of GA increased to nearly 100% by 52 weeks (Fig. 4O, arrow). Earlier reports of FA assessment of retinal vasculature in *Crb1*^{-/-} mice revealed vascular leakage and neovascularization after 18 months (Serge et al, 2007); no FA data are available for *Crb1*^{rd8/rd8} mice. We used FA to examine retinal vasculature in *Mthfr*^{+/+rd8/rd8} and *Mthfr*^{+/-rd8/rd8} mice to investigate whether the vascular alterations had an earlier onset in the presence of *Mthfr* deficiency. *Mthfr*^{+/+rd8/rd8} FA assessment revealed no marked vascular alterations at ages studied (Fig. 4B, 4F, 4J and 4N). At 8 weeks (*Mthfr*^{+/+rd8/rd8}, n=5; *Mthfr*^{+/-rd8/rd8}, n=5) and 12 (*Mthfr*^{+/+rd8/rd8}, n=6; *Mthfr*^{+/-rd8/rd8}, n=7) weeks, FA revealed no vascular abnormalities in *Mthfr*^{+/-rd8/rd8} mice (Fig. 4D and 4H). By 24 weeks (*Mthfr*^{+/+rd8/rd8}, n=5; *Mthfr*^{+/-rd8/rd8}, n=6), however ~60% of *Mthfr*^{+/-rd8/rd8} mice examined displayed variable ischemia (Fig. 4L, arrow) and vascular tortuosity (Fig. 4L, star). By 52 weeks (*Mthfr*^{+/+rd8/rd8}, n=4; *Mthfr*^{+/-rd8/rd8}, n=4), vascular abnormalities were observed in all *Mthfr*^{+/-rd8/rd8} mice studied (Fig. 4P, arrow). Higher magnification of retinal vascular alterations in *Mthfr*^{+/-rd8/rd8} is shown in Fig 5. The vascular alterations included vessel tortuosity (Fig. 5A, white arrow heads), ischemia (Fig. 5B area demarcated by white

boundary), beading (Fig. 5C, arrow), neovascularization (Fig. 5C, arrow heads) and vascular leakage (Fig. 5C, star). The data suggest that the combination of rd8 mutation and *Mthfr* deficiency results in severe retinal vascular alterations that worsen with age.

Morphology and morphometric analysis

To evaluate the consequences of the combination of rd8 mutation with *Mthfr* deficiency on retinal histology, retinas from *Mthfr*^{+/+rd8/rd8} and *Mthfr*^{+/-rd8/rd8} mice (8–52 weeks) were subjected to systematic morphological analysis. Focal areas of retinal disruption limited to the ONL and RPE layers are morphological features of rd8 mutation, which present as small half-rosettes or disruption in the ELM (Baehr and Frederick, 2009). Our analysis confirmed the presence of half-rosettes in *Mthfr*^{+/+rd8/rd8} retinas throughout the ages studied (Fig. 6A and 6B arrows). In *Mthfr*^{+/-rd8/rd8} mice, the incidence and extent of rd8-like half-rosettes increased markedly with age (Fig. 6C arrow). At 8 wks (*Mthfr*^{+/+rd8/rd8}, n=7; *Mthfr*^{+/-rd8/rd8}, n=6) retinal disruption was observed in ~50% of *Mthfr*^{+/-rd8/rd8} mice compared to ~25% *Mthfr*^{+/+rd8/rd8} mice. At 12 wks (*Mthfr*^{+/+rd8/rd8}, n=5; *Mthfr*^{+/-rd8/rd8}, n=10), retinal disruption, especially retinal rosettes, increased to ~70% *Mthfr*^{+/-} mice compared to ~40% *Mthfr*^{+/+rd8/rd8} mice. At 24 wks (*Mthfr*^{+/+rd8/rd8}, n=7; *Mthfr*^{+/-rd8/rd8}, n=11), retinal disruption was observed in ~80% of *Mthfr*^{+/-rd8/rd8} mice compared to ~30% *Mthfr*^{+/+rd8/rd8} mice. There were additional features observed in *Mthfr*^{+/-rd8/rd8} mice that have not been reported previously in association with rd8 mutation. For example profound retinal disorganization, characterized by extremely exaggerated rosettes, was frequently observed (Fig. 6D). In extreme cases, areas of retinal dysplasia characterized by the outer nuclear layer with more than 20 rows of photoreceptor nuclei versus the typical 10–12 rows were frequent. The incidence of excessive rows increased with age in the *Mthfr*^{+/-rd8/rd8} mice (~14 % at 12 weeks; ~25% at 24 weeks and ~33% at 52 weeks). These retinal dysplastic features were not observed in *Mthfr*^{+/+rd8/rd8} mice. In addition, fewer nuclei were seen in the GCL layer (Fig. 6D, 6E and 6F, arrows). The areas of dysplasia were focal; the non-involved regions appeared normally organized. It is noteworthy, that in our earlier study of the retinal phenotype of *Mthfr*^{+/-} mice (with no rd8 mutation), we did not observe rosettes, half-rosettes or excessive rows of photoreceptor nuclei in the ONL (Markand et al, 2015).

We quantified the retinal morphology in non-dysplastic regions by performing comprehensive morphometric analysis. This included quantification of the number of cells in the ganglion cell layer (GCL expressed as cell count per 100 μ m, the number of cell rows in the inner nuclear layer (INL) and outer nuclear layer (ONL), the measurement of the thickness of the inner and outer plexiform layers (IPL and OPL), the thickness of the inner and outer segments of photoreceptor cells (IS and OS), measurement of cell height of the retinal pigment epithelium (RPE) and total retinal thickness. A significant decrease in the number of cells in the GCL was observed at 24 and 52 weeks in *Mthfr*^{+/-rd8/rd8} mice in comparison with the *Mthfr*^{+/+rd8/rd8} mice (Fig. 7). The number of cell rows in the INL was similar between both groups of mice. Apart from outer segment thinning at 24 weeks in *Mthfr*^{+/-rd8/rd8} mice, the other parameters measured in non-dysplastic regions were not altered in *Mthfr*^{+/-rd8/rd8} mice compared to *Mthfr*^{+/+rd8/rd8} over the age ranges studied (data not shown).

Evaluation of blood vessel integrity

To investigate the retinal vascular morphology, we performed retinal flatmount with isolectin-b4, a marker for blood vessels. In *Mthfr*^{+/+rd8} mice, there is evidence of mild vasculopathy (Fig. 8A, arrow) although many areas appeared normal (Fig. 8C). In contrast age-matched, *Mthfr*^{+/-rd8} mice showed abnormal blood vessels characterized by (Fig. 8B arrow), loss of retinal blood vessel integrity (Fig. 8B arrow) and the presence of capillary tufts indicating abnormal neovascularization (Fig. 8D, arrows).

Discussion

Mutations of the *CRB1* gene in humans can lead to a devastating retinal dystrophy in the form of LCA and rod-cone dystrophies (Hollander et al, 2004; McKay et al, 2005; Mehalow, et al 2003). The clinical presentation is highly variable, with some individuals demonstrating significantly worse visual disorders than others. The variable presentation suggests that genetic factors, including mutations of other genes, may underlie the severity of retinal degenerations due to *CRB1* mutations. As described by Tuo et al, (2007), phenotype-genotype correlation suggests that disease severities associated with *CRB1* mutations are in fact a continuum of the same clinical entity with possible additional modifying factors influencing disease onset and progression. There is increasing evidence of the involvement of multiple alleles in the patient's phenotype, as has been shown for the Bardet-Biedl patients (Katsanis, et al, 2001) and more recently for a PRPH2-associated macular dystrophy family, where the phenotype has been modulated by additional heterozygous mutations in ABCA4 (MIM# 601691) and ROM1 (MIM# 180721) (Poloschek et al, 2010). There are likely to be mutations of other genes that alter *CRB1* and given the prevalence of mutations of *MTHFR* in the human population, it was relevant to investigate, in an animal model, whether deficiency of the *Mthfr* gene altered the *Crb1* (rd8) retinal phenotype. As summarized below, the data from the present study suggest that *Mthfr* does modify *Crb1*, though it remains to be determined in human patients with *CRB1* mutations whether the phenotype is modulated by mutations in *MTHFR*.

The rd8 mouse manifests as focal retinal dystrophy affecting the outer retina that manifests in the first few months. The functional consequences of the mutation are minimal as evidenced by normal ERG through the first 18 months (Aleman et al, 2011). Alterations in the retina due to a second genetic mutation are amenable to analysis since the rd8 phenotype is easily recognizable yet relatively mild. It is apparent from recent work that mutations of two genes *CCl2* (CC-chemokine ligand 2) and *Cx3cr1* (CX3C chemokine receptor 1) do interact with *Crb1* (at least in mice) and present an AMD-like retinal phenotype (Mattapallil et al, 2012; Tuo et al, 2007). However, not all genes modify *Crb1* as has been reported by Sahu et al (2015). Their study of *Ctrp5* mutant mice demonstrated that the rd8 mutation did not alter the retinal phenotype in their mutant mouse model. Clearly, not all mutations that affect retina are modifiers of rd8. Thus, genetic interactions relevant to *Crb1* must be tested on a case by case basis.

This study analyzed the effects of a deficiency (heterozygous mutation) of the *Mthfr* gene on the retinal phenotype of the rd8 mutant mouse. The underlying cause of retinal degeneration in rd8 mice is spontaneous frameshift mutation, c.3481delC, in the *Crb1* gene (Mehalow et

al, 2011). The data obtained from the current study suggest that deficiency of *Mthfr* modifies the rd8 retinal phenotype. Our analysis compared the electrophysiological function and retinal architecture of the rd8 mutant mouse with that of *Mthfr* heterozygous mice carrying the rd8 mutation. While the nomenclature in this report has been *Mthfr*^{+/+} rd8/rd8 to represent the rd8 mutant and *Mthfr*^{+/-} rd8/rd8 to represent the 'double mutant', for simplicity of this discussion, we will refer to the mice as either rd8 or the *Mthfr*-rd8.

We tested the hypothesis that the rd8 mutation would be accelerated in onset and severity in the presence of *Mthfr* deficiency. Our data support this hypothesis. We observed a significant decrease in a-, b- and c-wave amplitudes of the ERG in the *Mthfr*-rd8 mice by 12 months, whereas the rd8 mouse has a normal ERG through 18 months. The ERG analysis of the a-, b- and c-waves in *Mthfr*^{+/-} mice (with no rd8 mutation) are comparable to the WT mice at 24 wks (Markand et al, 2015). Thus, the ERG data on rd8 containing *Mthfr*^{+/-}rd8/rd8 mice support the notion of accelerated retinal dysfunction when Hhcy and rd8 coexist.

We observed vascular changes in the *Mthfr*-rd8 retina including vascular leakage, impaired vessel integrity and ischemia as early as 24 weeks, whereas vascular alterations were minimal in rd8 retinas. The retinal morphology of the *Mthfr*-rd8 mice demonstrated retinal rosettes, which were reminiscent of the typical rd8 mutation; however the disruption was exaggerated in the *Mthfr*-rd8 mice. There were more rosettes and they spanned a larger retinal region. Nevertheless, as with rd8 mice, the disruptions in *Mthfr*-rd8 mice were focal and there remained retinal areas that had normal stratification. In these areas, the thicknesses of retinal layers were similar to rd8 mice. Interestingly, there were retinal regions that showed excessive rows of inner or outer nuclear layer cells. These were not observed in the rd8 retina. Taken collectively, the data suggest that rd8 mutation in the presence of the *Mthfr* deficiency has a more profound retinal disruption that affects function.

Interestingly, though not a focus of this study we observed a significant increase in body weight in the *Mthfr*-rd8 mice by 12 months. No evidence exists linking rd8 or *Mthfr* mutations with weight gain although Hhcy due to *Mthfr* mutation is implicated in dysregulation of lipid metabolism (Adinolfi et al, 2005). The *Mthfr*^{+/-} mice have lipid deposits in the aorta (Chen et al, 2001). Interestingly, *Mthfr*^{+/-} mice (without rd8 mutation) have body weights similar to the WT (*Mthfr*^{+/+}) mice (Markand et al, 2015). Whether Hhcy with rd8 mutation influences body weight is a question that remains to be investigated.

Our laboratory has investigated mice with deficiency of *Mthfr* (Markand et al, 2015). Mutations of this gene are associated with increased levels of homocysteine and we have had a longstanding interest in the effects of hyperhomocysteinemia on retinal structure and function. We have examined the consequences on retina when homocysteine was injected into the vitreal cavity, as an acute injury (Moore et al, 2001) and we have assessed the consequences when metabolic defects have led to moderately excessive endogenous homocysteine levels (Ganapathy et al, 2009; Yu et al, 2011; Tawfik et al, 2014 and Markand et al, 2015). Much of our recent work focused on the effects of deficiency of enzymes involved in the homocysteine-methionine pathway. Deficiencies of cystathionine-β-synthase (*Cbs*) and methylene tetrahydrofolate reductase (*Mthfr*) and the subsequent moderate hyperhomocysteinemia are accompanied by loss of retinal ganglion cells (Ganapathy et al,

2009; Yu et al, 2011; Tawfik et al, 2014 and Markand et al, 2015). The hyperhomocysteinemic retinal phenotype we have observed is quite different from that described for the rd8 mouse, which manifests involvement of the outer retina in the form of focal rosettes and minimal functional changes for the first 18 months (Aleman et al, 2011). In the combined mutation, the *Mthfr-rd8* mice still have the loss of ganglion cells, which we presume is due to the *Mthfr* mutation since the rd8 mice do not manifest this phenotype.

There are limitations of the present study. For example, several of our observations regarding the effects of the rd8 mutation developed after 24 wks of age and future studies could compare the findings to *Mthfr*^{+/-} mice in the absence of the rd8 mutation to see which of the findings can be segregated to just the MTHFR haploinsufficiency. This would be relevant to outer retinal changes, which seem to be triggered by the rd8 mutation, while the inner retinal changes seem to be triggered by the MTHFR haploinsufficiency. Nevertheless, the data suggesting that the outer retinal changes caused by the rd8 mutation (e.g. focal areas of retinal rosettes and retinal disorganization) may be increased by the MTHFR haploinsufficiency, which by itself does not cause those changes, is of interest and worthy of comprehensive investigation. Also, the appearance of “geographic atrophy” by 24 wks of age in *Mthfr-rd8* mice is intriguing since it is not a prominent finding of either 24 wk old *Mthfr*^{+/-} mice or 52 wk old rd8 mice. It is unclear if the vascular pathology, some level of which is already seen in *Mthfr*^{+/-} mice at a young age, could all be due to this haploinsufficiency combined with the increased age of the mice in this study. A similar caveat could be argued with the ERG changes observed.

In conclusion, we found that *Mthfr* deficiency modified the rd8 retinal phenotype. Considering the variability of clinical features in LCA and rod-cone dystrophy patients, there is a need to identify genetic modifiers of *CRB1*. The present work may provide important clues as to yet another gene that should be investigated as a modifier of *CRB1* in humans.

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Highlights

The rd8 mouse is a model of *Crb1* mutation. *CRB1* mutations are associated with LCA and RP in humans.

Mutations of MTHFR, a homocysteine metabolic enzyme, are prevalent in humans.

We investigated the possibility that *Mthfr* deficiency modifies retinal phenotype in (*Crb1*) rd8 mice.

Mthfr deficiency altered retinal function, morphology and vasculature in rd8 mice.

Our data suggest that *Mthfr* deficiency may be a modifier in *CRB1* related retinopathies.

Figure 1. Body weight in *Mthfr*^{+/-}rd8/rd8 mice
 Body weight in *Mthfr*^{+/+}rd8/rd8 and *Mthfr*^{+/-}rd8/rd8 mice at 8–52 weeks. **Significantly different from *Mthfr*^{+/+}rd8/rd8 mice; p<0.001. Abbreviations: g, grams.

Figure 2. Summary of scotopic ERG responses in *Mthfr*^{+/+}*rd8/rd8* and *Mthfr*^{+/-}*rd8/rd8* mice at 32 weeks

Mthfr^{+/+}*rd8/rd8* and *Mthfr*^{+/-}*rd8/rd8* mice were subjected to ERG analysis at 32 weeks to assess retinal function. X-axis represents different intensities of luminance and Y-axis represents the amplitudes (A) the a-, (B) the b- (C) the c-waves in microvolts (μV).

Figure 3. Summary of ERG responses in *Mthfr*^{+/rd8/rd8} and *Mthfr*^{+/-rd8/rd8} mice at 52 weeks
Mthfr^{+/rd8/rd8} and *Mthfr*^{+/-rd8/rd8} mice (52 weeks) were subjected to ERG analysis. (A) Average amplitudes of the a-, (B) the b-, (C) the c-waves. (D) Peak time of the a-, (E) the b- and (F) the c-wave. (G) Average amplitude of Kernel, (H) Absolute phase and (I) latency. Results of two-way ANOVAs for intensity and genotype are shown, with “reject” indicating significant effects.

Figure 4. Fundoscopy and fluorescein angiography in *Mthfr*^{+/+rd8/rd8} and *Mthfr*^{+/-rd8/rd8} mice Representative fundus images and fluorescein angiography (FA) data from *Mthfr*^{+/+rd8/rd8} and *Mthfr*^{+/-rd8/rd8} mice at ages 8 weeks (A–D), 12 weeks (E–H), 24 weeks (I–L) and 52 weeks (M–P). At 8–12 weeks both *Mthfr*^{+/+rd8/rd8} and *Mthfr*^{+/-rd8/rd8} mice displayed rd8 spots on the fundus and a normal appearing FA (A–H). At 24 and 52 weeks, the fundus of *Mthfr*^{+/-rd8/rd8} mice displayed marked retinal disruption including geographic atrophy (arrows K, O). This was accompanied by ischemia (arrow, L), fluorescein leakage (arrow, P), beading and tortuosity in retinal blood vessels (star, P) in *Mthfr*^{+/-rd8/rd8} retinas.

Figure 5. Vascular alterations in *Mthfr*^{+/-rd8/rd8} mice by FA
Marked retinal vasculopathy was observed in *Mthfr*^{+/-rd8/rd8} mice from 24 weeks onwards. (A) Vascular tortuosity (arrow heads); (B) ischemia (marked by white boundary), (C) beading (white arrow); neovascularization (arrowhead) and vascular leakage (star).

Figure 6. Histology of retinas of *Mthfr*^{+/+rd8/rd8} and *Mthfr*^{+/-rd8/rd8} mice

Representative light photomicrographs of hematoxylin and eosin-stained retinal cryosections from *Mthfr*^{+/+rd8/rd8} and *Mthfr*^{+/-rd8/rd8} mice. Representative retinal morphology of *Mthfr*^{+/+rd8/rd8} mice at 16 weeks (A) and 52 weeks (B; arrows indicate pseudorosette (A, B)). Representative retinal morphology of *Mthfr*^{+/-rd8/rd8} mice at 52 weeks (C). Profound disruption observed in retinas from *Mthfr*^{+/-rd8/rd8} mice at 24 weeks (D), retinal dysplasia observed in *Mthfr*^{+/-rd8/rd8} mice at 24 weeks (E) and at 52 weeks (F). Black arrows point to regions of dropout of cells in the GCL at 24 (D, E) and 52 weeks (F). Scale bar: 50 μ m.

Figure 7. Number of nuclei in the GCL in $Mthfr^{+/-rd8/rd8}$ mice at 8–52 weeks
 Morphometric analysis of the number of cell bodies in the GCL in $Mthfr^{+/-rd8/rd8}$ mice at 8–52 weeks. (** Significantly different from $Mthfr^{+/+rd8/rd8}$ mice, $p < 0.001$, *** significantly different from $Mthfr^{+/+rd8/rd8}$ mice, $p < 0.0001$). Abbreviations: GCL, ganglion cell layer.

Figure 8. Assessment of retinal blood vasculature in retinal flatmount from $Mthfr^{+/-rd8/rd8}$ mice at 56 weeks

Retinal blood vessels from $Mthfr^{+/+rd8/rd8}$ and $Mthfr^{+/-rd8/rd8}$ mice were visualized by the blood vessel marker, isolectin-b4. (A) Disrupted blood vessel in $Mthfr^{+/+rd8/rd8}$ (arrow); (B) Disrupted blood vessels in $Mthfr^{+/-rd8/rd8}$ (arrow); (C) Normal appearing blood vessel in $Mthfr^{+/+rd8/rd8}$ and (D) Neovascularization (arrows) in $Mthfr^{+/-rd8/rd8}$ mice.

Table I

Animals used in this study.

Mouse Genotype	<i>n</i>	Age (wks)
<i>ERG</i>		
<i>Mthfr</i> ^{+/+} <i>rd8</i> / <i>rd8</i>	3	32
<i>Mthfr</i> ^{+/-} <i>rd8</i> / <i>rd8</i>	3	32
<i>Mthfr</i> ^{+/+} <i>rd8</i> / <i>rd8</i>	7	52
<i>Mthfr</i> ^{+/-} <i>rd8</i> / <i>rd8</i>	6	52
<i>Body weight and Morphometric analysis</i>		
<i>Mthfr</i> ^{+/+} <i>rd8</i> / <i>rd8</i>	7	8
<i>Mthfr</i> ^{+/-} <i>rd8</i> / <i>rd8</i>	6	8
<i>Mthfr</i> ^{+/+} <i>rd8</i> / <i>rd8</i>	5	12
<i>Mthfr</i> ^{+/-} <i>rd8</i> / <i>rd8</i>	10	12
<i>Mthfr</i> ^{+/+} <i>rd8</i> / <i>rd8</i>	7	24
<i>Mthfr</i> ^{+/-} <i>rd8</i> / <i>rd8</i>	11	24
<i>Mthfr</i> ^{+/+} <i>rd8</i> / <i>rd8</i>	7	52
<i>Mthfr</i> ^{+/-} <i>rd8</i> / <i>rd8</i>	4	52
<i>Fundoscopy and FA analysis</i>		
<i>Mthfr</i> ^{+/+} <i>rd8</i> / <i>rd8</i>	5	8
<i>Mthfr</i> ^{+/-} <i>rd8</i> / <i>rd8</i>	5	8
<i>Mthfr</i> ^{+/+} <i>rd8</i> / <i>rd8</i>	6	12
<i>Mthfr</i> ^{+/-} <i>rd8</i> / <i>rd8</i>	7	12
<i>Mthfr</i> ^{+/+} <i>rd8</i> / <i>rd8</i>	5	24
<i>Mthfr</i> ^{+/-} <i>rd8</i> / <i>rd8</i>	6	24
<i>Mthfr</i> ^{+/+} <i>rd8</i> / <i>rd8</i>	4	52
<i>Mthfr</i> ^{+/-} <i>rd8</i> / <i>rd8</i>	4	52
<i>Immunohistochemistry to examine Isolectin-b4</i>		
<i>Mthfr</i> ^{+/+} <i>rd8</i> / <i>rd8</i>	4	56
<i>Mthfr</i> ^{+/-} <i>rd8</i> / <i>rd8</i>	4	56