Optic Nerve Hypoplasia Is a Pervasive Subcortical Pathology of Visual System in Neonates

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CL and AK contributed equally to the work presented here and should therefore be regarded as equivalent authors.

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Citation: Liang C, Kerr A, Qiu Y, et al. Optic nerve hypoplasia (ONH) is the most common cause of childhood congenital blindness in developed nations, yet the fundamental pathobiology of ONH remains unknown. The objective of this study was to employ a ‘face validated’ murine model to determine the timing of onset and the pathologic characteristics of ONH.

METHODS. Based on the robust linkage between X-linked CASK haploinsufficiency and clinically diagnosed ONH, we hypothesized that heterozygous deletion of CASK in rodents will produce an optic nerve pathology closely recapitulating ONH. We quantitatively analyzed the entire subcortical visual system in female CASK+/− mice using immunohistochemistry, anterograde axonal tracing, toluidine blue staining, transmission electron microscopy, and serial block-face scanning electron microscopy.

RESULTS. CASK haploinsufficiency in mice phenocopies human ONH with complete penetrance, thus satisfying the ‘face validity’. We demonstrate that the optic nerve in CASK+/− mice is not only thin, but is comprised of atrophic retinal axons and displays reactive astrogliosis. Myelination of the optic nerve axons remains unchanged. Moreover, we demonstrate a significant decrease in retinal ganglion cell (RGC) numbers and perturbation in retinothalamic connectivity. Finally, we used this mouse model to define the onset and progression of ONH pathology, demonstrating for the first time that optic nerve defects arise at neonatally in CASK+/− mice.

CONCLUSIONS. Optic nerve hypoplasia is a complex neuropathology of the subcortical visual system involving RGC loss, axonopathy, and synaptopathy and originates at a developmental stage in mice that corresponds to the late third trimester development in humans.

Keywords: optic nerve hypoplasia, CASK, axonopathy, synaptopathy

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the X-linked gene CASK encoding a peripheral scaffolding protein have been strongly associated with ONH\textsuperscript{17} and mutations in CASK therefore represent a potential avenue to generate a novel model of ONH for mechanistic and pathologic evaluation.

CASK was initially described as a candidate gene for X-linked ONA\textsuperscript{18} and mutations in CASK are associated with ONH.\textsuperscript{17,19,20} We report new subjects with CASK mutations and ONH, further confirming the association, and we show that heterozygous deletion of CASK causes optic nerve pathology indicating that these phenotypes represent CASK loss of function. Furthermore, our results demonstrate that the optic nerve of CASK\textsuperscript{+/−} mice have atrophic axons with increased interaxonal space and astrogliosis. The numbers of retinogeniculate synapses are reduced with specific loss of smaller boutons; furthermore the large boutons exhibit an overall decrease in number of active zones. In retina, we observe a decrease in number of RGCs in CASK\textsuperscript{+/−} mice. Finally, we demonstrate that the optic nerve pathology in CASK\textsuperscript{+/−} mice occurs very early in postnatal rodent developmental, a stage considered equivalent to the late third trimester and neonatal period of human fetal development.\textsuperscript{21} Haploinsufficiency of CASK therefore produces a combination of retinopathy, optic nerve pathology, as well as synaptopathy. Thus, our results demonstrate that a face validated mouse model provides new insight into the pathogenesis and timing of ONH.

**METHODS**

**Ethical Statement**

The Virginia Tech Universities institutional review board approved the collection and use of data from subjects. Informed consent was obtained from the family prior to participation. All animal work was done in accordance to with the guidelines for the animal care of laboratory animals issued by Virginia Tech and adhered to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

**Identification of a Splice Mutation in CASK**

We performed whole-exome sequencing (WES) on genomic DNA of the patient. Using the Illuma HiSeq2000 platform (Illumina, San Diego, CA, USA) and the acquired reads were aligned to the reference human genome (UCSC Genome Browser hg19). Data processing was performed with the Genome Analysis ToolKit (GATK) and variants were annotated using Annovar (http://annovar.openbioinformatics.org/en/latest/, in the public domain) and an in-house developed web interface called Annotate-it.\textsuperscript{22} In the first stage, we analyzed all genes that were previously associated with microcephaly. The heterozygous splice variant in exon 24 (NM_001126054: exon24:c.2233+1G>A) of CASK, was predicted to lead to exon skipping, and was confirmed by Sanger sequencing. The variant arose de novo and was absent in both biological parents. To further test the effect of the mutation, we performed PCR with primers in exons 25 and 23 on cDNA made from RNA extracted from Epstein-Barr virus-transformed lymphocytes of the patient.\textsuperscript{23} The RT-PCR on the patient clearly showed two bands and sequencing of the smallest band confirmed exon 24 skipping.

**Mouse Breeding and Genotyping**

CASK\textsuperscript{−/−} female mice, generated in house,\textsuperscript{24} were crossed with C57BL6 male mice to propagate CASK\textsuperscript{−/−} female pups and their CASK\textsuperscript{+/−} female littermates. Genotyping was done using a PCR-based method with following primer pair (forward: TTTGGGGACTAGTGGGTGTTG, reverse: CTTGGTGCGACCTTTGGAGTA).

**Immunohistochemistry (IHC)**

Mice were anesthetized and perfused with PBS and then 4% paraformaldehyde (PFA). Brain, retina, or optic nerve were dissected and postfixed in 4% PFA. Tissues were cryopreserved in 30% sucrose before embedding in Neg50 matrix (Richard-Allan Scientific, San Diego, CA, USA) and cryosectioned using Leica Cryostat (Leica Biosystems, Wetzlar, Germany) to generate 16- to 25-μm thick tissue sections. Sections were permeabilized and blocked with 0.25% Triton X-100 and 5% BSA in PBS. Immunostaining were performed using primary antibodies at an appropriate concentration followed by labeling with fluorophore conjugated secondary antibodies. Primary antibodies include: Calretinin (rabbit polyclonal Ab, Cat. No. AB5054, 1:2000; Millipore, Billerica, MA, USA), RNA-binding protein with multiple splicing (RBPS; rabbit polyclonal Ab, Cat. No. 1830, 1:500; PhosphoSolution, Aurora, CO, USA), glial fibrillary acidic protein (GFAP; mouse monoclonal Ab, Cat. No. 75240, 1:1000; Neuromab, UC Davis, CA, USA). Secondary antibodies include: Alexa Fluor 488 (goat anti-rabbit, Cat. No. A-11008, 1:1000; Invitrogen) and Dylight 633 (anti-mouse IgG, Cat. No. 35512, 1:1000; Thermo Scientific, Waltham, MA, USA). Sections were mounted with VectaShield containing 4’,6-diamidino-2-phenylindole (DAPI; Vector Laboratories, Burlingame, CA, USA) and were imaged using a Zeiss 700 or 710 laser scanning confocal microscope (Oberkochen, Germany).

**Immunoblotting**

Optic nerves dissected from young female wild-type and CASK\textsuperscript{−/−} mice (postnatal day [P] 40) were boiled in sample buffer and proteins separated by 8% SDS-PAGE. Proteins were then transferred onto nitrocellulose membrane and blocked in 5% skimmed milk for 2 hours and incubated using appropriate primary and secondary antibodies. For quantitative immunoblots, secondary antibodies conjugated with fluorophore were used and images were captured using appropriate filter in Chemidoc (Biorad, Hercules, CA, USA). Antibodies used are CASK (mouse monoclonal Ab, Cat. No. 75000, 1:1000; NeuroMab), neurofilament-associated antigen (NFAA; mouse monoclonal Ab, Cat. No. 5A10, 1:1000; DSHB, Iowa City, IA, USA), and tubulin (mouse monoclonal Ab, Cat. No. 12G10 anti-alpha-tubulin, 1:1000; DSHB).

**Optic Nerve Samples Preparation for Toluidine Blue Staining and Transmission Electronic Microscopy (TEM)**

Mice were anesthetized and perfused with PBS and then sodium cacodylate (NaCa) buffer (2% glutaraldehyde/2% PFA [2G/2PF] in 0.1 M sodium cacodylate in PBS, pH 7.4). After perfusion, optic nerves were dissected and immersed in NaCa buffer overnight and then put in 2% OSO4 for 2 hours for postfixation. Dehydration was achieved by sequentially adding 15% to 100% ethanol. Samples were embedded in 100% epoxy resin for 24 hours and then placed in oven at 58°C for 2 days. Sectioning of samples and successive toluidine blue stain or TEM was performed at Virginia-Maryland College of Veterinary Medicine at Virginia Tech.

**G-Ratio Distribution Spectra**

G-ratio, defined as the ratio of inner axonal diameter to the outer axonal diameter (including myelin sheath),\textsuperscript{25} of 240 optic
nerve axons were measured on TEM images (<4000) using ImageJ software (http://imagej.nih.gov/ij/; provided in the public domain by the National Institutes of Health, Bethesda, MD, USA). The measurements of inner and outer axonal diameters (d) were calculated from inner and outer axonal areas (A), respectively, to reduce error because the sections were not perfectly round in shape, where \( d = \sqrt{\frac{A}{\pi}} \). Probability density distribution of G-ratio values was plotted based on a smooth kernel density estimate by using the function of “SmoothHistogram” offered in the software of Mathematica (Wolfram, Champaign, IL, USA). The smooth kernel density estimate based probability density function is given by a linearly interpolated version of

\[
\frac{1}{nh} \sum_{i=1}^{n} k \left( \frac{x - x_i}{b} \right),
\]

where \( x \) is the value of G-ratio, \( n \) is the sample size, width \( b \) is selected as “Automatic,” and kernel smoothing function \( k(x) \) was given by a Gaussian (see Eq. 1), \( u \in \mathbb{R} \) in Mathematica. Above functions can be found in any advanced level statistics textbook. The Kolmogorov-Smirnov test was used to determine if the two probability density distributions are from the same population.

**Serial Block-Face Scanning Electron Microscopy**

Control and CASK<sup>+/−</sup> mice were transcardially perfused with PBS and 4% paraformaldehyde/2% glutaraldehyde in 0.1 M cacodylate buffer. Brains were removed, 500-μm coronal sections were obtained using vibrotome and dorsolateral geniculate nucleus (dLGN) were dissected. Tissues were stained, embedded, sectioned, and imaged as described previously<sup>26</sup> by Renovo Neural, Inc. (Cleveland, OH, USA). Images were acquired at a resolution of 6 nm/pixel and image sets included greater than 200-serial sections (with each section representing 65 nm in the z axis). Data sets were analyzed in TrakEM2 and retinal terminals were unequivocally identified by the presence of synaptic vesicles and pale mitochondria.<sup>26,27</sup>

**Intraocular Injections of Anterograde Tracers and Quantification**

Intraocular injection of cholera toxin subunit B (CTB) conjugated to AlexaFluor 555 (Invitrogen) was performed as described previously.<sup>28</sup> After 2 days, mice were euthanized, perfused with 4% PFA, and brains were dissected and postfixed in 4% PFA overnight. On a vibratome, 80- to 100-μm coronal slices were sectioned and mounted using VectaShield (Vector Laboratories). Confocal Z-stack images from coronal dLGN sections were acquired on a Zeiss LSM 700 confocal microscope. Z-stacks were obtained with a Zeiss x20 Plan-Apochromat objective and contained 14- to 20-optical sections (in 3-μm steps). Confocal images were obtained from four adult CASK<sup>+/−</sup> mutants and four littermate controls. The size and quantity of synapses in each dLGN (from the center of its rostral-caudal extent), was performed in ImageJ, blind to the genotype of the sample. Five images of each dLGN Z-stacks were randomly selected, images were manually thresholded, and the quantity and surface area of the isolated puncta was measured. Data was exported into Microsoft Excel (Redmond, WA, USA), binned by size, and plotted into a histogram.

**Cell Counts and Lamina Quantification**

Cell counts were performed on 16-μm cryosectioned 4% PFA-fixed retinal tissue as described previously.<sup>29,30</sup> Slides were stained with DAPI (diluted 1:5000 in PBS) for 60 seconds and then mounted with VectaShield (Vector Laboratories). Images of central retina were acquired on a Zeiss LSM 700 confocal microscope or a Zeiss Axio Imager A2 fluorescent microscope. All DAPI and RBPMs-positive cells in the ganglion cell layer were counted manually under ×20 objective. Cell counts were analyzed from at least four animals for each age and genotype. Five images per retina per mouse were analyzed. Retinal lamination was quantified for each genotype in the central retina in DAPI-stained retinas using ImageJ.

**Description of Coarse and Fine Axons**

Because the axons in CASK<sup>+/−</sup> mice are overall small, it is difficult to apply the same threshold for fine and coarse axons on these mice as with those of wild type. On examining the distribution of the axonal area we found they are distributed in a bimodal manner (2 different slopes; see Fig. 3C). Because the CASK<sup>+/−</sup> axons were smaller we applied a different threshold for each genotype, the value of the threshold was the point where the data points start to lose linearity (see Fig. 3C).

**Results**

**Mutations in the CASK Gene are Associated With ONH**

Mutations in the CASK gene have been previously associated with ONH, ONA, and glaucoma.<sup>17,20</sup> Here we describe a 3-year-old girl with a heterozygous splice site mutation in CASK (NM_001126054:exon24:c.2233+1G>A) leading to skipping of exon 24 that exhibits microcephaly, global developmental delays, and spastic quadriaparesis (Figs. 1A–C). Magnetic resonance imaging (MRI) did not reveal any specific midline structural defect as seen in septo-optic dysplasia or any white matter lesion (Figs. 1D, 1E). Although truncated proteins may be generated in the subject from the mutated CASK allele, they are unlikely to be functional in absence of the Hook motif and the guanylate kinase domain, especially because the interdomain interaction with the guanylate kinase domain is critical for proper folding and function of membrane-associated guanylate kinase proteins.<sup>31,32</sup> Furthermore, the transcripts with premature stop codon are likely to get degraded due to nonsense-mediated decay. Thus, the pathogenic mutation described here is likely to produce haploinsufficiency. The girl displays ONH and other ophthalmic conditions including hyperopia, nystagmus, and infantile esotropia. Fundoscopy revealed a bilaterally normal anterior segment, however both optic discs were pale, small, and hypoplastic (Fig. 1F). Hypoplastic optic nerves have also been observed in both MRI and fundoscopic examination of a second girl with heterozygous nonsense mutation in the N-terminal calcium/calcmodulin-dependent kinase domain of CASK (c.661G>T; p.G221X [het]) (personal communications). This highly premature stop codon would either result in a nonfunctioning small peptide or no CASK protein product due to nonsense-mediated decay. The second girl displays severe visual impairment as well as nystagmus and intermittent esotropia (Mukherjee K, personal communications, 2017). These observations, and a previously published report,<sup>17</sup> indicate that heterozygous loss of function mutations of CASK gene associates with ONH.

**Heterozygous Deletion of CASK in Mice Produces Optic Nerve Pathology**

To test whether heterozygous mutation of CASK is sufficient to alter the ON and subcortical visual system in mice, we analyzed...
female mouse mutants with a single allele of CASK deleted (CASK\(^{+/−}\) mice). Previously, we documented that CASK\(^{+/−}\) mice exhibit secondary microcephaly, disproportionate cerebellar hypoplasia, as well as thinning and hypoplasia of the optic nerve indicating that it recapitulates the human phenotypes associated with CASK mutation or loss.\(^{21}\) We examined optic nerves following toluidine blue staining of semithin cross-sections—a method that labels myelin and

![Genetic, brain, and retinal alterations associated with heterozygous CASK mutation.](http://tvst.arvojournals.org/)

**Figure 1.** Genetic, brain, and retinal alterations associated with heterozygous CASK mutation. (A) Sanger sequences showing the de novo heterozygous splice variant in exon 24 (NM_001126054:exon24:c.2233+1G>A). (B) The schematic of exon skipping. (C) CASK. Reverse-transcription PCR with primers (arrows in panel B) in exons 25 and 23 on cDNA of the patient and subsequent sequencing confirmed exon 24 skipping (a lower band indicated by the arrow compared with control). (D, E) Magnetic resonance images of brain indicate no specific midline structural defect or white matter lesion. (F) A fundoscopic image from this haploinsufficient 3-year-old girl who was diagnosed with ONH. Note the pale, hypoplastic optic disc (as compared with a fundoscopy image from a 3.5-year-old girl without ONH; Supplementary Fig. S1).
allows the quantification of myelinated retinal axons in the ON. Surprisingly, besides being small (Figs. 2A, 2B), the optic nerve displayed an overall decrease in axonal density (Figs. 2C, 2D). The number of axons in a given area was reduced by approximately 25% and the interaxonal space was expanded (Fig. 2E). Histopathology on a single ONH case suggested that there might be an increase in number of astrocytes in ONH. We therefore examined the optic nerve of CASK(+/−/−) mice for levels of GFAP, a marker of astrocytes. We found a large increase in the GFAP staining in the optic nerve of CASK(+/−/−) mice compared with sex-matched littermate controls suggesting that haploinsufficiency of CASK gene may be associated with increased astrocytes in optic nerve (Fig. 2F).

Axonopathy is Present in Optic Nerves of CASK(+/−/−) Mice
A reduced axonal density in the CASK(+/−/−) mice optic nerve may reflect not only a loss of RGC axons, but also a thinning of individual axons of RGCs. Very small atrophic axons may escape counting from toluidine blue–stained semithin sections. We therefore performed TEM on cross sections of ON and quantified axons in CASK(+/−/−) mutants (Figs. 3A, 3B). Indeed, our data indicate that the cross-sectioned area of individual axons was significantly decreased in CASK(+/−/−) mice (Figs. 3A–D), with the presence of many very small axons in mutant ON. Because optic nerves contain populations of coarse and fine retinal axons, we measured the area of coarse and fine axons separately. Our data demonstrate that the area of both the types of axons are decreased in CASK(+/−/−) mice (Figs. 3E, 3F).

We next turned our attention to myelin content in mutant ON, to assess whether the reduction in ON diameter reflects myelin loss. G-ratio distribution spectra, which assess the ratio of axonal diameter to the diameter of the myelinated axon, were applied to compare myelin of wild-type littermate mice with that of the CASK(+/−/−) mice. The probability density distribution of G-ratios in mutant mice appears shifted to the left, indicating a higher myelin-to-axon ration compared with controls (Fig. 3G). We interpret these results to indicate that while CASK haploinsufficiency influences axon diameter it...
FIGURE 3. CASK<sup>+/−</sup> ON display axonal thinning. (A, B) Representative transmission electron micrograph of optic nerves from indicated genotypes. (C) Scatter plot of individual axonal areas from control and mutant ON obtained from three mice per genotype. Arrows indicate the “threshold” where the data points start to lose linearity (correlation coefficient $R^2$ value starts and continue decreasing). Axons with area values that are greater than or equal to the threshold are considered as coarse axons, and axons with volume values that are smaller than the threshold are considered as fine axons. Threshold value for (+/+) is 4 and (+/−) is 2 μm$^2$. (D) Mean and SDs of all axons from (C) are plotted, $N = 240$. (E, F) Comparisons of fine and coarse axons area in mutant (+/−) and control (+/+) ON ($n = 3$ mice per genotype). Data are plotted as mean ± SD; $N = 240$. (G) G-ratio probability density distributions of myelinated optic nerve axons of (+/+) and (+/−), $N = 240$. (H) Immunoblot of optic nerves from six randomly selected animals of indicated genotype. The antigens are indicated, NFAA is neurofilament associated antigen, which is axonal marker. (I) Quantitation of the blots normalized to ponceau staining. Data are relative to wild-type levels and is plotted as mean ± SEM, $n = 3$ (all panels, *$P < 0.05$; **$P < 0.01$).
defects in synapse formation. We initially examined RGC RGCs would have produced a favorable skewing of X- was approximately 47%. Secondary selection of atrophic axons. However, the CASK content within optic nerve indeed be an overestimate due to omission of very small gauge statistical significance. Our data thus indicate that the decrease zones (release sites) per unit volume of RGC terminals are affected by CASK loss. Our data indicate the lamination of retinal layers is not perturbed in CASK(−/−) mice (Supplementary Fig. S3).

To assess whether there was a specific loss of retinal ganglion cells in CASK(−/−) mice we counted the total number of cells within the ganglion cell layer of the retina, by labeling nuclei with DAPI and staining for RBPMS, a RNA binding protein selectively expressed by RGCs in the rodent retina(Fig. 5A). Together these data reveal a significant loss of RGCs in CASK(−/−) mutants (Figs. 5A, 5B). Taken together, we find that ONH in CASK(−/−) mice is a complex developmental disorder involving a decrease in the number of RGCs, thinning of individual axons, reactive astrogliosis within the ON, and alterations in retinogeniculate synapses. Thus, mutation of CASK appears to phenocopy many of the features observed in human patients with ONH, and therefore represents an appropriate mouse model to elucidate the mechanisms underlying ONH.

ONH Develops Postnatally in CASK(−/−) Mice

The first mechanistic question regarding ONH pathogenesis that has eluded our understanding is when exactly the disease is initiated. The decrease in RGCs and their axons could occur pre- or postnatally. While it is difficult to parse these differences out in humans, in this mouse model the difference can be studied by observing ON diameter throughout development. We therefore examined toluidine blue–stained semithin section of CASK(−/−) mice and control optic nerves during the first 3 weeks of postnatal mouse development (Fig. 6A). It is important to point out that the first 2 postnatal weeks of rodent development correspond to developmental milestones that occur in the third trimester of human fetal development. Moreover, neonatal mice do not open their eyes until the end of the second postnatal week of development. Surprisingly, the ON of P1 CASK(−/−) mice was of similar size to that of the wild-type controls (although, a slight trend toward being smaller was observed). A more clear and significant difference between the size optic nerves became apparent by the end of the first week of postnatal development and the difference from the wild type reaches the difference observed in adults by P22 (Fig. 6B). Although myelination starts around P5 in mouse ON it peaks during the third and fourth postnatal week. By P22 myelinated axons are easily quantifiable in the ON. To confirm that the decrease in axonal density occurs at an early age and not as a part of later degeneration, we examined the number of axons in P22 optic nerve per unit area. Our data indicate that axonal density is already reduced (at par as noted in adults Fig. 2C) by this stage suggesting that CASK-linked ONH can clearly be classified as a developmental and not a
CASK haploinsufficiency produces ONH.

**Figure 4.** CASK<sup>+/−</sup> mice display a decrease number of retinogeniculate synapses and a reduced number of active zones in the remaining synapses. (A) Quantification of the area of retinal terminals in dLGN by anterograde CTB-labeling. Red arrows represent distinct CTB-labeled retinal terminals of different sizes. Data represents mean ± SEM, n = 4. (B, C) Representative image of Serial Block-Face Scanning Electron Microscopy (SBFSEM) ultramicrograph with a retinogeniculate presynapse (identified by presence of pale mitochondria indicated with yellow arrows) labeled red and a postsynapse labeled green. Examples of reconstructed retinal terminals from a series of SBFSEM micrographs are shown on the right. Volumetric quantitation (D), number of active zones (E), and active zone density (F), of the presynaptic boutons from 60 reconstructed retinogeniculate.
synapses (SBFSEM analysis) from control and mutant mice. Data represent mean ± SEM, n = 3 mice per genotype. (CP < 0.05; **P < 0.01). (G) Scattered plot of terminal volumes showing two linear regressions for (+/+) and (+/-). Arrows indicate the threshold where the data points start to lose linearity (correlation coefficient R² value starts and continue decreasing). Terminals with volumes greater than or equal to threshold are classified as large terminals, and terminals with volume values that are smaller than the threshold are considered as small terminals. Threshold value for (+/+) is 7 and (+/-) is 10 µm³. (H) Comparisons of small and large axons between CASK⁶/⁶⁺ and CASK⁶/⁶⁻ by terminal volume (bouton), data are plotted as mean ± SD; n = 3.

degenerative process. Because, we observed thinning of optic nerve only from P6, we repeated the measurement of RGCs at P6. Our data indicates that at P6 there is no reduction in RGC numbers in CASK⁶/⁶⁻ mice, indicating a potential loss of RGCs early in development (Figs. 6D, 6E). Overall, our data demonstrate that ONH in CASK⁶/⁶⁻ mice occurs early in postnatal development (P6–P22), prior to the full maturation of the subcortical visual system.

DISCUSSION

Optic nerve hypoplasia is the most common cause of childhood blindness in developed nations, and its prevalence is growing at an alarming rate.⁴² A validated genetic animal model of ONH recapitulating the clinical condition with high fidelity will serve not only as a necessary tool to understand the mechanism of disease pathogenesis, but also to test potential therapeutic interventions. Optic nerve hypoplasia may occur with midline defects as observed in SOD, with other brain malformations like atrophic brain or even as isolated condition.⁵ The coincidence of ONH with other brain malformations such as midline defects associated with microcephaly is not surprising given that the retina and optic nerves are a part of our central nervous system and subject to the same developmental processes as the brain. Therefore, a clear understanding of ONH may also help unlock the pathophysiology of other neurodevelopmental disorders. In fact, subjects with ONH display a high incidence of autistic phenotype such as stereotypy.⁴⁸,⁴⁹ In this regard, mutations in CASK gene as a causal factor for ONH is particularly important because CASK mutations are also known to produce autistic traits.⁵⁷ CASK directly binds and phosphorylates neuronal adhesion molecule neurexin.⁵⁹,⁶⁰ It is pertinent to note that mutations in both neurexins and their transsynaptic interacting partners also are associated with autism.⁶⁰ Despite known interactions between CASK and neurexin, no synaptic ultrastructural defect has been shown to be associated with CASK mutation in any animal model. Here, we show for the first time clear morphologic defects at retinogeniculate synapse involving changes in size and number of release site. Such changes align with the idea that CASK may be involved in trafficking of molecules and/or acts as a molecular scaffold at the synapse. Although it may also be possible that changes in synapses reflect the atrophy in axons. Mutations in the CASK gene are also associated with postnatal microcephaly.²⁴ It is possible to argue that ONH may simply be a part of microcephaly. However, other monogenic syndromes associated with postnatal microcephaly such as Rett or Angelman syndrome do not present with ONH.⁵¹,⁵² indicating that ONH is an independent manifestation of CASK mutation.

CASK is a membrane associated guanylate kinase protein, which interacts with a large number of other molecules. Specifically, CASK has been considered to be a synaptic scaffolding molecule both at the pre- and postsynaptic

**Figure 5.** Decreased number of retinal ganglion cells in CASK⁶/⁶⁻ mice. (A) Immunostaining of retina with antibodies against calretinin reveals normal lamination in CASK⁶/⁶⁻ mutants at P26. (B) Representative images of retinas stained with DAPI and RBPMS (a marker of RGCs) reveal a significant loss of cells in the ganglion cell layer in CASK⁶/⁶⁻ mutants compared with controls. Scale bar: 25 µm. Lower panel is a grayscale image for better visualization. (C) Quantification of cells in the ganglion cell layer in indicated staining. (CP < 0.05). Data are relative to wild-type levels and is plotted as mean ± SEM, n = 4.
compartments. We have recently made two independent observations, first, CASK stabilizes neuronal adhesion molecule neurexin and links it to the signaling molecule liprin-a. Liprin-a's are critical for photoreceptor axonal targeting in drosophila as well as for forming active zones. Neurexins themselves may play a critical role in intercellular adhesion and signaling. A reduction in neurexin signaling and defect in axonal targeting may produce ONH, secondary to defect at the retinogeniculate synapses due to retrograde degeneration. Second, CASK may regulate cellular metabolism including mitochondrial respiration. Retina and optic nerve are highly susceptible to metabolic defects, and mitochondrial damage and mutations in mitochondrial genes are frequently associated with optic neuropathies. In fact, as many as 12% cases of nonsyndromic mitochondrial cytopathies may display ONH. Thus, a change in metabolic status of retinal cells may also lead to ONH in CASK<sup>+/−</sup> mice and CASK haploinsufficient girls.

Mutations in CASK have been associated with diagnosis of both ONH and ONA. The difference between these two conditions is often blurred and it has been speculated that better imaging techniques may differentiate these conditions. Many authorities even suggest that both ONH and ONA may be similar pathology with the only difference being in their

**Figure 6.** CASK<sup>+/−</sup> mice optic nerve display secondary reduction in size. (A) Representative images of toluidine blue–stained semithin-sections derived from optic nerve of indicated ages and genotypes. Scale bars: 100 μm. (B) Quantitation of optic nerve areas from mice of indicated age and genotype. Data are plotted as mean ± SEM, n = 3. (C) Quantitation of axonal density. Data are plotted as mean ± SD. (P < 0.05; **P < 0.01). (D) Representative images of retinas from P6 mice stained with DAPI and RNA-RPMS reveal no change in cell number in the ganglion cell layer of CASK<sup>+/−</sup> mutants compared with controls. Scale bar: 25 μm. (E) Quantification of DAPI- and RPMS-positive cells in the ganglion cell layer in P6 mutant and control retina.
decrease in optic nerve area in CASK mutants have ONH, which was not diagnosed earlier. In fact, many infants that are diagnosed with ONA may actually simply stem from the timing when originally uncovered. CASK neuropathies may not be amenable to straightforward binary classification simply by morphology. We suggest that mutations in CASK is typically associated with ONH, the diagnosis of ONH may simply stem from the timing when originally uncovered. In fact, many infants that are diagnosed with ONA may actually have ONH, which was not diagnosed earlier.

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