Non-Cell Autonomous Roles for CASK in Optic Nerve Hypoplasia

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PURPOSE. Heterozygous mutations in the essential X-linked gene CASK associate with optic nerve hypoplasia (ONH) and other retinal disorders in girls. CASK+/− heterozygous knockout mice with mosaic CASK expression exhibit ONH with a loss of retinal ganglion cells (RGCs) but no changes in retinal morphology. It remains unclear if CASK deficiency selectively affects RGCs or affects other retinal cells. Furthermore, it is not known if CASK expression in RGCs is critical for optic nerve (ON) development and maintenance.

METHODS. The visual behavior of CASK+/− mice was assessed and electoretinography (ERG) was performed. Using a mouse line with a floxed CASK gene that expresses approximately 40% CASK globally in all cells (hypo/hemaporph) under hemizygous and homozygous conditions, we investigated effects of CASK reduction on the retina and ON. CASK then was completely deleted from RGCs to examine its cell-autonomous role. Finally, for the first time to our knowledge, we describe a hemizygous CASK missense mutation in a boy with ONH.

RESULTS. CASK+/− heterozygous mutant mice display reduced visual contrast sensitivity, but ERG is indistinguishable from wildtype. CASK hypomorph mice exhibit ONH, but deletion of CASK from RGCs in this background does not exacerbate the condition. The boy with ONH harbors a missense mutation (p.Pro673Leu) that destabilizes CASK and weakens the crucial CASK–neurexin interaction.

CONCLUSIONS. Our results demonstrate that mosaic or global reduction in CASK expression and/or function disproportionately affects RGCs. CASK expression in RGCs does not appear critical for cell survival, indicating a noncell autonomous role for CASK in the development of ON.

Keywords: optic nerve hypoplasia, CASK, retinal ganglion cells, non-cell autonomous

Optic nerve hypoplasia (ONH) is the most common cause of childhood blindness in developed nations, and its incidence is on the rise.1,2 ONH involves thinning of the optic nerve (ON) and typically is associated with loss of retinal ganglion cells (RGCs) and their axons.3 Most cases of ONH are nongenetic in nature, and therefore, the etiogenesis of ONH has remained obscure. Genetically identified forms of ONH are associated with transcription factors that are directly involved in the development of RGCs.4 In many instances of ONH, however, RGCs initially begin to develop and connect with the brain, but development stalls or RGCs undergo atrophy before complete ON development.5,6 Therefore, to better understand the etiopathogenesis of ONH, face-validated animal models of this disease must be investigated.

We demonstrated that haploinsufficiency of the X-linked gene CASK (calcium/calmodulin activated serine kinase) produces ONH in humans and mice.7 Like many forms of human ONH, CASK-linked ONH does not affect formation of RGCs.7 The protein product of this gene (also CASK) is a peripheral scaffolding protein,8 and its role in RGC development or survival remains unknown. In addition to ONH, mutations in CASK also are associated with other retinopathies, including retinal dystrophy, indicating that retinal cells distal to RGCs also may be affected.9–11 In previous work7 on mice exhibiting CASK-linked ONH, we did not observe any change in lamination or loss of non-RGC retinal cells. Here, we investigated the effect of CASK haploinsufficiency on the function of retinal cells and determined if CASK deficiency produces an RGC-specific pathology in a cell-autonomous fashion.

Our results demonstrated that CASK haploinsufficiency produces isolated RGC pathology. We also demonstrated that global reduction of CASK by approximately 60% as observed in CASK−/− mice (mice with floxed Cask gene; stock #006382; Jackson Laboratories, Bar Harbor, ME, USA)12 is sufficient to induce RGC loss. Surprisingly, we find that despite this
disproportionate sensitivity of RGCs to CASK deficiency. CASK expression in an RGC is not important for its survival or maintenance. Finally, using a human case report, we demonstrated that a hemizygous partial loss-of-function mutation in CASK is sufficient to produce ONH. Taken together, these results suggest that global reduction in CASK expression or function adversely affect RGC survival and CASK deficiency produces a developmental RGC pathology via a noncell autonomous mechanism.

METHODS

Statement of Ethics

All experiments were performed in accordance with Virginia Tech Institutional Animal Care and Use Committee, the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research, and institutional review board guidelines and approved protocols.

Visual Behavior Tasks

The two-alternative forced-swim task was performed as described previously. The method is based on finding a hidden platform in response to visual cues. See Supplementary Material for full methods.

Electroretinography (ERG)

Stimulus-dependent transcorneal potential changes from both eyes were simultaneously recorded using the UTAS BigShot system (LKC Technologies, Gaithersburg, MD, USA). Photopic recordings ensued immediately after scotopic recordings by exposing the animals to a white background light of 30 Cd/m² intensity for 10 minutes followed by flashes of 25 Cd/s/m² in intensity and presented at 1 Hz frequency for 90 seconds. See Supplementary Material for full methods.

Analysis of Human CASK Sequence Variant

The human CASK sequence variant, p.Pro673Leu, was examined biochemically and in silico as described previously by us. Details of the methodology are in the Supplementary Material.

Immunoblots and Immunohistochemistry (IHC)

Detailed methodology is described in the Supplementary Material. IHC was performed as described previously.

In Situ Hybridization (ISH)

ISH was performed on 16-μm sections as described previously. Sense and antisense riboprobes were generated against full-length CASK IMAGE Clone (catalog number MM1013-202761641; Dharmaco, Lafayette, CO, USA). Riboprobes were synthesized using digoxigenin (DIG; Roche, Mannheim, Germany) and the MAXI-Script In Vitro Transcription Kit (Ambion, Austin, TX, USA). Probes were hydrolyzed to approximately 500 base pairs. Images were obtained on a Zeiss LSM700 confocal microscope (Carl Zeiss Meditec, Jena, Germany).

Animals

Cask+/− female mice generated in-house were crossed with C57BL6 male mice to generate CASK+/− female pups and their Cask+/+ female littermates. Calb2-Cre (stock #010774), Rosa-stop-tdT (Ai9; stock #007909), Cask+/− (stock #006382) all were obtained from Jackson Laboratories. Genotyping was done using a PCR-based method with primer pairs given in Supplementary Table 1. All mice used in these experiments were between 3 and 6 months old.

ON Toluidine Blue Staining and Electron Microscopy [TEM]

ON analysis was performed as described previously. Details of the methodology are in the Supplementary files.

RESULTS

Heterozygous Loss of CASK Produces Behavioral But Not ERG Deficits in Vision

We described previously that heterozygous loss of Cask (Cask+/−) in mice results in ONH. To assess visual function in Cask+/− mice, we used a two-alternative forced-swim task. In this task, mice learned to associate a visual cue of a sine-grating with a hidden platform used to escape the water (Fig. 1A). Mice genetically devoid of RGCs are unable to perform this task. We first assessed the ability of Cask+/− mice (and female wild-type [WT] littermates) to learn this task. Cask+/− mice and controls were trained for 8 days on a vertical grating (0.17 cycles per degree [cpd]) on the S+ monitor above a hidden platform (compared to a gray screen on the S− monitor). When their ability to locate the hidden platform in 10 trials (per day) exceeded 70%, they were considered to have successfully discriminated between the visual cues. Cask+/− mice and female littermate controls performed at similar rates through the 8 days of training, and by the end of this training both genotypes were performing at near 100% accuracy (Fig. 1B).

By changing the spatial frequency of the S+ sine-grating, we were able to test visual acuity of mutant and control mice. Although Cask+/− mice performed slightly worse than littermate controls, there was no significant difference in performance regardless of the spatial frequency of the gratings (Fig. 1C). We then altered the contrast of the gratings. Cask+/− mice performed statistically worse than littermate controls in these tasks, and their performance consistently worsened (compared with littermates) as the contrast of the grating decreased (Fig. 1D). Thus, in addition to ONH and reduced RGCs, Cask+/− mice exhibited readily noticeable decreased visual performance.

To assess whether this behavioral deficit was due to ONH (and RGC loss) or from defects within the retina, ERGs were recorded on Cask+/− mice and controls. ERG waveforms are well-documented to reflect neural activity from the outer retina, with the a-wave reflecting photoreceptor activity and the b-wave reflecting activity of the depolarizing bipolar cells. Under photopic conditions, ERG b-wave responses appeared similar between Cask+/− and WT littermates (Fig. 1E). Moreover, the average photopic b-wave amplitudes elicited by light flashes of 25 Cd/sec/m² in intensity showed no difference between genotypes (Fig. 1F). Likewise, scotopic a-wave amplitudes also were comparable between Cask+/− and WT littermates (Figs. 1G, 1H).

By fitting the ensemble scotopic b-wave amplitudes versus retinal illuminance to a modified Naka-Rushton function for maximum rod- and cone-driven responses and their half-saturating light intensities, we further found their ERG responses to be very similar (Fig. 1I). Taken together, these results indicated that outer retinal function is normal in Cask+/− mice and, thus, suggested that the observed deficit in visual behavior is likely due to the loss of RGCs and their axonal thinning.
Global Reduction in CASK Expression Produces ONH

Due to random X-inactivation, CASK heterozygous mutations result in a mosaic condition, with approximately 50% of brain cells generating 100% of CASK and approximately 50% of cells being CASK-null. In our experiments designed to understand the cellular role of CASK in RGCs and ON development, such mosaicism is a confounding variable. A global CASK loss-of-expression mutation, in either a hemizygous male or homozygous female mouse, is a useful model to help disentangle this confound.

Cask^+/0 mice do not survive, but a conditional allele of Cask (Cask^fl/fl) generated previously was shown to cause a global reduction in CASK expression (~40% of WT expression) due to selection cassette interference.12 This Cask^fl/fl mouse also has been reported to have microcephaly and cerebellar hypoplasia.18,26

We confirmed that CASK expression in the ON of Cask^0/0 mice (Fig. 2A) is similarly reduced; therefore, we used these mice to determine if uniform reduction in CASK expression impacts the developing visual system. Toluidine blue sections showed that Cask^+/0 ONs are thin and hypoplastic compared to littermate Cask^++/++ controls (Fig. 2B), demonstrating that global reduction of CASK in all cells results in ONH.

Next, we evaluated RGC numbers in Cask^+/0, Cask^fl/+, and controls using a marker for RGCs (RBPMS) and a Nissl stain. We saw a dose-dependent decrease in RGCs in the presence of the floxed allele of CASK (Fig. 2D): one floxed allele (Cask^fl/+) significantly reduced RGC numbers compared to controls (Cask^++/++), and homozygous floxed alleles (Cask^0/0) significantly reduced RGC numbers compared to heterozygotes (Cask^fl/+) and controls (Cask^++/++; Fig. 2E). This decrease is comparable to what we previously found in Cask^++/0 retinas (27.2 ± 3.1 RBPMs cells; Cask^0/0 30.6 ± 2.9 RBPMs cells).7 We also evaluated total numbers of cells in the RGC layer using a...
NeuroTrace stain to verify that RBPMS expression is not sensitive to CASK deficiency. Cask<sup>fl/fl</sup> mice have a reduction in total numbers of NeuroTrace-positive cells in the RGC layer compared to Cask<sup>+/+</sup> mice (Fig. 2F). We saw no difference in the average area of each RGC, based on measurement of RBPMS signal (WT 57.8 ± 8.6 µm<sup>2</sup>; CASK 61.4 ± 8.7 µm<sup>2</sup>). Finally, we explored potential axonopathy in Cask<sup>fl/fl</sup> mice. RGC axon number and diameter were evaluated using TEM of ON cross-sections from Cask<sup>fl/fl</sup> and control mice (Fig. 2G). No reduction in the density of axons in Cask<sup>fl/fl</sup> samples was observed compared to Cask<sup>+/+</sup> ONs (Fig. 2H), but we did observe a significant decrease in the average cross-sectional area of axons in Cask<sup>fl/fl</sup> ON (WT 1.54 ± 0.70 µm<sup>2</sup>, CASK 1.20 ± 0.52 µm<sup>2</sup>; P < 0.05). RGC axons in the ON can be classified as fine or coarse. Previous analysis in Cask<sup>+/+</sup>/C0 mice revealed a loss of fine and coarse RGC axons. When plotting RGC axon diameter, we could identify an inflection point between axon types in Cask<sup>fl/fl</sup> and controls (Fig. 2I; 2.208 µm<sup>2</sup> in controls). By separating the fine and coarse axons and averaging axon size for each type, we...
observed that fine (Fig. 2J) and coarse axons (Fig. 2K) were significantly reduced in Caskfl/fl ON. Myelination patterns were evaluated in TEM images, and Caskfl/fl ON myelination appeared normal in adulthood. However, analysis of the ON at P12 showed a reduction in total myelinated axon numbers compared to Cask+/+ (Supplementary Fig. S1). This difference was no longer apparent by P18, suggesting that global reduction in CASK delays the onset of myelination. Overall, our data suggested RGCs are extremely sensitive to level of CASK expression.

**CASK is Expressed in RGCs**

To analyze the role of CASK in RGC development, we first asked if RGCs express detectable levels of CASK using in situ hybridization (Fig. 3A). Riboprobes against Cask mRNA were developed and revealed widespread expression in all three nuclear layers (Fig. 3B); no appreciable reactivity in these regions (versus synaptic regions) was observed with a sense riboprobe (Fig. 3C). Importantly, we observed significant reactivity of the CASK antisense riboprobe in the ganglion cell layer (GCL), suggesting CASK is generated by RGCs. Results were validated by showing CASK immunoreactivity in the GCL using IHC (Fig. 3D).

**RGC-Derived CASK is Not Required for RGC Survival**

Due to the aforementioned sensitivity of RGCs to CASK levels, we next wanted to evaluate whether RGCs were dependent upon CASK expression for survival. To remove CASK from RGCs, we sought a driver line that generated Cre recombinase in RGCs to excise the floxed Cask allele. One option was calretinin-Cre (Calb2-Cre). Calretinin is generated by RGCs, as well as some types of horizontal and amacrine cells in the developing and adult mouse retina.30–32 Importantly, calretinin is not generated by thalamic relay cells. Furthermore, calretinin (and Cre in Calb2-Cre mice) is generated by RGCs at neonatal ages in mice.13,33 The percentage of RGCs in which Calb2-Cre exhibited recombination activity was quantified by crossing with a reporter line (Rosa-stop-tdT A9). As expected, widespread expression of tdT was observed in the retinas of Calb2-Cre::Rosa-stop-tdT mice (Fig. 4A, 4A'). Cross-section analysis showed that tdT expression is present in cells of the inner nuclear layer and GCL (Fig. 4B; 77 ± 2.5% of 4'6-diamidino-2-phenylendole (DAPI)-labeled cells in the GCL were tdT+; n = 4 mice). Since misplaced amacrine cells also reside in the GCL and also may express Cre in this driver line (Fig. 4C), we used immunostaining for RBPMS to assess the percent of RGCs expressing Cre in this line (Fig. 4C'). We found 91.1 ± 2.4% (n = 4 mice) of RBPMS cells were tdT+ in Calb2-Cre::Rosa-stop-tdT. Furthermore, 87.6 ± 5.1% of the tdT+ cells were immunoreactive for RBPMS. It remains possible that approximately 9% of RGCs that do not generate Cre in Calb2-Cre mice belong to a small number of distinct subtypes of RGCs, such as intrinsically photosensitive RGCs (ipRGCs). This was not expected, based on scRNAseq analysis of murine RGCs, which has shown Calb2 (also known as calretinin) in all subtypes of RGCs, but to address this possibility, we used IHC to show that ipRGCs and other subtypes of RGCs are labeled in Calb2-Cre mice (Supplementary Fig. S2).

To assess the requirement for CASK in RGCs, Caskfl/fl::Calb2-Cre+/− mice were generated. These mutants have a global reduction in CASK expression (as described above), as well as a complete loss of CASK in approximately 91% of RGCs. We compared the ON of Caskfl/fl::Calb2-Cre+/− mice with Caskfl/fl::Calb2-Cre−/− mice using toluidine blue staining as described earlier (Fig. 5A). Surprisingly, there was no further reduction in the size of ON when CASK was abolished from most RGCs (Fig. 5B). Furthermore, when RGCs were labeled with RBPMS and NeuroTrace (Fig. 5C), no significant loss of RGCs or overall cells in the GCL layer in the Caskfl/fl::Calb2-Cre−/− mice was observed compared to Caskfl/fl::Calb2-Cre+/−, suggesting that lack of CASK expression in remaining RGCs does not affect their survival (Figs. 5D, 5E). Finally, axonal morphology in the ON of these mutants (Fig. 5F) was assessed; there were no differences in axonal density of Caskfl/fl::Calb2-Cre+/− or Caskfl/fl::Calb2-Cre−/− mice (Fig. 5G). Overall our data indicated that CASK expression in RGCs is not a requisite for RGC development or survival.

**Partial Loss of Function Mutation of CASK in a 3-Year-Old Boy With ONH**

CASK loss in males is neurodevastating and can be associated with ONH35 and epileptic encephalopathies.36 Missense mutations that decrease CASK function, however, are frequently present in males with X-linked intellectual disability who survive into adulthood. These cases typically have been reported to display nystagmus, strabismus, and reduced visual acuity. An analysis of the optic nerve in these cases, however, has not been described. We reported a 3-year-old boy with a milder condition exhibiting microcephaly and severe pontocerebellar hypoplasia (MICPCH) similar to haploinsufficient females (Fig. 6A) and ON thinning (Fig. 6B). This subject presented with decreased visual acuity (20/130; Teller acuity test) and horizontal nystagmus (with a pendular jerk of small amplitude and frequency) that did not dampen with convergence, indicating that it may be central in origin. These phenotypes (thin ON, nonretinal defects) led to the diagnosis of ONH. Exome sequencing uncovered a CASK variant c.2018C>T (p.Pro673Leu) that was absent from the parents and had not been identified or reported in any other database.

We expressed recombinant CASK containing the P673L variation (CASKP673L) fused with GFP protein for biochemical analysis. WT CASK (CASKWT) expressed in HEK293 cells diffusely fills the cytosol (Fig. 6C), whereas expression of CASKP673L results in an aggregation of protein (Fig. 6D), indicating that this protein may be partially misfolded or prone to aggregation compared to WT. CASKP673L also exhibits higher insolubility, supporting the notion that this protein variant may have a propensity to misfold (Fig. 6E).

We used molecular dynamics (MD) simulations to simulate the impact of the P673L mutation on the structure of CASK. Three 100 ns MD simulation trajectories were run using a model of the CASK WT and CASKP673L PDZ-SH3-GK (PSG) supradomain.15 The radius of gyration of a protein structure is an important observation given CASK's role in binding to other proteins. We found that CASKP673L has a smaller radius of gyration (2.1 nm) than WT (2.4 nm), an important observation given CASK's role in binding to other proteins. We determined that the P673L mutation has a significant effect on the structure of CASK, leading to aggregation of protein (Fig. 6D).

To further specify the impact of a given mutation; in the case of CASKP673L, of particular interest is the notable absence in
helical propensity and mobility of the α-C helix of the CASK P673L PDZ domain; this particular alpha helix is purported to be involved in coupling the PDZ and SH3 domains to allow for ligand binding. The P673L variation in CASK’s SH3 domain disrupts packing between all three of the domains, causing a reorientation of the domains with respect to each other (Fig. 6F). This disruption in packing may indicate that CASK P673L is more likely to adopt a conformation that oligomerizes. In sum, these structural differences point to a CASK P673L structure that is slightly less compact than its...
Upon coexpressing neurexin-1 b quantitatively measure the change in CASK P673L affinity revealing that neurexin is unable to efficiently recruit the neurexin-1 revealed that the affinity of CASKP673L for neurexins is reduced towards neurexin, we performed GST pulldown assays using the cytosolic tail of neurexin-1 fused to the GST protein, which did not colocalize on the membrane (Fig. 6H), CASK P673L and Cask also did not colocalize on the membrane (Fig. 6H), neurexin is unable to efficiently recruit the remaining soluble fraction of CASKP673L from the cytosol. To quantitatively measure the change in CASKP673L affinity towards neurexin, we performed GST pulldown assays using the cytosolic tail of neurexin-1 fused to the GST protein, which revealed that the affinity of CASKP673L for neurexins is reduced by approximately 80% compared to CASKWT (Figs. 6I, 6J).

Overall, these results suggested that the P673L sequence variation affects the structure of CASK sufficiently to increase misfolding and impair but not abolish its interaction with neurexins. Such a reduction in functional CASK is sufficient to affect projection neurons, such as those in pontocerebellar circuitry.9

Since the CASK hypomorph line is based on a floxed Cask allele, we also used this line to delete all CASK expression from RGCs. Surprisingly, total loss of CASK from RGCs did not exacerbate the ONH phenotype, indicating RGC-derived CASK is not critical for RGC survival. How, then, does CASK reduction produce ONH? There are at least three possible explanations. First, although the discernible pathology of ONH appears late in the course of development, it is possible that the biological insult required for ONH occurs earlier in development before the Cre-mediated deletion of the CASK gene. This, however, does not explain why RGCs are more susceptible. Secondly, similar to the experimental models of glaucoma, it is possible that certain RGC subtypes are highly susceptible to CASK deficiency and already are lost in the CASK hypomorph line; hence, no further decrease would be observed upon targeted deletion of CASK in the remaining RGCs. This would, however, suggest that the mosaic Cask-/- mice display a postnatal decrease in the number of RGCs, thinning of the ON, and atrophy of RGC axons.7 Despite deficits in RGCs, no defects are observed in other cells or in laminination of the Cask-/- retina.7 Cask-/- mice also exhibit significantly decreased contrast sensitivity. Based on retinal morphology and ERG data, we suggest that this visual deficit is due to RGC pathology and not due to defects in retinal circuitry. Thus, CASK haploinsufficiency specifically affects RGCs to produce ONH. Furthermore, we demonstrated that global reduction of CASK expression by approximately 60% also is sufficient to induce ONH, indicating that RGCs are extremely sensitive to CASK loss. Why are RGCs disproportionately affected by CASK loss? The anatomy and physiology of RGCs are unique; they are the predominant action potential-generating neurons within the retina and have extremely long, myelinated axons, unlike other retinal cells. In other brain regions, Cask loss or mutation appears to disproportionately affect projection neurons, such as those in pontocerebellar circuits.8

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DISCUSSION

Although the prevalence of ONH is on the rise, the mechanism that underlies its onset and progression remains unknown. ONH is likely a heterogeneous disorder with differing etiologies that share similar downstream pathogenesis. Many cases of ONH arise much later in perinatal and postnatal development and are associated with environmental factors.46,47 The mechanism of this later-onset ONH is not clear. Mouse models that target genes involved in RGC development often lead to complete nondevelopment of eyes (anophthalmal)48 or of the ON itself49 and, thus, do not recapitulate ONH. Heterozygous deletion of CASK produces ONH and microcephaly in girls and female mice.7,9,18,50 We have shown that microcephaly linked to CASK mutation may stem from loss of PDZ domain-mediated interactions with proteins, such as neurexin.15 Analysis of a CASK variant (CASKP673L) in a male subject with microcephaly and ONH suggests that microcephaly and ONH are likely to result from disruption of PDZ domain-mediated interactions of CASK. CASKP673L's interaction with neurexin, however, is not entirely abolished but merely reduced, indicating that this mutation causes partial loss of function in CASK. Such global CASK partial loss-of-function is compatible with survival but sufficient to produce ONH. Intriguingly, CASK mutations also have been associated with retinopathies.9,10 These observations pose an interesting question: do CASK mutations produce isolated ONH or are the ON pathologies reflective of a broader retinal disorder? Answering this question is not only critical for more accurately classifying the ocular phenotypes seen with CASK mutations, but also is crucial for validating CASK mutant mice as tools to investigate mechanisms underlying ONH.

**FIGURE 4.** Calb2-Cre labels RGCs. (A) Retinal whole mount of the GCL of Calb2-Cre::Rosa-stop-tdT (Calb2-Cre::tdT). Note the large number of cells labeled with tdT. (B) Retinal cross-section of Calb2-Cre::tdT shows tdt⁺ cells in the INL and GCL. (C) tdt⁺ cells in the GCL of Calb2-Cre::tdT co-label with RBPMS. Scale bar: 50 μm, all Figures.

WT counterpart and has alterations in the region predicted to interact with known binding partners, such as neurexin.39,41-45

Based on the structural changes predicted by MD in CASKP673L, we next tested these in silico predictions by determining whether the mutation indeed impairs CASK-neurexin binding in vitro by using a previously described recruitment assay where distribution of GFP-CASK can be altered from cytoplasmic to membrane-bound upon coexpression of neurexin-1β (Figs. 6C, 6G). For this recruitment assay, analysis was done only on cells where GFP-CASKP673L was not aggregating. Upon coexpressing neurexin-1β, CASKP673L and neurexin-1β did not colocalize on the membrane (Fig. 6H), revealing that neurexin is unable to efficiently recruit the remaining soluble fraction of CASKP673L from the cytosol. To quantitatively measure the change in CASKP673L affinity towards neurexin, we performed GST pulldown assays using the cytosolic tail of neurexin-1 fused to the GST protein, which revealed that the affinity of CASKP673L for neurexins is reduced by approximately 80% compared to CASKWT (Figs. 6I, 6J).

Overall, these results suggested that the P673L sequence variation affects the structure of CASK sufficiently to increase misfolding and impair but not abolish its interaction with neurexins. Such a reduction in functional CASK is sufficient to produce ONH manifestation, indicating RGCs may be highly sensitive to CASK functional deficiency. Furthermore, our data pointed to the strong possibility that other boys with CASK missense mutations also may have ONH.
Figure 5. RGC-derived CASK is not required for RGC survival. (A) Toluidine blue staining shows no reduction in ON size in Cask<sup>fl/fl</sup>::Calb2-Cre<sup>+/+</sup> compared to Cask<sup>fl/fl</sup>. Scale bar: 50 μm. (B) Quantification of cross-sectional area in Cask<sup>fl/fl</sup>::Calb2-Cre<sup>+/+</sup> compared to Cask<sup>fl/fl</sup>. (n = 4 mice per genotype). (C) No reduction in number of cells in GCL layer using RBPMS (RNA-binding protein with multiple splicing) or NeuroTrace. Scale bar: 50 μm. (D) Quantification of number of cells in GCL using RBPMS in Cask<sup>fl/fl</sup>::Calb2-Cre<sup>+/+</sup> compared to Cask<sup>fl/fl</sup>. (n = 10 mice per genotype). (E) Quantification of number of cells in GCL using NeuroTrace in Cask<sup>fl/fl</sup>::Calb2-Cre<sup>+/+</sup> compared to Cask<sup>fl/fl</sup>. (n = 6 mice per genotype). (F) TEM of RGC axons in the ON in Cask<sup>fl/fl</sup>::Calb2-Cre<sup>+/+</sup> compared to Cask<sup>fl/fl</sup>. Scale bar: 5 μm. (G) Quantification of density of axons per image in Cask<sup>fl/fl</sup>::Calb2-Cre<sup>+/+</sup> compared to Cask<sup>fl/fl</sup>. (n = 4 mice per genotype). (H–J) Analysis of axon area in Cask<sup>fl/fl</sup>::Calb2-Cre<sup>+/+</sup> ON compared to Cask<sup>fl/fl</sup>. Quantification of the average size of fine axons (I) and course axons (J). For all panels data are plotted as mean ± SEM.
FIGURE 6. Partial loss of function of CASK in a 3-year-old boy produces ONH. (A, B) CASK variant in a 3-year-old boy with microcephaly and severe pontocerebellar hypoplasia with CASK variant c.2018C>T (p.Pro673Leu) mutation. T2-weighted magnetic brain resonance images show small cerebellum (black arrow) and thinning of optic nerve (red arrow). (C) Expression of WT-CASK-GFP fusion protein in HEK293 cells shows localization of CASK in cytosol. (D) CASK\textsuperscript{P673L}-GFP fusion protein shows cotton-wool aggregates (white arrows). (E) Blot for CASK in soluble and insoluble fractions of cell lysate show that CASK\textsuperscript{P673L} is predominantly present in the insoluble fraction, similar to CASK\textsuperscript{L209P}, described previously to
have aggregation tendencies. (F) Structural models of the PDZ-SH3-GK supradomain of CASKWT (left) and CASKP673L (right). Site of P673L variation shown in orange, PDZ domain in purple, α-C helix in green, SH3 domain in yellow, hook/hinge region in salmon, and GK domain in gray. Arrows indicate structural differences between WT and variant structures. (G) Recruitment assay using a known CASK substrate, neurexin-1, shows that CASKWT colocalizes with neurexin. (H) CASKP673L is mislocalized, with some colocalization with neurexin but substantial amounts visible in the cytosol. (I) GST-NRX is able to capture CASKWT but not CASKP673L. (J) Effectiveness of capture for CASKWT and CASKP673L is plotted after normalization to WT. Data are plotted as mean ± SEM; *P < 0.05 by 2-way ANOVA. N = 3. Scale bar = 5 μm.

mutant should have a milder phenotype than the global Cask−/− mutant, which is not the case. A third, more plausible explanation may be that the effect seen on RGCs is non-cell-autonomous in origin. A strong argument for this possibility is that in Cask−/− mice, we do not observe secondary selection (apoptosis) of neurons resulting from random X-linked inactivation, suggesting that neurons lacking CASK do not exhibit reduced survival.7,18 Further experiments are required in the future to fully investigate these three alternative mechanisms.

Importantly, CASK is present not only in neurons but also in glial and endothelial cells.55,56 Aberrant functioning of these cell populations is likely to affect survival and health of RGCs by producing changes in retinal metabolism and vascularization. ONH has been associated with mitochondrial cytopathies,57,58 peroxisomal disorders,57 and nutritional deprivation,58 all of which point toward a possible metabolic derangement. Similarly, altered vasculature, such as vascular tortuosity, has been suggested to be one of the hallmarks of ONH.47,59,60 Overall our data suggested that an in-depth study of CASK mutant mice may uncover a common underlying pathobiogenes of ONH.

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