

Gene Therapy Targeting the Inner Retina Rescues the Retinal Phenotype in a Mouse Model of CLN3 Batten Disease

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The neuronal ceroid lipofuscinoses (NCLs), often referred to as Batten disease, are inherited lysosomal storage disorders that represent the most common neurodegeneration during childhood. Symptoms include seizures, vision loss, motor and cognitive decline, and premature death. The development of brain-directed treatments for NCLs has made noteworthy progress in recent years. Clinical trials are currently ongoing or planned for different forms of the disease. Despite these promising advances, it is unlikely that therapeutic interventions targeting the brain will prevent loss of vision in patients as retinal cells remain untreated and will continue to degenerate. Here, we demonstrate that *Cln3*^{Δex7/8} mice, a mouse model of CLN3 Batten disease with juvenile onset, suffer from a decline in inner retinal function resulting from the death of rod bipolar cells, interneurons vital for signal transmission from photoreceptors to ganglion cells in the retina. We also show that this ocular phenotype can be treated by adeno-associated virus (AAV)-mediated expression of *CLN3* in cells of the inner retina, leading to significant survival of bipolar cells and preserved retinal function. In contrast, the treatment of photoreceptors, which are lost in patients at late disease stages, was not therapeutic in *Cln3*^{Δex7/8} mice, underlining the notion that CLN3 disease is primarily a disease of the inner retina with secondary changes in the outer retina. These data indicate that bipolar cells play a central role in this disease and identify this cell type as an important target for ocular AAV-based gene therapies for CLN3 disease.

Keywords: gene therapy, retina, Batten disease, CLN3, bipolar cells

INTRODUCTION

THE NEURONAL CEROID LIPOFUSCINOSES (NCLs), also known as Batten disease, are a group of fatal, inherited lysosomal storage disorders predominantly affecting children. The incidence is as high as 1:12,500 in some countries, while worldwide it is estimated to be 1:100,000.¹ NCLs are collectively characterized by the gradual accumulation of auto-fluorescent waste material in the lysosome of neuronal and non-neuronal cells, resulting in severe neuronal and retinal degeneration. Patients present with blindness, seizures, loss of motor and cognitive function, behavioral abnormalities, and premature death.² NCLs are typically inherited in an autosomal recessive manner and 13 disease-causing genes were identified that encode soluble or transmembrane proteins, residing mostly in the lysosome or endoplasmic reticulum.³

The most prevalent form of NCL is CLN3 disease, which is caused by mutations in *CLN3*, a gene with elusive function despite more than a decade of research. The ubiquitous

CLN3 protein is membrane-bound and primarily present in endosomes and lysosomes.⁴ In neurons, expression was also reported in axonal extensions and synaptosomes.⁵

Classic CLN3 disease presents during early childhood with progressive visual decline from as early as 4 years of age, usually leading to complete loss of vision within less than 2 years. Decline in cognitive abilities, behavioral problems, seizures, and motor defects follow. Cardiac abnormalities and death occur in the second and third decade of life.⁶ Approximately 85% of all CLN3 disease cases are caused by a homozygous 1 kb deletion mutation of exons 7 and 8, leading to a truncated protein (for a complete list of mutations see www.ucl.ac.uk/ncl/mutation). Other mutations and compound heterozygosity can have milder clinical manifestation. Of note, adult-onset non-syndromic CLN3 disease with ocular failure devoid of neurological involvement was found in several unrelated families.⁷⁻⁹

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Four genetically different *Cln3*-deficient mouse models are available. Unlike most other NCL animal models, all mouse models mimicking the CLN3 disease show a mild disease phenotype with a late-onset, slow progression, and minimally reduced life span, hindering the development of treatment.¹⁰

Cln3^{Δex7/8} mice, a knockin mouse line bearing the 1 kb deletion mutation commonly found in CLN3 disease patients, have been studied most extensively. The accumulation of lysosomal storage material and neuropathological changes precedes region-specific neuronal loss in these mice, which becomes evident from 12 months.^{11,12} A moderate decrease in retinal function with an electronegative response was measured in electroretinography (ERG) recordings, indicating that the function of inner retinal cells was reduced in *Cln3*^{Δex7/8} mice, while the function of the outer retina was not compromised.¹²

In line with these findings, the thickness of the inner retina, including cells of the inner nuclear layer (INL), ganglion cell layer, and nerve fiber layer, was decreased in another *Cln3*-deficient mouse line. The thickness of the photoreceptor layer and the retinal pigment epithelium (RPE) was not affected.¹³ The retinal histology was not assessed in detail in the other mouse models.

There is currently no cure for CLN3 disease. Several therapeutic interventions mostly targeting the brain were tested in preclinical studies using animal models for CLN3 disease or other forms of NCL.^{14,15} A handful of treatments were translated to the clinic. Most notably, an enzyme replacement therapy using biweekly intravitreal infusions of tripeptidyl peptidase (TPP1) showed efficacy in CLN2 disease patients¹⁶ and has now been approved by the Food and Drug Administration (FDA) and European Medicines Agency (EMA). A phase I/II clinical trial using central nervous system (CNS)-directed adeno-associated virus (AAV)-mediated gene supplementation therapy is currently ongoing for CLN6 disease patients (NCT02725580). A similar clinical trial was recently started for CLN3 disease (NCT03770572).

As the translation of brain-directed treatments for NCL progresses, the demand for therapies targeting other affected organs such as the eye is increasing. This is particularly relevant for transmembrane protein deficiencies of NCL as an effective correction of the ocular phenotype is unlikely upon AAV delivery to the CNS. Recently, we reported that ocular administration of an AAV-based gene therapy rescues the photoreceptor degeneration in *Cln6*-deficient mice, a model for CLN6 disease caused by defects in a membrane-bound protein.¹⁷

Here, we establish that *Cln3*^{Δex7/8} mice show a loss of bipolar cells, interneurons located in the INL of the retina, and confirm the selective dysfunction of the inner retina. We also show that the ocular phenotype can be treated by ubiquitous expression of *CLN3* in the inner retina or cell-type-specific expression in bipolar cells of

mutant mice. These data provide proof-of-concept supportive of ocular gene therapy targeting the inner retina in CLN3 disease, paving the way toward future clinical translation.

MATERIALS AND METHODS

Mice

Cln3^{Δex7/8} and C57BL/6J mice were purchased from Harlan Laboratories (United Kingdom). All mice were maintained under cyclic light conditions (12-h light/dark). Animal experiments were performed in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research, and UK Home Office and institutional regulations.

Plasmid construct

Mouse *Cln3* (mCln3) cDNA was cloned into a pD10 vector in between the cytomegalovirus (CMV) promoter and an internal ribosome entry site (IRES) cassette followed by enhanced green fluorescent protein (eGFP). Human CLN3 (hCLN3) cDNA was cloned into a pD10 vector containing the CMV promoter. To drive expression in all ON bipolar cells, hCLN3 cDNA was also cloned into the pAAV vector carrying the 4xGrm6 (4x enhanced mGluR6) promoter kindly provided by Botond Roska (FMI, Basel). pD10.CMV.eGFP and pAAV.Grm6.eGFP plasmids were used to produce control vector.

Production of rAAV

Both recombinant AAV 7m8 and AAV2/8 vectors were produced through a triple transient transfection method and purified by affinity-based AVB Sepharose column (GE Healthcare, United Kingdom) as described previously.¹⁸ Viral genome (vg) titers were determined by quantitative real-time PCR using a probe-based assay annealing to the SV40 poly A tail of the genomic plasmid. Final titers are stated as total vg per eye.

Viral vector administration

Viral vector administration was performed under general anesthesia using an operating microscope (Carl Zeiss) and a 34-gauge needle (Hamilton). For intravitreal injections, the needle was inserted through the sclera, positioned toward the nasal retina in the vitreal cavity, and the viral particles were released. To deliver virus subretinally, the needle was inserted tangentially through the sclera into the subretinal space and the viral particles were released. The needle was withdrawn under great care to support self-sealing. For intravitreal injections, a total volume of 1 μL rAAV was administered in early postnatal (P5-P6) mice, and for subretinal injection, a total volume of 1.5 μL rAAV was administered in juvenile (P14) mice. Left and right eyes were selected for vector administration in an alternating manner to prevent bias.

Electroretinography

ERG recordings were obtained from both eyes in mutant and wild-type mice using commercially available equipment (Espion ERG Diagnosys System) as described previously.¹⁹ After dark adaptation overnight, scotopic examinations were performed under single-flash recording using increasing light intensities. Photopic single-flash recordings were obtained following 5 min of light adaptation at a background light intensity of 30 cd.s/m².

Tissue preparation and immunohistochemistry

Mouse eyes were enucleated and the cornea, iris, and lens were removed. Eye cups were fixed in 4% PFA for 1–2 h, cryoprotected in 20% sucrose, and flash-frozen in optimal cutting temperature (OCT) medium. All eyes were sectioned in a sagittal orientation (12 μ m), collected on glass slides, and stored at -20°C . Before use, the slides were briefly thawed, washed in phosphate-buffered saline (PBS), and a blocking buffer was used containing 5% normal goat serum, 1% bovine serum albumin, and 0.1% Triton-X100. The samples were incubated in a primary antibody diluted in blocking buffer at 4°C overnight [anti-PKC α 1:500 (Sigma-Aldrich), anti-GFAP 1:300 (Merck Chemicals), anti-eGFP 1:300 (Life Technologies), anti-Calbindin 1:100 (Abcam), anti-cone-arrestin 1:300 (Millipore), and anti-CRALBP 1:300 (Abcam)]. Sections were washed in PBS and incubated in a secondary antibody diluted in blocking buffer [Alexa Fluor[®] 546 anti-mouse IgG 1:500 and Alexa Fluor 633 anti-rat IgG 1:500 (both Life Technologies)]. Sections were washed in PBS, incubated with 600 nM 4',6-diamidino-2-phenylindole for 5 min, and mounted in DAKO mounting media. The slides were stored in the dark at 4°C until imaging by a confocal microscopy (Leica DM5500Q).

Quantification of retinal cross sections

For quantifications of rod bipolar and horizontal cells, merged confocal images (consisting of 12–13 z-stacks) of the same size (275 \times 275 μ m) were taken from the superior and inferior midcentral retina of three sagittal cross sections (in total six images per eye). The number of protein kinase C alpha-subunit (PKC α)-positive and Calbindin-positive cells was counted in all images and averaged for each eye. Researchers performing counts were masked to the genotype of the animals and treatment received.

Statistical analyses

Data are represented as mean \pm standard deviation with the appropriate *n* (number of samples) values as indicated. Statistical analyses were performed using GraphPad Prism 5 for Windows. The appropriate tests and *p*-values are provided.

RESULTS

Cln3 ^{Δ ex7/8} mice have reduced inner retinal function accompanied by the loss of rod bipolar cells

Cln3 ^{Δ ex7/8} mice were reported to have a mild decline in retinal function, yet no morphological phenotype of the retina was described.¹² To confirm the retinal phenotype and to establish parameters to measure the therapeutic outcome, we performed ERG recordings in *Cln3* ^{Δ ex7/8} mice and age-matched wild-type controls over time. In agreement with previous work, at 12 months we observed a moderate but significant reduction in the size of the scotopic and photopic b-wave amplitudes, indicative of a decline in rod- and cone-mediated inner retinal function. At 15 months, this decrease progressed slightly, while the scotopic and photopic a-wave amplitudes remained unchanged (Supplementary Fig. S1). A table with an overview of the scotopic and photopic b-wave amplitudes at 12 and 15 months can be found in Supplementary Tables S1 and S2.

We investigated the retinal morphology in mutant mice and stained for PKC α , a marker for rod bipolar cells, which are retinal interneurons of the INL that predominantly contribute to the scotopic b-wave amplitude.²⁰ Rod bipolar cells receive direct input from rod photoreceptors and subsequently transmit signals to retinal ganglion cells. In 12-month-old mutants, the midretina appeared to have fewer PKC α -positive cells. At 15 months, the lack of rod bipolar cells became more apparent in the midretina and was very obvious in the periphery of the retina when compared with age-matched controls (Fig. 1A, B).

To assess the loss of bipolar cells, we counted the number of PKC α -positive cells in the midretina and found a significantly reduced number of cells in mutants compared with wild-type controls at 15 months (*p* = 0.0079) (Fig. 1C). In contrast, quantification of Calbindin-positive horizontal cells, a cell type that together with ON bipolar cells forms the postsynaptic entity of the photoreceptor ribbon synapses, did not reveal a significant difference between mutant and wild-type retinas (Fig. 1D, E). We also stained for glial fibrillary acidic protein (GFAP), a common marker for astrocytes and reactive gliosis that is upregulated in retinas of other NCL models.^{17,21} At both time points, we did not observe a severe increase in GFAP staining in the midretina. A mild GFAP upregulation in Mueller glial cells, spanning the width of the retina, and in astrocytes, located in the nerve fiber layer of the retina, was present in the mid- and peripheral retina in mutants at 15 months (Fig. 1B).

Immunohistochemical staining for cellular retinaldehyde binding protein showed a similar pattern of Mueller glial cell bodies and processes in mutant and wild-type retinas, indicating that there was no obvious cell loss

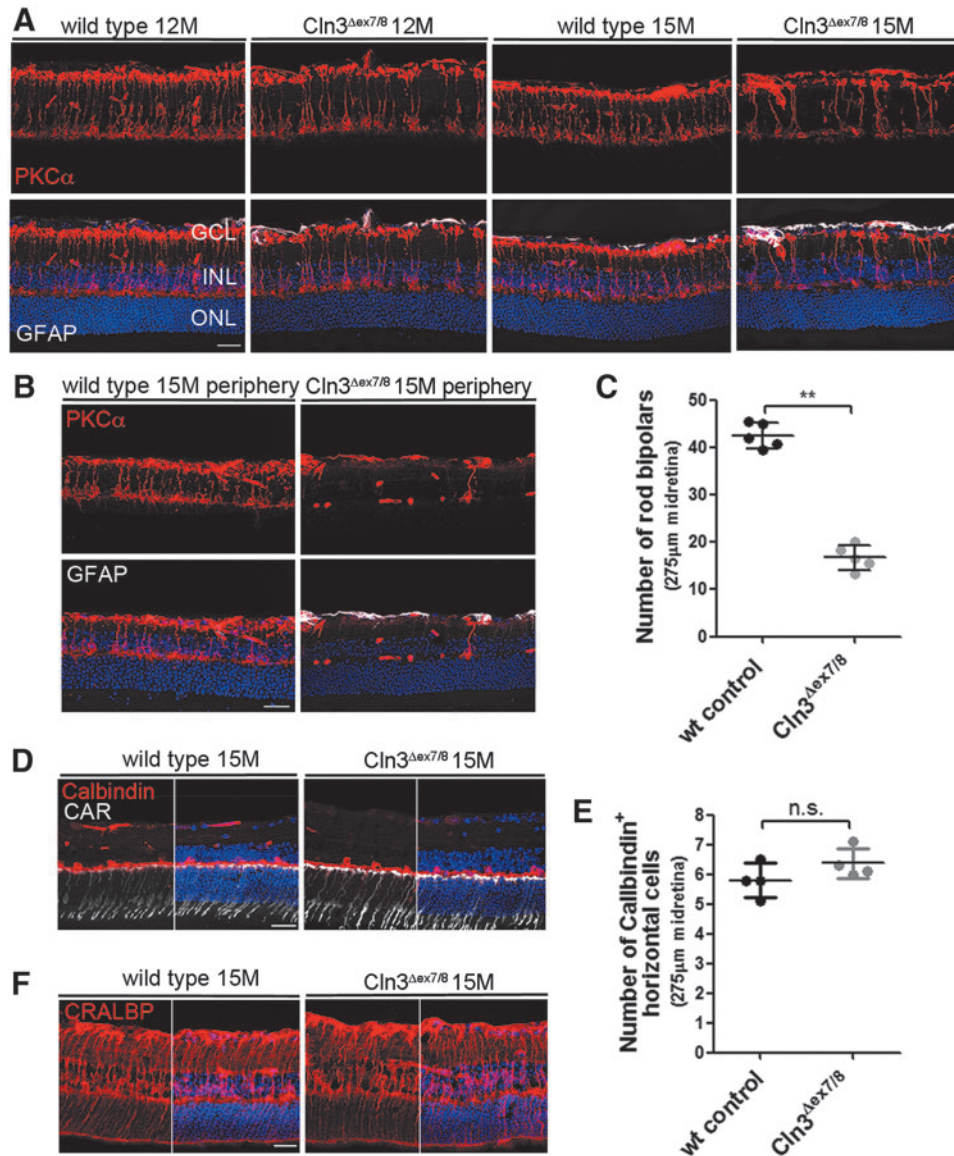


Figure 1. *Cln3*^{Δex7/8} mice show a progressive loss of rod bipolar cells. **(A)** Representative confocal images of the midretina from *Cln3*^{Δex7/8} and wild-type mice immunostained with antibodies against PKC α and GFAP. At 12 months, fewer PKC α -positive cells were detected in mutant than wild-type retinas, which progressed to an obvious loss of cells at 15 months. GFAP staining did not appear to be elevated. **(B)** PKC α staining revealed a pronounced lack of rod bipolar cells in the periphery of the retina. **(C)** Quantification of the number of PKC α -positive rod bipolar cells in the midretina of mutant and wild-type retinas at 15 months. Wild-type and *Cln3*^{Δex7/8}. $n=5$ eyes. Data are presented as mean \pm SD and analyzed by a nonparametric Mann–Whitney test (** $p=0.0079$). **(D, F)** Representative images of 15-month-old mutant and wild-type midretina immunostained for Calbindin, CAR, and CRLABP. No obvious loss of horizontal, cone-photoreceptor, and Mueller glial cells was observed. **(E)** Quantification of the number of Calbindin-positive cells in the INL did not reveal any significant difference between mutant and wild-type retinas at 15 months. Wild-type and *Cln3*^{Δex7/8}. $n=4$ eyes. Data are presented as mean \pm SD. Scale bar: 25 μ m. CAR, cone-arrestin; CRLABP, cellular retinaldehyde binding protein; GCL, ganglion cell layer; GFAP, glial fibrillary acidic protein; INL, inner nuclear layer; ONL, outer nuclear layer; PKC α , protein kinase C alpha-subunit; SD, standard deviation.

in *Cln3*-deficient mice (Fig. 1F). In line with normal photoreceptor function demonstrated by unaffected scotopic and photopic a-wave amplitudes, the thickness of the outer nuclear layer (ONL) and the number of cone-arrestin-positive cells did not appear to be noticeably decreased in *Cln3*^{Δex7/8} retinas (Fig. 1D and Supplementary Fig. S1A, B).

7m8-mediated delivery of mCln3 to the inner retina rescues retinal phenotype of *Cln3*^{Δex7/8} mice

Due to the lack of a good anti-Cln3 antibody and the low protein abundance, we were not able to assess the endogenous *Cln3* expression in the different cell types of the retina. In *Cln3*^{lacZ/lacZ} mice, a knockin reporter mouse

line harboring β -galactosidase in the *Cln3* locus, the expression of the lacZ reporter was first detected in bipolar cells and later became apparent in photoreceptors.²² Microarray and RNAseq analyses found *Cln3* expression in bipolar cells, photoreceptors, and microglia in the adult mouse and human retina (<https://www.fmi.ch/roska.data/index.php>).^{23,24}

Because *Cln3* ^{Δ ex7/8} mice showed an inner retinal dysfunction and a loss of bipolar cells, we focused on developing an AAV-based gene therapy using AAV 7m8, a variant of AAV2/2,²⁵ engineered to target the inner retina and able to transduce bipolar cells across the width of the retina.¹⁷ We performed intravitreal injections with 7m8 carrying the CMV promoter, the mCln3 transgene, and an internal ribosome entry site (IRES) cassette followed by eGFP. We administered the vector in postnatal day (P) 5–6 *Cln3* ^{Δ ex7/8} mice at a high dose of 1×10^{10} total vg per eye or a low dose of 1×10^9 vg per eye. Contralateral eyes received injections with 7m8.CMV.eGFP at 1×10^{10} vg or were left uninjected as controls.

At 12 months, the scotopic and photopic b-waves were significantly higher at a light intensity of 1 cd.s/m^2 in mCln3-treated than in untreated or eGFP-treated mutant eyes with a larger effect in eyes that received the high-dose vector. The size of the b-wave amplitudes decreased in untreated and eGFP-treated eyes at 15 months, while the amplitudes were maintained in *Cln3*-treated eyes in a

dose-dependent manner, indicating that the therapeutic effect was sustained over time (Fig. 2A, Supplementary Fig. S2A, and Supplementary Tables S1 and S2). Representative scotopic ERG traces of a mutant mouse that received injections with the therapeutic vector at high dose in one eye (red traces) and no treatment in the contralateral eye (black traces) are shown in Fig. 2B.

In the absence of an antibody detecting Cln3, we performed immunostaining with an anti-eGFP antibody and observed a low fluorescent signal in various cell types, including photoreceptors and bipolar cells, in treated retinas at 15 months. The low expression level of eGFP can most likely be explained by the presence of the IRES cassette. Comparison with 7m8.CMV.eGFP-treated retinas demonstrates the widespread transduction of cells throughout all layers of the retina, consistent with previous reports.^{17,25} Immunostaining for PKC α revealed that there were a significantly higher number of rod bipolar cells in the midretina of mutants treated with high-dose 7m8.CMV.mCln3-ires-eGFP than eGFP-treated or untreated mutant retinas (Fig. 2C, D). Similar to the findings in the midretina, the therapeutic effect on the survival of rod bipolar cells was also apparent in the peripheral retina of treated *Cln3* ^{Δ ex7/8} mice and representative images are shown in Supplementary Fig. S2B.

Intravitreal injections of 7m8 lead to the transduction of $\sim 25\%$ of photoreceptors in mouse retinas.¹⁷ To exclude

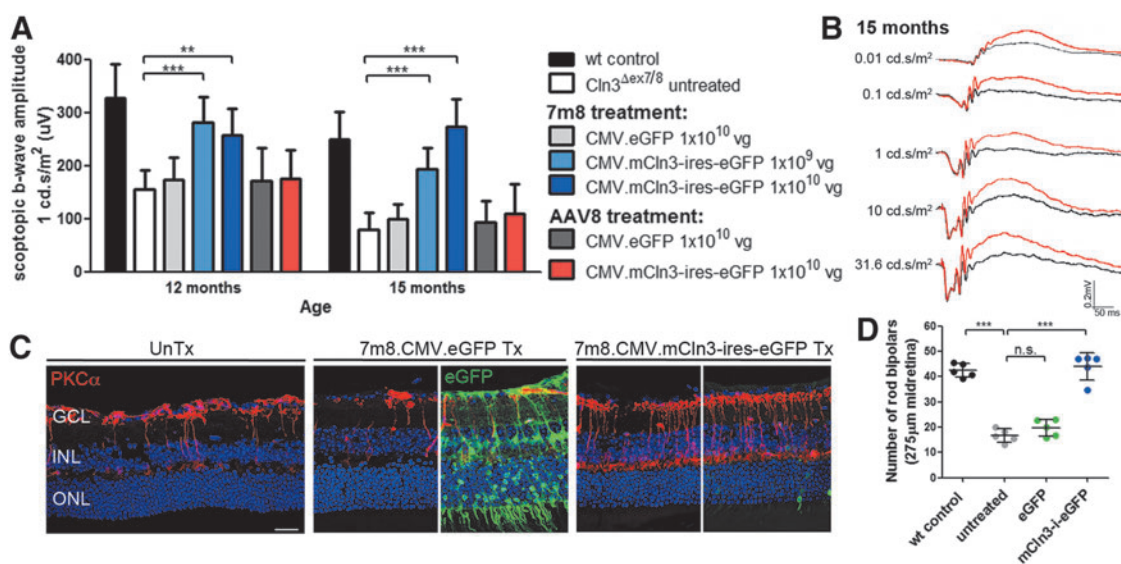


Figure 2. Intravitreally delivered 7m8.CMV.mCln3-ires-eGFP treats the ocular phenotype in *Cln3*-deficient mice. **(A)** Scotopic ERG b-wave recordings of *Cln3* ^{Δ ex7/8} mice at a light intensity of 1 cd.s/m^2 following the intravitreal and subretinal administration of 7m8.CMV.mCln3-ires-eGFP and AAV8.CMV.mCln3-ires-eGFP, respectively. Wild type: $n=7-8$ eyes, *Cln3* ^{Δ ex7/8} untreated: $n=19$ eyes, 7m8.CMV.eGFP: $n=5-6$ eyes, 7m8.CMV.mCln3-ires-eGFP 1×10^9 vg: $n=5-6$ eyes, 7m8.CMV.mCln3-ires-eGFP 1×10^{10} vg: $n=5$ eyes, AAV8.CMV.eGFP: $n=3$ eyes, AAV8.CMV.mCln3-ires-eGFP: $n=4$ eyes. Wild-type recordings from Fig. 1 were added as a reference. **(B)** Scotopic ERG traces of a mutant mouse treated with 1×10^{10} vg 7m8.CMV.mCln3-ires-eGFP in one eye (red). The contralateral eye did not receive treatment (black). **(C)** PKC α staining on untreated, 7m8.CMV.eGFP-, and 7m8.CMV.mCln3-ires-eGFP-treated midretinas at 15 months. eGFP-staining was performed to visualize mCln3-ires-eGFP-transduced cells. **(D)** Quantification of the number of rod bipolar cells in the midretina of mutant mice treated with 7m8. Data are presented as mean \pm SD and analyzed by **(A)** two-way ANOVA with Bonferroni or **(D)** one-way ANOVA with Kruskal–Wallis test (** $p < 0.01$, *** $p < 0.001$). Scale bar: $25 \mu\text{m}$. ANOVA, analysis of variance; CMV, cytomegalovirus; eGFP, enhanced green fluorescent protein; ERG, electroretinography; ires, internal ribosome entry site; mCln3, mouse Cln3; vg, viral genome.

that transgene expression in photoreceptors contributed to the therapeutic effect, we also performed subretinal injections with AAV2/8, an AAV serotype commonly used to target photoreceptors and RPE.²⁶ *Cln3*-deficient pups received injections in one eye with a 1.5×10^{10} vg AAV2/8, harboring mCln3-ires-eGFP under the control of the CMV promoter. Contralateral mutant eyes received injections with AAV2/8.CMV.eGFP or remained uninjected. At 12 and 15 months, no preservation of the scotopic b-wave amplitudes was detected in *Cln3*-treated compared with eGFP-treated or uninjected eyes, indicating that transgene expression in photoreceptors was not therapeutic in *Cln3* ^{Δ ex7/8} mice (Fig. 2A, Supplementary Fig. S2A, and Supplementary Tables S1 and S2). Histological assessment of AAV2/8.mCln3-treated retinas at 15 months confirmed transgene expression in photoreceptors and the loss of PKC α -positive bipolar cells, comparable with untreated retinas (Supplementary Fig. S2B, C).

Overexpression of hCLN3 is therapeutic in *Cln3* ^{Δ ex7-8} mice

To investigate whether the delivery of the human transgene, which shares over 80% sequence homology with the mouse gene,²⁷ corrected the retinal phenotype, we also produced 7m8 carrying the CMV promoter and the hCLN3 gene. No IRES cassette or fluorescent reporter was included because poor eGFP expression levels were observed in retinas treated with 7m8.CMV.mCln3-ires-eGFP. *Cln3*-deficient pups received intravitreal injections

with 7m8.CMV.hCLN3 at 1×10^{10} vg, and ERG recordings were performed to measure retinal function. At 12 months, the scotopic and photopic b-waves were significantly higher in treated compared with untreated mutant eyes and they remained higher than untreated eyes at 15 months (Fig. 3A, Supplementary Fig. S3A, and Supplementary Tables S1 and S2).

Figure 3B shows example traces of scotopic ERG recordings from a 15-month-old *Cln3* ^{Δ ex7/8} mouse with a treated (red traces) and an untreated eye (black traces). Histological assessment indicated that there were more PKC α -positive bipolar cells in treated than untreated mutant retinas at 15 months in the midretina (Fig. 3C) and the peripheral retina (Supplementary Fig. S3C). Quantification of the cell numbers established that a significantly higher number of rod bipolar cells were present in mutants following the treatment (Fig. 3D).

Bipolar cell-specific expression of hCLN3 rescues retinal phenotype of *Cln3*-deficient mice

Untreated *Cln3* ^{Δ ex7/8} mice show a significant decline in the ERG b-waves and a loss of bipolar cells. To assess whether *CLN3* overexpression in bipolar cells was sufficient to treat the ocular phenotype in mutant mice, a 7m8 vector was produced that carried *CLN3* under the control of the Grm6 promoter, predominantly driving expression in rod bipolar cells.²⁸ P5-P6 mutant mice were injected intravitreally with 7m8.Grm6.hCLN3 at either 1×10^9 vg

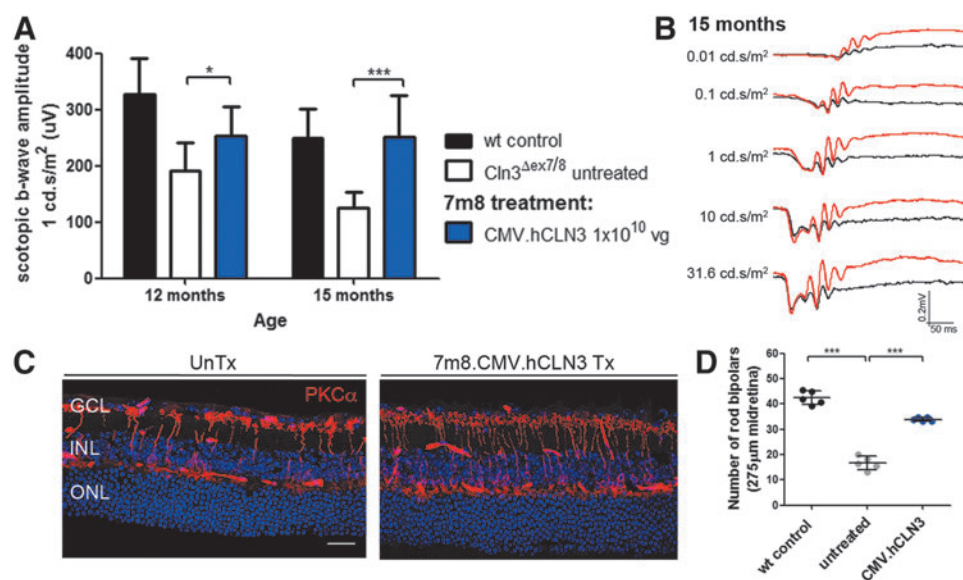


Figure 3. Intravitreal administration of 7m8.CMV.hCLN3 prevents the loss of retinal function and bipolar cells in *Cln3* ^{Δ ex7/8} mice. **(A)** Scotopic ERG b-wave amplitudes of 7m8.CMV.hCLN3-treated mutant mice. Wild type: $n=7-8$ eyes, *Cln3* ^{Δ ex7/8}: $n=8$ eyes, 7m8.CMV.hCLN3: $n=10$ eyes. Wild-type recordings from Fig. 1 were added as a reference. **(B)** Scotopic ERG traces of a *Cln3* ^{Δ ex7/8} mouse that received treatment in one eye (red) and no treatment in the contralateral eye (black). **(C)** Confocal images of the midretina from untreated and 7m8.CMV.hCLN3-treated eyes stained for PKC α at 15 months. **(D)** Counts of PKC α -positive cells in the midretina at 15 months. Data are presented as mean \pm SD and analyzed by **(A)** two-way ANOVA with Bonferroni test and **(D)** one-way ANOVA with Kruskal–Wallis test (* $p < 0.05$, *** $p < 0.001$). Scale bar: 25 μ m. hCLN3, human CLN3.

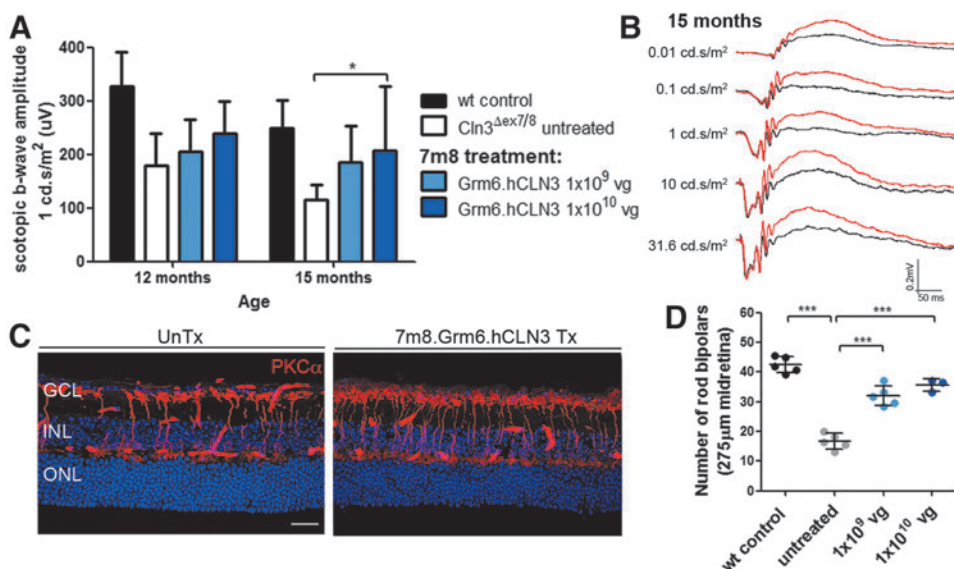


Figure 4. (A) Scotopic ERG b-wave amplitudes of 7m8.Grm6.hCLN3-treated mutant mice. Wild type: $n=7-8$ eyes, *Cln3*^{Δex7/8}: $n=8-9$ eyes, 7m8.CMV.hCLN3 1×10^9 vg: $n=5-6$ eyes, 7m8.CMV.hCLN3 1×10^{10} vg: 3–4 eyes. Wild-type recordings from Fig. 1 were added as a reference. (B) Scotopic ERG traces of a mutant mouse that received treatment in one eye (red). The contralateral eye was left uninjected (black). (C) Representative confocal images of the midretina from untreated and a high-titer 7m8.Grm6.hCLN3-treated eyes stained for PKC α at 15 months. (D) Counts of PKC α -positive cells in the midretina at 15 months. Data are presented as mean \pm SD and analyzed by (A) two-way ANOVA with Bonferroni test and (D) one-way ANOVA with Kruskal–Wallis test (* $p < 0.05$, *** $p < 0.001$). Scale bar: 25 μ m.

or 1×10^{10} vg. Contralateral eyes were not injected to provide internal age-matched controls.

ERG recordings at 12 and 15 months demonstrated that the scotopic b-waves were higher in treated mutant eyes in a dose-dependent manner at the light intensity of 1 cd.s/m². Statistical significance was reached at 15 months in eyes that received high-titer 7m8.Grm6.hCLN3 compared with untreated mutant eyes (Fig. 4A and Supplementary Table S1). The overlay of the scotopic ERG traces underlines the therapeutic effect in a high-titer treated eye (red traces) compared with an untreated contralateral eye (black traces) across a range of light intensities (Fig. 4B). The analysis of the photopic b-wave amplitudes did not show a significant difference between Grm6.hCLN3-treated and untreated eyes (Supplementary Fig. S3B and Supplementary Table S2), unlike CMV.hCLN3-treated mutant eyes (Supplementary Fig. S3A and Supplementary Table S2). The reason for this may be the poor transduction efficiency of cone bipolar cells by 7m8²⁹ in combination with perhaps lower transgene expression levels due to the Grm6 promoter. In line with the scotopic ERG recordings, a significantly higher number of PKC α -positive bipolar cells were detected in eyes that received low- and high-titer 7m8.Grm6.hCLN3 than untreated mutant eyes (Fig. 4C, D, and Supplementary Fig. S3C).

DISCUSSION

The development of therapies for NCLs targeting the brain has seen big advances over the last decade. CNS-directed

enzyme replacement therapy received FDA and EMA approval for CLN2 disease, and two phase I/II gene therapy clinical trials, one for CLN6 and one CLN3 disease patients, are currently ongoing (NCT02725580) (NCT03770572).

Although the studies are encouraging to combat neurodegeneration, these therapeutic interventions are unlikely to treat retinal degeneration and NCL patients will most likely continue to go blind. To address this need for ocular treatments, we studied the retinal phenotype of *Cln3*^{Δex7/8} mice, a model for CLN3 disease, and found that retinal function can be maintained long term by pre-symptomatically delivered ocular gene therapy. We confirm that *Cln3*-deficient mice suffer from a late and mildly progressing decline of inner retinal function and we discovered a significant decrease in rod bipolar cell counts, especially evident in the peripheral retina. Ubiquitous AAV-mediated *CLN3* expression in cells of the inner retina or bipolar-specific expression successfully prevented the loss of retinal function and bipolar cells in mutant mice, establishing the inner retina as the prime target for ocular gene therapy in CLN3 disease.

Inherited retinopathies due to gene defects in photoreceptors and the RPE are a common cause of blindness. Several ocular gene therapies targeting the outer retina have been translated into the clinic, including Leber congenital amaurosis,^{30–33} retinitis pigmentosa,³⁴ and achromatopsia.³⁵ Retinal diseases caused by mutated genes in cells of the inner retina, leading to loss of inner retinal function, are rare. Electronegative ERG responses characterized by a more pronounced loss of the b- than the a-wave were described in

early symptomatic CLN3 disease patients preceding the complete loss of retinal function at advanced disease stages.^{36–38} Postmortem tissue of patients revealed gross disturbances of the retinal morphology, severe thinning of all retinal layers, and a complete lack of photoreceptors in the mid- and central retina.³⁹

As *CLN3* was reported to be expressed in photoreceptors in mice^{22,23} and humans²⁴ and photoreceptors are absent at disease end stage, it was suggested that gene supplementation therapy targeting the outer retina by subretinal injections could be an effective strategy to treat ocular failure in CLN3 disease.⁴⁰ Here, we show that the AAV2/8-mediated overexpression of *Cln3* in photoreceptors is not therapeutic in *Cln3*^{Δex7/8} mice and that instead the transduction of inner retinal cells, especially bipolar cells, is crucial for successful ocular *CLN3* gene therapy. Of note is that the current CNS-directed gene therapy clinical trial for CLN3 disease uses AAV2/9, an AAV serotype similar to AAV2/8 transduces photoreceptors and the RPE well, yet does not target cells in the inner retina.⁴¹ In line with other reports,^{12,13} we did not observe any reduction in photoreceptor function or obvious photoreceptor death in *Cln3*-deficient mice.

Why mutant mice fail to reproduce the secondary loss of photoreceptors observed in patients remains unclear. It could be speculated that the photoreceptor loss in (end stage) patients is entirely secondary to bipolar cell defect (note that such a mechanism is also present in *Cln6*^{nclf} mice, where treatment of bipolar cells rescued the loss of photoreceptor cells¹⁷). As the degeneration in *Cln3*^{Δex7/8} mice is slow, there may not be enough time for bipolar cell death to substantially affect the ONL. Why the rate of degeneration is so different in mice is not clear, but this observation is not uncommon in ophthalmic research. For example, the *rd1* mouse, a model that carries a mutation in the photoreceptor phosphodiesterase 6b (*Pde6b*) gene, shows a much faster disease progression than patients⁴² and the *RPGR* mouse, a model that carries a mutation in the retinitis pigmentosa GTPase regulator gene, shows a far slower degeneration than patients.⁴³

While it cannot be fully excluded that there is a primary photoreceptor loss in CLN3 disease patients that is not replicated in the mice, which would not be treated with our approach, the electronegative ERG in patients together with our study data strongly suggests that bipolar cell loss in patients is very likely a direct effect and that gene transfer to these cells is a prerequisite for successful treatment in patients.

Recently we reported another ocular gene therapy targeting the inner retina in *Cln6*^{nclf} mice, a model for variant late-infantile CLN6 disease. Although *Cln6*-deficient mice predominantly lose photoreceptors and bipolar cells only die at late disease stages, 7m8-mediated gene supplementation of bipolar cells prevented the photoreceptor degeneration in these mice.¹⁷ Similar to *CLN3*, *CLN6* is

expressed in bipolar and photoreceptor cells,^{17,23} encoding a membrane-bound protein of unknown function. Despite these similarities, *CLN3* and *CLN6* are located in different cell organelles and have been implicated in different cell functions.³ The retinal disease mechanism in *CLN3* and *CLN6* disease is not understood, however, our studies demonstrate that in both forms of the disease, cells in the inner retina and in particular bipolar cells play a central role. We therefore recommend that efforts to unravel the function of retinal degeneration-associated genes and their disease mechanisms, such as *in vitro* disease modeling using induced pluripotent stem cell-derived retinal cultures, should not be just limited to the study of photoreceptors but should also focus on cells of the inner retina to uncover relevant disease features.

Intravitreal administration of 7m8 and other AAVs, including AAV2/2 and tyrosine-mutated AAV2/2, transduces bipolar cells and other cells of the inner retina well in mice. However, the transduction efficiency of these vectors is lower in larger animals due to the substantially thicker inner limiting membrane (ILM) and the vitreous humor, both acting as physical barriers for AAVs.^{25,44} Surgical peeling of the ILM and vitrectomy to remove vitreous before intravitreal injections resulted in a localized, enhanced retinal transduction in nonhuman primate (NHP) retinas.^{45,46} However, it remains to be established if ILM removal in diseased eyes in combination with intravitreal AAV injections poses additional inflammatory challenges that would need to be addressed in the clinic.

Optimization of bipolar cell transduction from the subretinal space has been explored and the synthetic AAV8BP2 was shown to target bipolar cells in mice,²⁸ yet the transduction efficiency of the INL appeared to be low in NHPs.⁴⁷ It is important to point out that in these NHP studies, ubiquitous promoters are commonly used, driving expression in all cell types of the INL, which makes it difficult to specifically identify bipolar cells and estimate their transduction efficiency. Large-animal studies using bipolar cell-type-specific promoters could facilitate the search and optimization of AAVs targeting bipolar cells. While there is currently no AAV available to achieve widespread expression in the INL of large animals, intravitreal administration of 7m8 in NHPs results in a good transduction of the fovea and parafovea,²⁵ a region that was reported to be spared or affected late in CLN3 disease patients.⁸ Although 7m8 may not be the ideal candidate vector to achieve an extensive transduction of the inner retina and the development of more efficacious vectors should be considered, the 7m8 vector could provide a successful strategy to maintain central vision and thereby could improve the quality of life of patients substantially.

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No competing financial interests exist.

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SUPPLEMENTARY MATERIAL

Supplementary Figure S1
 Supplementary Figure S2
 Supplementary Figure S3
 Supplementary Table S1
 Supplementary Table S2

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