Photoreceptor Degeneration, Azoospermia, Leukoencephalopathy, and Abnormal RPE Cell Function in Mice Expressing an Early Stop Mutation in *CLCN2*

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PURPOSE. To determine the molecular basis and the pathologic consequences of a chemically induced mutation in a mouse model of photoreceptor degeneration, *nmf240*.

METHODS. Mice from a G3 *N*-ethyl-*N*-nitrosourea mutagenesis program were screened by indirect ophthalmoscopy for abnormal fundi. A chromosomal position for the recessive *nmf240* mutation was determined by a genome-wide linkage analysis by use of simple sequence length polymorphic markers in an F2 intercross. The critical region was refined, and candidate genes were screened by direct sequencing. The *nmf240* phenotype was characterized by histologic analysis of the retina, brain, and male reproductive organs and by electroretinogram (ERG)-based studies of the retina and retinal pigment epithelium (RPE).

RESULTS. Clinically, homozygous *nmf240* mutants exhibit a grainy retina that progresses to panretinal patches of depigmentation. The mutation was localized to a region on chromosome 16 containing *Cln2*, a gene associated with retinal degeneration. Sequencing identified a missense C-T mutation at nucleotide 1063 in *Cln2* that converts a glutamine to a stop codon. Mice homozygous for the *Cln2*^{*nmf240*} mutation experience a severe loss of photoreceptor cells at 14 days of age that is preceded by an elongation of RPE apical microvilli. Homozygous mutants also experience leukoencephalopathy in multiple brain areas and male sterility. Despite a normal retinal histology in *nmf240* heterozygotes, the ERG light peak, generated by the RPE, is reduced.

CONCLUSIONS. The *nmf240* phenotype closely resembles that reported for *Cln2* knockout mice. The observation that heterozygous *nmf240* mice present with a reduced ERG light

peak component suggests that *CLCN2* is necessary for the generation of this response component. (*Invest Ophthalmol Vis Sci.* 2010;51:3264–3272) DOI:10.1167/iovs.09-4887

Normal cell function depends on the maintenance of appropriate intra- and extracellular ion concentrations as well as control of ionic flow, which is achieved by the action of many ion channels. Cl[−] is one of the most abundant ions used by cells, and the CLC family of Cl[−] channels includes nine members that, based on their crystal structure, organize as dimers to form an ion pore.¹ The CLC channels are localized to the cellular plasma membrane or to the membranes of intracellular compartments where they regulate cellular functions such as membrane excitability, salt transport, cell cycle progression, apoptosis, and cell volume.^{2–6} In view of this wide range of functions, it is not surprising that many inherited human disorders involve CLC genes. Mutations in *CLCN1* cause myotonia and hyperexcitability of the muscle membrane,⁷ mutations in *CLCN5* lead to kidney stones,⁸ and *CLCN2* mutations have been linked to epilepsy.⁹ Valuable information regarding the function of CLC proteins has been gained through mouse models lacking functional copies of these genes. For example, targeted deletion of *Cln3* leads to hippocampal and photoreceptor cell loss,¹⁰ whereas mice lacking *Cln2* experience severe, early-onset loss of retinal photoreceptors and male germ cells^{11,12} and also have progressive leukoencephalopathy.¹³

In the present report, we describe a mouse mutant identified through an ocular screen of mice mutagenized with *N*-ethyl-*N*-nitrosourea (ENU) in the Neuromutagenesis Facility (NMF) and studied in the Translational Vision Research Models (TVRM) program at The Jackson Laboratory (Bar Harbor, ME). Genetic mapping and sequencing of *nmf240* DNA identified a point mutation early in the *Cln2* gene that leads to a premature stop codon. As described herein, *Cln2*^{*nmf240*} homozygotes share many of the abnormalities described in *Cln2* knockout mice,^{11,13} including early-onset photoreceptor degeneration, male germ cell loss, and leukoencephalopathy. We also report that *Cln2*^{*nmf240*} heterozygotes, expressing a single *Cln2* functional allele, have a selective reduction in the ERG light peak (LP), implicating this Cl[−] channel in the generation of this component.

MATERIALS AND METHODS

Generation of Mutants by The Jackson Laboratory Neuroscience Mutagenesis Facility

Male C57BL/6J (B6) mice were administered ENU in three intraperitoneal injections at 80 mg/kg over a 3- to 4-week period. After returning to fertility, these G0 mice were mated to B6 females to produce G1 male mice that were subsequently crossed with B6 females to generate

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G2 progeny. G3 mice, generated by backcrossing G2 females to G1 sires, were screened for retinal abnormalities by indirect ophthalmoscopy at 12 weeks of age.

Mouse Husbandry and Genotyping

All experimental procedures were performed according to Animal Care and Use Committee approved protocols and the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. Mice were fed a National Institutes of Health 6% fat diet and acidified water and were maintained on a 12-hour light/12-hour dark cycle. The mouse colony at The Jackson Laboratory was maintained by heterozygous matings. Heterozygosity at the *Cln2* locus was established by polymerase chain reaction (PCR) amplification of the region encompassing the mutation, followed by restriction digestion of the product to determine genotypes. PCR amplification was performed as follows: 94°C for 3 minutes, 94°C for 30 seconds, annealing temperature of 55°C for 45 seconds, 72°C for 1 minute, steps 2 through 4 were repeated for 35 cycles followed by one cycle at 72°C for 2 minutes. The following oligonucleotides were used for PCR amplification: 5'-ATTGCTAGTGGCTTCGGGGGAGCCCTCT-3' and 5'-CAGCTTCTGCAGTGCTGAGATCCT-3'. Salt was removed by filtration, and the *Bst*NI restriction enzyme (New England Biolabs, Ipswich, MA) was used for restriction digestion of the resulting product. After 4 to 12 hours at 60°C, the digested products were electrophoresed on a 1.5% agarose gel.

Genetic Mapping and Analysis

B6-*nmf240* homozygous female mice were mated to male DBA/2J mice to generate F1 progeny, which were subsequently intercrossed. The phenotype of F2 progeny was assessed at 12 weeks of age by indirect ophthalmoscopy, and DNA was isolated from tail snips by using a modified version of published methods.¹⁴ A genome-wide scan to determine the chromosomal location of *nmf240* was performed with simple sequence length polymorphic markers. PCR amplification was performed as just described. PCR products were separated by electrophoresis on a 4% agarose gel (MetaPhor; FMC, Rockland, ME), stained with ethidium bromide, and visualized under ultraviolet light.

For sequencing of candidate genes, RNA and cDNA were prepared from three mutant mice and three control B6 mice. RNA was isolated from snap-frozen eyes (TRIzol; Invitrogen, Carlsbad, CA), according to the manufacturer's instructions. cDNA was generated with a reverse transcription kit (Retroscript; Ambion, Austin, TX). The *Cln2* coding region was amplified from cDNA using PCR amplification, and purified products were sequenced.

Fundus Examination

Pupils were dilated with 1% atropine, and mice were dark-adapted before viewing retinas with an indirect ophthalmoscope and a 60-D aspheric lens. Fundus photographs were taken with a fundus camera (Kowa, Nagoya, Japan) and 400 ASA film and a 60-D superfield lens (Volk Optical Inc., Mentor, OH), held 2 in. from the eye.

ERG Testing

After overnight dark adaptation, the mice were anesthetized (ketamine, 80 mg/kg; xylazine, 16 mg/kg), the cornea was anesthetized (1% proparacaine HCl), and the pupils were dilated (1% tropicamide, 2.5% phenylephrine HCl, 1% cyclopentolate). The mice were placed on a temperature-regulated heating pad throughout each recording session.

The protocols used to record ERG components generated by the outer neural retina or the RPE have been described.¹⁵⁻¹⁷ In brief, responses of the outer retina were recorded with a stainless-steel electrode referenced to a needle electrode placed in the cheek in response to strobe flash stimuli presented in the dark or superimposed on a steady rod-desensitizing adapting field. The amplitude of the a-wave was measured 8 ms after flash onset from the prestimulus

baseline. The amplitude of the b-wave was measured from the a-wave trough to the peak of the b-wave or, if no a-wave was present, from the prestimulus baseline. Implicit times were measured from the time of flash onset to the a-wave trough or the b-wave peak.

Components of the dc-ERG generated by the RPE were recorded with an Ag/AgCl electrode bridged to the corneal surface with Hanks' balanced salt solution in response to stimuli presented for 7 minutes. The amplitude of the c-wave was measured from the prestimulus baseline to the peak of the c-wave. The amplitude of the fast oscillation (FO) was measured from the c-wave peak to the trough of the FO. The amplitude of the LP was measured from the FO trough to the asymptotic value. The amplitude of the off-response was measured from the LP asymptote to the peak of the off-response.

Histologic Assessment of the Retina

The mice were euthanatized by carbon dioxide asphyxiation, and the enucleated eyes were placed in methanol acetic acid (37.5% methanol, 12.5% acetic acid in phosphate buffered saline [PBS]) overnight. After fixation, the eyes were embedded in paraffin rings and cut into 5-μm sections through the ora serrata and optic nerve. The sections were deparaffinized and rehydrated before staining with hematoxylin-eosin (H&E) or use in immunohistochemical analysis. For immunohistochemistry, nonspecific binding was blocked with 1:50 horse serum in PBS containing 0.3% Triton X-100 for 20 minutes. The sections were incubated in anti-ezrin (1:500; Sigma-Aldrich, St. Louis, MO) overnight in blocking solution at 4°C followed by 1.5 hours of incubation with the cyanine 3-conjugated secondary anti-mouse antibody (1:200; Jackson ImmunoResearch, West Grove, PA). Staining was visualized with a fluorescence microscope (DMRXE; Leica, Deerfield, IL) equipped with a CCD camera (SPOT; Diagnostic Instruments, Sterling Heights, MI). Apical RPE processes stained with ezrin were measured with Image J software (developed by Wayne Rasband, National Institutes of Health, Bethesda, MD; available at <http://rsb.info.nih.gov/ij/index.html>). For wild-type (WT) retinas, the intensity of staining and the small size of processes prevented measurement of individual apical processes; therefore, the length of the ezrin-labeled area was used. Measurements were repeated in a minimum of 10 areas across a retinal image taken with a 40× objective. The average length of the apical processes was assessed for a minimum of four mutant and WT retinas. Student's *t*-test was used to determine statistical significance between the WT and mutant retinas.

Western Blot Analysis

Enucleated eyes from three WT and three mutant postnatal day (P)10 mice were snap frozen in liquid nitrogen. Whole eyes were digested in RIPA buffer (1% NP-40, 0.1% SDS, and 0.5% sodium deoxycholate in PBS), left on ice for 30 minutes, and centrifuged for 20 minutes at 4°C. The supernatants were collected, and the total protein in each sample was determined (Bradford kit; Bio-Rad, Hercules, CA) according to the manufacturer's instructions. Samples containing 200 μg total protein with loading dye and reducing agent were heated to 90°C for 10 minutes to denature the samples before loading on 4% to 12% Bis-Tris gel (Invitrogen). Samples were run on gels in MOPS running buffer (Invitrogen) and transferred to nitrocellulose membrane with transfer buffer (Invitrogen). Nonspecific binding to the membrane was blocked with 5% powdered milk in Tris-buffered saline (TBS) containing Tween 20. The primary antibody was applied in blocking solution at a 1:2000 (ezrin; Sigma-Aldrich) or 1:2500 (β-actin; Sigma-Aldrich) dilution overnight at 4°C. After washes in TBS with Tween 20, the peroxidase-conjugated anti-mouse secondary antibody (Jackson ImmunoResearch) was applied in blocking solution at a 1:5000 dilution for 1 hour at room temperature. Proteins were detected by enhanced chemiluminescence (Western Lightning kit; Perkin-Elmer, Waltham, MA).

Histologic Assessment of the Central Nervous System

After deep anesthesia with avertin (0.02 mL/mg body weight), mice were perfused transcardially with PBS, followed by 4% paraformaldehyde.

hyde in 0.1 M sodium phosphate buffer (PB). The brains were dissected, immediately removed from the cranium, and transferred to fresh 4% paraformaldehyde for overnight fixation at 4°C. The brains were then cryoprotected by sinking in 30% sucrose in 0.125 M PB and left at 4°C overnight. After bisection along the midline, the brains were embedded in OCT compound. Cryostat sections (10 μ m) were cut and allowed to air dry on glass slides overnight¹⁸ (SuperPlus; Fisher Scientific, Pittsburgh, PA), followed by staining with cresyl violet (0.5% cresyl violet acetate in 0.3 M glacial acetic acid).

Analysis of Fertility, Reproductive Physiology, and Gonadal Histology

Fertility was assessed in spontaneous mating experiments with age-matched, adult male and female homozygous mutant, heterozygous, and WT mice ($n = 6-8$ /genotype and sex). Mice were mated to either 6-week-old female or 12-week-old male B6 mice, and the number of litters generated was recorded over the ensuing 12 weeks. A second group of male mice was used for timed-mating experiments to determine whether behavioral abnormalities had caused infertility in homozygous mutants. For these experiments, adult female B6 mice were injected with pregnant mare serum gonadotropin (PMSG; 5 IU, IP; Sigma-Aldrich), followed 44 to 46 hours later by injection of human chorionic gonadotropin (hCG; 5 UI, IP; Sigma-Aldrich), to induce ovulation, and mated overnight with homozygous mutant or WT male mice ($n = 6-7$ per genotype). Hormone-primed females were assessed for successful mating behavior by the presence of a copulatory plug the following morning, and subsequent pregnancy was verified by palpation. The percentage of male mice that successfully mated and sired offspring was recorded and analyzed in χ^2 tests. In vitro fertilization tests were conducted to confirm that germ cell (spermatozoa) dysfunction underlies infertility in homozygous mutants. Caudal epididymal spermatozoa extracted from 12- to 16-week-old homozygous mutant ($n = 3$) and WT ($n = 3$) males were used to inseminate 40 to 60 ovulated B6 oocytes per donor male. The inseminated oocytes were cultured for 4 hours at 37°C in miniature CO₂ incubators (New Brunswick Scientific, Edison, NJ) in minimum essential medium prepared with Earle's balanced salt solution, both essential and nonessential amino acids (Invitrogen), 0.23 mM pyruvic acid, 75 mg/L penicillin G, 50 mg/L streptomycin sulfate, 0.01 mM tetra sodium EDTA (Sigma-Aldrich), and 3 mg/mL BSA (Sigma-Aldrich). After fertilization, all oocytes were collected, washed, and cultured overnight until the cleaved embryo stage.^{19,20} For histologic verification of germ cell dysfunction, testes and epididymides were collected, cleaned of all fatty tissue, weighed, and immersed overnight in Bouin's fixative. The tissues were rinsed, paraffin embedded, sectioned at 5 μ m on a microtome, and processed with the cytological stains, periodic acid-Schiff, H&E, and toluidine blue. Images were collected with a digital camera attached to a microscope (DMRB; Leica), under a 20 $\times$ objective.

RESULTS

Mutation Underlying Disease Phenotypes in *nmf240* Homozygous Mice

Linkage analysis demonstrated that the gene mutated in *nmf240* mice resided on chromosome 16 in a region containing the *Cln2* gene. The *Cln2* coding region was sequenced to reveal a C-T nonsense mutation at base pair 1063 (Fig. 1) that was present in a homozygous state in affected mice. This mutation changes glutamine at amino acid 355 to a stop codon. From this point forward, the *nmf240* allele will be referred to as *Cln2*^{*nmf240*}.

Retinal Degeneration in *Cln2*^{*nmf240*} Homozygotes

As early as 3 weeks of age (the earliest time point at which the fundus was examined clinically), the retinas of the *Cln2*^{*nmf240*} homozygotes had a grainy appearance when viewed by indi-

FIGURE 1. Positional cloning and direct sequencing revealed that *nmf240* mice carry a mutation in *Cln2*, producing a premature stop signal. Chromatographs showing sequencing of *Cln2*, beginning at base pair 1054, depict the C-T point mutation at base pair 1063 observed in homozygous *nmf240* mice. Sequencing of cDNA from F1 mice demonstrates the C/T heterozygosity at nt1063. This mutation converts amino acid 355 from a glutamine to a stop codon.

rect ophthalmoscopy (data not shown). By 12 weeks of age, this graininess progressed into large white patches distributed across the entire retina (Fig. 2).

At P10, the morphology of the homozygous *Cln2*^{*nmf240*} retinas (Fig. 3B) was similar to that of WT littermates (Fig. 3A). By P14, however, several abnormalities were noted in the retinas of the homozygous *Cln2*^{*nmf240*} mice (Fig. 3D) that were not observed in the WT littermates (Fig. 3C). Specifically, the outer nuclear layer (ONL) of the mutant retina was reduced in thickness and contained several pyknotic nuclei, and the photoreceptor outer segments were disorganized and shortened (Fig. 3D). By 3 weeks of age, the photoreceptor layer was reduced to one to two layers of cells and outer segments were not visible (Fig. 3F). Retinas of the heterozygous *Cln2*^{*nmf240*} mice were similar to WT at all ages examined (data not shown).

FIGURE 2. Fundus examination of the *Clcn2^{nmf240}* mutant demonstrated areas of depigmentation. Indirect ophthalmoscopy revealed periretinal patches of depigmentation, indicative of retinal degeneration, in 12-week-old *Clcn2^{nmf240}* homozygotes (B) that were not observed in the control animals (A).

ERG Amplitude in *Clcn2^{nmf240}* Homozygotes

On the basis of the morphologic alterations of early-onset photoreceptor degeneration in the *Clcn2^{nmf240}* homozygotes, we recorded strobe flash ERGs between P14 and P30. Figure 4 presents representative strobe flash ERGs recorded in P14 mice under (Fig. 4A) dark- or (Fig. 4B) light-adapted conditions. Responses obtained from the *Clcn2^{nmf240}* heterozygotes were comparable to those noted in other studies of the developing

WT retina,^{17,21} indicating that a single *Clcn2* allele is sufficient to support normal outer retinal development. In comparison, responses obtained from the *Clcn2^{nmf240}* homozygotes were clearly reduced in amplitude at all ages, indicating that the photoreceptor degeneration noted anatomically affects both rod- and cone-driven responses. The time course over which the ERG response declined was characterized by recording the amplitude of the response of the homozygous *Clcn2^{nmf240}* mutants to a high-intensity stimulus presented to the dark-adapted eye as a percentage of the corresponding response obtained from the heterozygous *Clcn2^{nmf240}* littermates (Fig. 4C). From P14 to P26, there was a progressive decline in the response of the *Clcn2^{nmf240}* homozygotes, consistent with the loss of photoreceptors in the retinas of affected mice that was noted morphologically. Little additional decline in the ERG amplitude of the affected animals was observed from P26 to P30, indicating that the few cells that were retained in the homozygous *Clcn2^{nmf240}* retina remained functional. These results are consistent with the exponential model proposed for application to the time course of photoreceptor degeneration in multiple models.²²

FIGURE 3. Histologic assessment of retinas revealed a loss of outer segments and photoreceptor cells. At P10, the mutant retina (B) resembled that of WT littermates (A). At P14, the mutants (D) showed a reduction in outer segment length compared with those in WT littermates (C). Pyknotic cells and disorganization were also noted in the ONL of the mutants. At P21 (E, F), the ONL in the mutant retina was reduced to one to two nuclei in thickness (F).

Apical Microvilli of the RPE in *Clcn2^{nmf240}* Homozygotes

The apical processes of the RPE were elongated in the *Clcn2^{nmf240}* homozygous mice relative to the WT mice at P10, before the age when photoreceptors degenerated. In WT mice, ezrin labeled the apical processes of the RPE cells (Figs. 5A, 5C) but because of the intensity of the staining, individual microvilli were visible only at magnifications of 100 $\times$ (Fig. 5C). In contrast, ezrin labeling of the apical face of the RPE in the homozygous *Clcn2^{nmf240}* retinas was diffuse, and individual microvilli were readily observable at 20 $\times$ magnification and clearly depicted with a 40 $\times$ objective (Fig. 5B). Statistical analysis of apical microvilli length demonstrated a significant elongation in the *Clcn2^{nmf240}* homozygotes relative to WT (Figs. 5D, 5E). In addition to the observed elongation, some ezrin staining was also seen in the basal RPE, indicating a potential disruption in cell polarity (Fig. 5B). At 3 weeks of age, when the outer segments and photoreceptors had degenerated, the apical RPE processes in the mutants were shorter than those observed at P10 but were still elongated in comparison to those in the WT littermates (data not shown). Despite the differences noted in ezrin labeling between the WT and homozygous *Clcn2^{nmf240}* retinas, Western blot analysis at P10 indicated no apparent difference in the amount of this protein in the homozygous *Clcn2^{nmf240}* retina (Fig. 5F).

Effect of a Single WT *Clcn2* Allele in Mice

Because the components of the dc-ERG are generated by the RPE secondary to rod photoreceptor activity,¹⁶ it was not possible to record dc-ERGs from the *Clcn2^{nmf240}* homozygotes

FIGURE 4. ERG amplitudes were reduced in the *Clcn2*^{nmf240} homozygotes. Representative strobe flash ERGs recorded under (A) dark- or (B) light-adapted conditions from a *Clcn2*^{nmf240} heterozygote (left) and homozygote (right) at P14. (C) Age-related changes in the *Clcn2*^{nmf240} homozygous response. Maximum dark-adapted b-wave amplitude for the *Clcn2*^{nmf240} homozygous mice are plotted as a percentage of the response obtained from the *Clcn2*^{nmf240} heterozygous littermates. Data points indicate the average ($\pm$ SD) for two to five mice.

in which photoreceptors had degenerated. Therefore, we examined the *Clcn2*^{nmf240} heterozygotes to determine whether the loss of a single functional copy of CLCN2 affects the generation of any ERG component. Figure 6 presents strobe flash responses obtained from 9-month-old WT and *Clcn2*^{nmf240} heterozygotes under (Figs. 6A, 6B) dark- or (Figs. 6C, 6D) light-adapted conditions. Consistent with the normal morphologic appearance of the retina in the *Clcn2*^{nmf240} heterozygotes, ERGs were not different from those of the WT, indicating that these animals do not develop late-onset photoreceptor degeneration. The fact that the dark-adapted a-waves of the *Clcn2*^{nmf240} heterozy-

gotes were comparable to those of the WT mice indicates that the signal from rod photoreceptors to the RPE to initiate the generation of dc-ERG components is also comparable. Figure 7A presents average dc-ERGs obtained from the WT mice ($n = 11$) and the *Clcn2*^{nmf240} heterozygotes ($n = 9$) in response to a 2.4-log cd/m² stimulus, which evokes a maximum response in WT animals.¹⁶ The amplitude of the LP component was reduced in the *Clcn2*^{nmf240} heterozygous mice, whereas the amplitude of the other major dc-ERG components (c-wave, FO, off-response) appeared to be comparable between the WT and *Clcn2*^{nmf240} heterozygotes. Figure 7B summarizes the average ($\pm$ SEM) measures for each component. The average LP of the *Clcn2*^{nmf240} heterozygotes was 66.5% of that of the WT mice, a difference that was statistically significant ($P < 0.02$). There was no statistically significant difference in any other dc-ERG component assessed.

FIGURE 5. Apical microvilli were elongated in the *Clcn2*^{nmf240} homozygotes. Images of ezrin staining demonstrated that apical microvilli were elongated in (B, D) the P10 *Clcn2*^{nmf240} homozygotes compared with (A, C) the P10 *Clcn2*^{+/+} littermates. (B, arrows) ezrin-positive staining observed on the basal RPE in the mutant retinas. Magnification: (A, C) $\times 40$; (B, D) $\times 100$. NFL, nerve fiber layer; INL, inner nuclear layer; ONL, outer nuclear layer. (E) The length of the labeled band (WT) or the length of individual processes (mutant) was measured at a minimum of 10 areas in four WT and four mutant retinas, to determine the average apical RPE microvilli length. Error bars, SEM. (F) Western blot analysis demonstrated that levels of ezrin were the same in the *Clcn2*^{nmf240} homozygotes and WT mice. β -Actin served as the loading control.

FIGURE 6. The heterozygous *Clcn2*^{nmf240} mice had normal ERG responses. Representative strobe flash ERGs recorded under (A) dark- or (C) light-adapted conditions from 9-month-old WT and heterozygous *Clcn2*^{nmf240} mutants. Intensity-response functions for the major components of the (B) dark- or (D) light-adapted conditions. Data points indicate the average $\pm$ SEM for five mice.

Leukoencephalopathy in *Clcn2*^{nmf240} Homozygous Brains

Clcn2^{tm1Tj} homozygotes (*Clcn2*^{-/-}) develop a progressive leukoencephalopathy characterized by vacuoles in multiple brain regions.¹⁵ To determine whether similar abnormalities exist in *Clcn2*^{nmf240} homozygotes, we examined brain structure at 7 and 18 months of age. Figure 8 presents sections of a representative 18-month old *Clcn2*^{nmf240} heterozygote (Figs. 8A, 8C, 8E) and a *Clcn2*^{nmf240} homozygote (Figs. 8B, 8D, 8F). Although the overall brain architecture appeared similar, at low power vacuoles were apparent in the white matter tracts (Fig. 8B, a) and cerebellum (b) of the *Clcn2*^{nmf240} homozygote. These abnormalities are more clearly seen in the high-power images of these brain regions (Figs. 8D, 8F). Similar but less severe abnormalities were noted at 7 months of age (data not shown). Other gross morphologic abnormalities were not observed in the central nervous system of the *Clcn2*^{nmf240} homozygotes. Despite the vacuolization in the white matter tracts and cerebellum, no overt behavioral characteristics such as ataxia, tremors, or tics were observed. In addition, no brain abnormalities were observed in the *Clcn2*^{nmf240} heterozygotes when compared to the WT mice (data not shown).

Azoospermia in Fertility in Male *Clcn2*^{nmf240} Homozygotes

In spontaneous mating experiments, female *Clcn2*^{nmf240} homozygotes were fertile, and litter sizes and mating frequencies were not different from those of age-matched WT or *Clcn2*^{nmf240} heterozygous female mice (Fig. 9A). In contrast, male *Clcn2*^{nmf240} homozygotes failed to sire offspring (Fig. 9A). To determine whether male sterility was caused principally by a lack of reproductive behavior, we mated male mice overnight with hormone-primed female mice. Copulatory

plugs, the overt sign of successful reproductive behavior, were found in all the *Clcn2*^{nmf240} homozygotes and WT female mice (Fig. 9B). Unlike those induced by the WT males, no subsequent pregnancies were detected from the *Clcn2*^{nmf240} homozygous males (Fig. 9B). Based on these fertility tests, in vitro fertilization (IVF) was attempted to determine whether germ cell dysfunction causes infertility. No sperm was found in the caudal epididymides of the adult male *Clcn2*^{nmf240} homozygotes ($n = 3$), whereas the WT males ($n = 3$) contained motile sperm capable of inducing fertilization and producing embryos during IVF experiments (Fig. 9B).

The lack of sperm in the caudal epididymis suggests that *Clcn2*^{nmf240} homozygous males undergo severe atrophy of seminiferous tubules in the testes or physical blockage in the male reproductive tract, where sperm acquire competence to induce fertilization as they move from the lumen of the testes into the caudal epididymis. The weight of the testes as well as the epididymides from the *Clcn2*^{nmf240} homozygotes was reduced by 2- to 2.5-fold compared with those of the WT males (Fig. 9B), implicating testicular atrophy and germ cell loss as the primary cause underlying the male infertility. Histologic analysis revealed that as early as 6 weeks of age, when the first wave of spermatogenesis occurs, most of the *Clcn2*^{nmf240} homozygotes were azoospermic, with severe degradation of spermatogenesis, and were already devoid of spermatocytes, spermatids, and spermatozoa with elongated tails (Fig. 9E), whereas the WT mice showed all stages of spermatogenesis (Fig. 9C). Two homozygous mutants, however, retained a few spermatozoa in the lumen of the seminiferous tubule (Fig. 9D), although degeneration of spermatogenesis was already apparent in the presence of substantially fewer sperm than in the WT (Fig. 9C). By 20 weeks of age, all the *Clcn2*^{nmf240} homozygotes contained only a few spermatogonial stem cells, although Sertoli cells, somatic cells that support developing germ cells, remained along with

FIGURE 7. The LP was reduced in the heterozygous *Clcn2^{nmf240}* mice. (A) Average dc-ERGs recorded from WT ($n = 11$) and *Clcn2^{nmf240}* heterozygotes ($n = 9$) in response to a 7-minute, 2.4-log cd/m² stimulus. (B) Average $\pm$ SEM measures for the major components of the dc-ERG from the WT ($n = 11$) and *Clcn2^{nmf240}* heterozygous ($n = 9$) mice. There was no difference between the WT and *Clcn2^{nmf240}* heterozygous mice in the amplitude of the c-wave, FO, or off-response. The LP component was significantly reduced in the heterozygous mutants.

hypertrophy of the hormone-producing Leydig cells, which lie between the seminiferous tubules (Fig 9F).

DISCUSSION

We have described the phenotype of *Clcn2^{nmf240}* mice expressing a form of CLCN2 that carries a premature stop codon located at amino acid 355, approximately a third of the way through the protein. Homozygous mutants share several features previously described in *Clcn2^{-/-}* mice.^{11,13} *Clcn2^{nmf240}* homozygotes develop an early-onset and severe photoreceptor degeneration, with only one layer of photoreceptor cells remaining at P21 and exhibit leukoencephalopathy and male sterility. These shared features suggest that *Clcn2^{nmf240}* is also likely to be a null mutation. One notable distinction between the *Clcn2^{nmf240}* mutant and the previously described *Clcn2^{-/-}* mice is that the former is on a B6 background, whereas the latter was generated on a mixed B6 and 129/SvJ background.¹¹ The availability of a *Clcn2* null on a B6 background eliminates the potential for modifier interactions that can be introduced on a segregating genetic background.

As previously noted in *Clcn2^{-/-}* mice,¹¹ our homozygous *Clcn2^{nmf240}* mutant retinas were comparable to those of littermate controls at P10. At this age, however, apical RPE microvilli were significantly longer in the *Clcn2^{nmf240}* homozygotes than in the WT littermate controls. In addition, ezrin

staining was more diffuse in the *Clcn2^{nmf240}* homozygotes than in the WT animals, with some mislocalization to the basal RPE. The elongation of apical microvilli may be a response to the improper development of outer segments that was noted at P10 in the *Clcn2^{-/-}* mice by transmission electron microscopy.¹¹ Elongation of apical microvilli has been reported in retinal degeneration (*rd*) mice after the loss of photoreceptor cell bodies.²³ In the *Clcn2^{nmf240}* homozygotes, however, the elongation of apical microvilli preceded photoreceptor loss, which suggests that the absence of functional CLCN2 is a primary factor underlying the observed microvilli elongation. It is likely that the absence of functional CLCN2 disrupts ionic homeostasis within RPE cells. Indeed, CLCN2 is activated by cell swelling²⁴ and may play an important role in cell-volume regulation. The elongation and the diffuse staining of apical processes in *Clcn2^{nmf240}* homozygote retinas could result from a loss of cell volume regulation in the RPE cells. The aberrant RPE apical microvilli, may in turn affect other ion channels including KCNJ13 and Na⁺, K⁺-ATPase, localized to the apical processes of the RPE.^{25,26} Cumulatively, these changes may contribute to the degeneration of outer segments and subsequent photoreceptor loss as the RPE is unable to perform its supportive roles. Indeed, Bosl et al.¹¹ point out that both retinal and sperm cell degeneration occurs around the time of blood-organ barrier formation, indicating the possible involvement of CLCN2 in ionic maintenance. Although not observed until older ages, the leukoencephalopathy observed in both the *Clcn2^{-/-}* and homozygous *Clcn2^{nmf240}* mutants is also consistent with such a role for CLCN2. Vacuolation is observed surrounding astrocyte/oligodendrocyte networks, which are sensitive to ionic changes.¹³

FIGURE 8. Homozygous *Clcn2^{nmf240}* mice exhibited leukoencephalopathy. Cresyl violet-stained sagittal section of brain from a representative 18-month-old *Clcn2^{nmf240}* heterozygous (A) and a *Clcn2^{nmf240}* homozygous (B) mouse demonstrates vacuolation in the white matter tracts of mutants. Arrows: areas of prolific vacuolation that were present in white matter tracts of the corpus callosum (a) and cerebellum (b) of the *Clcn2^{nmf240}* homozygotes but were not seen in the *Clcn2^{nmf240}* heterozygous mice or WT controls (data not shown). (C-F) Higher power images of the white matter tracts of the corpus callosum (C, D) and cerebellum (E, F) of the *Clcn2^{nmf240}* heterozygous (C, E) and the *Clcn2^{nmf240}* homozygous (D, F) mice. Scale bars: (A, B), 1 mm; (C-F), 10 μ m.

FIGURE 9. Homozygous *Clcn2^{nmf240}* mice were infertile due to nonobstructive azoospermia. (A) Fertility assessment of female and male mice by measuring the percentage of successful mating, number of litters, litter size, and total number of pups ($^*P < 0.01$, $P < 0.005$, median tests). (B) Behavioral mating tests, subsequent pregnancy, and testes and epididymides size were measured in WT and homozygous *Clcn2^{nmf240}* mice ($^*P < 0.01$, χ^2 test; $^{**}P < 0.001$, t -test). H&E-stained sections of the testes were examined for spermatogenesis in WT mice at 6 weeks (C) and in homozygous *Clcn2^{nmf240}* mice at 6 (D, E) and 20 (F) weeks of age. Magnification, $\times 20$.

The basal membrane of the RPE hyperpolarizes and then depolarizes in response to light stimulation of the retina.²⁷ These changes in membrane potential underlie the FO and LP components of the ERG,²⁷ which are clinically useful in the diagnosis of certain retinal disorders.^{28,29} Although it has been recognized for many years that Cl^- channel activity underlies the generation of the FO and LP,^{30,31} the specific Cl^- channel underlying these ERG components have yet to be identified, despite the evaluation of several candidates. Although LPs are reduced in patients with mutations in bestrophin³² and it is clear that bestrophin can form Cl^- channels when expressed in in vitro systems,³³ mice lacking bestrophin generate LPs of normal or supernormal amplitude.³⁴ Similarly, although cystic fibrosis transmembrane regulator (CFTR) can serve as a Cl^- channel,³⁵ FOs and LPs are retained in CFTR mutant mice.³⁶ These results indicate that another Cl^- channel is needed to generate the FO and/or LP in RPE cells. The observation that the expression of a single functional *Clcn2* allele is insufficient to generate a normal ERG LP component indicates that CLCN2 is intimately involved in LP generation. Further studies are necessary to determine the exact role that CLCN2 plays in the LP response. It is interesting to note that Bosl et al.¹¹ have shown homozygous *Clcn2^{-/-}* mice to have a significant reduction in active transepithelial transport, as evidenced by a reduction in both the transepithelial resistance and equivalent short circuit current in Ussing chamber experiments with RPE tissue from mutant and WT mice.

Azoospermia is diagnosed when no measurable level of spermatozoa are present in semen and ejaculate fluids and is associated with male infertility. There are two clinically distinct forms of azoospermia: obstructive and nonobstructive.³⁷ Obstructive azoospermia occurs when spermatozoa are produced but cannot mix with seminal fluids due to a physical, obstructive barrier, whereas nonobstructive azoospermia arises from a disruption in spermatogenesis. Azoospermia can also be congenital, as in the case of male patients with cystic fibrosis (CF). CFTR, the gene product that is disrupted in CF, normally functions as a Cl^- channel that affects male fertility possibly by regulating the physiological volume of semen and leading to azoospermia (for a review, see Ref. 38). In addition, disruption of Cl^- channels from other *Clcn* gene family members is associated with the male infertility caused by decreased fluid volume.³⁹ Little is known about the physiological contribution

or mechanisms relating the lack of CLCN2 to azoospermia and male infertility in either mice or humans. However, fertility, physiological, and IVF tests and histologic examination all indicate that the *Clcn2^{nmf240}* mutants have nonobstructive azoospermia, as spermatogenesis is arrested, and atrophy of the testes begins as early as 6 weeks of age and is uniformly present by 20 weeks of age. Our observations of the male reproductive phenotype are similar to those reported in genetically engineered homozygous *Clcn2^{-/-}* mice,¹¹ although the *Clcn2^{nmf240}* mutants appeared to have an earlier onset of infertility and azoospermia.

In conclusion, the present study presents a new mouse model with a point mutation in *Clcn2* leading to a premature stop codon that, if translated, is predicted to form a truncated protein. The *Clcn2^{nmf240}* mouse will be useful in studying the function of this gene in retinal development and disease. The early-onset and severe photoreceptor degeneration in the *Clcn2^{nmf240}* and *Clcn2^{-/-}* mice suggest that some forms of human retinal disease could involve CLCN2. Although no retinal diseases have been mapped near the CLCN2 locus (3q27-28) (www.sph.uth.tmc.edu/Retnet; <http://www.sph.uth.tmc.edu/RetNet>; University of Texas Houston Health Science Center, Houston, TX), it would be interesting to evaluate CLCN2 as a candidate gene in cases of early-onset photoreceptor degeneration accompanied by male sterility. Furthermore, *Clcn2^{nmf240}* mutants may provide a translational model to investigate nonobstructive azoospermia that frequently occurs in cases of male infertility in humans.

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