Cone Photoreceptor Function Loss-3, a Novel Mouse Model of Achromatopsia Due to a Mutation in Gnat2

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PURPOSE. To report a novel mouse model of achromatopsia with a cpfl3 mutation found in the ALS/LtJ strain.

METHODS. The effects of a cpfl3 mutation were documented using fundus photography, electroretinography (ERG), and histopathology. Genetic analysis was performed using linkage studies and PCR gene identification.

RESULTS. Homozygous cpfl3 mice had poor cone-mediated responses on ERG at 3 weeks that became undetectable by 9 months. Rod-mediated waveforms were initially normal, but declined with age. Microscopy of the retinas revealed progressive vacuolization of the photoreceptor outer segments. Immunochemistry with cone-specific markers showed progressive loss of labeling for α-transducin, but the cone outer segments in the oldest mice examined remained intact and positive with peanut agglutinin (PNA). The cpfl3 mapped to mouse chromosome 3 at the same location as human GNA2, known to cause achromatopsia. Sequence analysis revealed a missense mutation due to a single base pair substitution in exon 6 in cpfl3.

CONCLUSIONS. The Gnat2cpfl3 mutation leads to cone dysfunction and the progressive loss of cone α-transducin immunolabeling. Despite a poor cone ERG signal and loss of cone α-transducin label, the cones survive at 14 weeks as demonstrated by PNA staining. This mouse model of achromatopsia will be useful in the study of the development, pathophysiology, and treatment of achromatopsia and other cone degenerations. The gene symbol for the cpfl3 mutation has been changed to Gnat2cpfl3. (Invest Ophthalmol Vis Sci. 2006;47:5017–5021) DOI:10.1167/iovs.05-1468

Mice are valuable for the identification of novel gene mutations that lead to retinal disease. Mutant retinas are investigated to elucidate the pathophysiology and natural history of the disorders. During screening for mouse models of ocular diseases at The Jackson Laboratory (TJL; Bar Harbor, ME), a novel mutation, named cone photoreceptor function loss-3 (cpfl3), was identified by electroretinography. This mutant appears to be a good model for achromatopsia occurring in humans.

Rod monochromatism or achromatopsia is a group of autosomal recessive human congenital disorders. Achromatopsia (all types) has an estimated incidence of 1 in 30,000.1 Clinical manifestations usually present in infancy with horizontal pendular nystagmus, photophobia, total color blindness, and poor visual acuity (20/200–20/400), which is slightly improved at dusk and night.2 The nystagmus often improves as the patient moves from childhood to the teenage years.3 The primary deficit in achromatopsia is a lack of functional retinal cone photoreceptors. Clinical diagnosis of these conditions relies on electroretinography, as retinal examinations are often normal in early stages of the disease.4

Three independent mutations have been identified in humans as causes of achromatopsia (Table 1). Mutations in CNGB3 (cyclic nucleotide gated channel β-3), which encodes the β-subunit of the cone cyclic GMP-gated cation channel cause approximately 56% to 50% of cases.5–7 Patients with CNGB3 mutations present with colorblindness, nystagmus, and subnormal vision and may eventually have myopia. This mutation has been studied primarily among the Pingelap islanders and has been linked to Irish ancestry.8 Progressive cone dystrophy has also been reported in CNGB3 mutant families with a history of achromatopsia, linking this gene to multiple diseases.9 CNAG3 encodes the α-subunit of the cone cGMP-gated cation channel, and mutations within this gene account for approximately 41% of cases of achromatopsia.10,11 CNAG3 mutations are found in Moroccan, Iraqi, and Iranian Jews, with an increased frequency of cases in Denmark.12 CNAG3 mutations may result in complete, incomplete, or (rarely) severe progressive phenotypes. Patients with achromatopsia who have mutations in both CNAG3 and CNGB3 present with a similar clinical phenotype, confirming the essential function of both the α- and β-subunits of the cGMP-gated cation channel for normal cone function.13 CNBG3 mutations have also been reported in dogs. The third gene for human achromatopsia is GNA2 (guanine nucleotide binding protein [G protein]), which encodes for the α-subunit of transducin necessary for hyperpolarization of cones. Transducin mediates an initial step in phototransduction, whereas the cGMP-gated channels CNAG3 and CNGB3 moderate the final steps in the cascade. Kohl et al.14,15 reported six separate mutations in GNA2: one nonsense, four small insertions/deletions, and one larger recombination, all of which result in premature termination of translation. This termination prevents the carboxyl terminus of α-transducin from interacting with the excited photopigment. In addition to these mutations, Aligianis et al.16 noted a 4-bp insertion in exon 7 of GNA2 that results in a frameshift mutation. Finally, one case of achromatopsia was caused by maternal isodisomy of chromosome 14.16
We report the clinical appearance, electrophysiology, histopathology, gene localization, and identification of a mutation in a novel murine model of achromatopsia.

**METHODS**

**Animals**

The mice in this study were bred and maintained in standardized conditions in the Research Animal Facility at TJL. They were maintained on NIH31 6% fat chow and acidified water, with a 14-hour light/10-hour dark cycle in conventional facilities that are monitored regularly to maintain a pathogen-free environment. All experiments were approved by the Institutional Animal Care and Use Committee and conducted in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

**Clinical Retinal Evaluation**

Mice were studied at various ages and documented at 1, 2, and 9 months. Pupils were dilated with 1% atropine ophthalmic drops, and the retinas were evaluated by indirect ophthalmoscopy with a 78-D lens. Signs of retinal degeneration, such as vessel attenuation, alterations in the RPE, and presence or absence of retinal dots were noted. Fundus photographs were taken with a small-animal fundus camera (Kowa Genesis; Torrance, CA), and a standardized electroretinogram (ERG) was recorded from the corneal surface of one eye after pupil dilation (1% atropine sulfate) with a gold loop electrode referenced to a gold wire in the mouth. A needle electrode containing ketamine (15 mg/g) and xylazine (7 mg/g body weight) was placed in the tail served as the ground. All stimuli were presented in a novel murine model of achromatopsia.

**Histology**

Both eyes from cpfl3 mutant mice and age-matched control mice were examined at intervals from 4 to 36 weeks of age. The mice were deeply anesthetized and killed by cardiac perfusion with phosphate-buffered saline (PBS) followed by 4% paraformaldehyde in 0.1 M phosphate buffer. The retinas were cryosectioned and processed for immunofluorescence according to the method of Milam.19 Cones were identified with primary anti-cone-α-transducin raised in rabbit (1:1000; Santa Cruz Biotechnology, Santa Cruz, CA) and secondary anti-rabbit IgG (1:1000, Jackson Immunoresearch, West Grove, PA). Rod-dominated responses were recorded to short-wavelength (λmax = 470 nm; Wratten 47A filter; Eastman Kodak, Rochester, NY) flashes of light over a 4.0-log-unit-range of intensities (0.3-log-unit steps). Cone-dominated responses were obtained with white flashes using intensities of 4.3, 1.6, 0.82, 0.48, and 0.25 cd/m² on a white, rod-saturating background and were obtained after 10 minutes of exposure to the background light, to allow complete light adaptation.

**Known Genetic Mutations in Human Achromatopsia**

<table>
<thead>
<tr>
<th>Mutations</th>
<th>%</th>
<th>Protein</th>
<th>Presentation</th>
<th>Ethnic Association</th>
</tr>
</thead>
<tbody>
<tr>
<td>CNGB3</td>
<td>36</td>
<td>β-Subunit of cone photoreceptor</td>
<td>Total colorblindness with myopia, may be progressive.</td>
<td>Pingelap islanders; Irish ancestry; linked to cone dystrophy</td>
</tr>
<tr>
<td>CNGA3</td>
<td>41</td>
<td>α-Subunit of cone photoreceptor</td>
<td>Varies: complete, incomplete, and progressive (rare) phenotypes</td>
<td>Moroccan, Iraqi, and Iranian Jews; large concentration in Denmark</td>
</tr>
<tr>
<td>GNAT2</td>
<td>2–3</td>
<td>α-Subunit of transducin</td>
<td>Total colorblindness, may be progressive</td>
<td>Several documented families in Europe, one Pakistani family</td>
</tr>
</tbody>
</table>

**FIGURE 1.** (A) Linkage cross data. One hundred twenty-three mice from a backcross between ALS/LtJ-Gnat3<sup>−/−</sup> homoygotes and CAST/EiJ mice were phenotyped and genotyped. Linkage to several markers on mouse chromosome 3 was observed. The columns of squares represent haplotypes (■, affected cpfl3/cpfl3 allele; □, CAST/EiJ allele). The number of chromosomes with each haplotype is indicated below each column. (B) Genetic map of chromosome 3 in the cpfl3 region showing the closest markers and the region of human homology. (C) The nucleotide sequences around the single-base substitution at position 598 (G→A) in exon 6 are shown for the wild-type allele and the cpfl3 allele of the Gnat2 gene. A novel mutation changes codon 200 GAT to AAT (amino acid change: Asp200Asn) in the Gnat2 gene in Gnat3<sup>−/−</sup> mice. WT, wild-type.
Gene Mapping and Sequencing

To determine the chromosomal location of the cpfl3 gene, ALS/LtJ-cpfl3 mice were mated to CAST/EiJ mice. The F1 mice, which exhibit no retinal abnormalities, were backcrossed to ALS/LtJ-cpfl3 mice (Fig. 1). Amplification was performed on isolated tail DNAs, using 25 ng of DNA in a 10-µL volume containing 50 mM KCl, 10 mM Tris-Cl (pH 8.3), 2.5 mM MgCl2, 0.2 mM oligonucleotides, 200 µM dNTP, and 0.02 U DNA polymerase (AmpliTaq; Applied Biosystems, Foster City, CA). The reactions, were initially denatured for 3 minutes at 94°C and then subjected to 40 cycles of 15 seconds at 94°C, 1 minute at 51°C, 1 minute at 72°C, and a final 7-minute extension at 72°C. PCR products were separated by electrophoresis on 3% agarose gels (MetaPhor; FMC, Rockland, ME) and visualized under UV light after staining with ethidium bromide. Initially, a genome scan of microsatellite (Mit) DNA markers was performed on pooled DNA samples. After detection of linkage on chromosome 3, the microsatellite markers D3Mit6, D3Mit49, D3Mit286, D3Mit11, D3Mit288, and D3Mit350 were scored in individual DNA samples. To test the Gnat2 gene as a candidate, we designed two pairs of PCR primers based on the mouse coding sequence to amplify overlapping cDNA fragments. For direct sequencing, the PCR reaction was scaled up to 30 µL. Amplification was performed for 36 cycles with a 15-second denaturing step at 94°C, a 2-minute annealing step at 60°C, and a 2-minute extension step at 72°C. PCR products were purified from agarose gels with a kit (Qiagen, Valencia, CA). Sequencing reactions were performed with automated fluorescence tag sequencing. Total RNA was isolated from retinas of newborn mice (TRizol LS Reagent; Invitrogen-Gibco, Grand Island, NY) and a preamplification system (SuperScript; Invitrogen-Gibco) was used to make first-strand cDNA. The following primer pairs were used to amplify overlapping cDNA fragments and sequence directly: (Gnat2-1F)-AATGGGGAGTGGCATCAGTGCTG and (Gnat2-1R)-TAGCCGCAAGAAACCTTGG; (Gnat2-2F)-ATCGCATGCAGGAGTCTTTG and (Gnat2-2R)-CTAAACAGAACCAGCAGCTTTG.

PCR Methods for Genotyping cpfl3 Mutations

One pair of primers (Gnat2-dF CATCGAGACCAAGTTTTCTG; Gnat2-dR ACCATGTAGTAGGCACTGAG) was used to genotype and confirm the cpfl3 mutation. A 362-bp fragment was amplified for mutant and wild-type Gnat2. There is an MseI recognition site in the cpfl3 mutant Gnat2 that is not found in wild-type Gnat2. PCR amplification was performed in 36 cycles with a 15-second denaturing step at 94°C, a 1-minute annealing step at 51°C, and a 1-minute extension step at 72°C. MseI digestion was performed directly in a 10-µL volume by adding 8 µL of PCR products, 1 µL of 10 x buffer (NE Buffer 2), and 2 to 5 units of MseI (NE Enzyme).

Complementation Tests

Complementation tests between the cpfl3 strain and other strains with similar phenotype were performed. Affected homozygotes were mated to ALS/LtJ mice that were homozygous for the cpfl3 mutation. The cone function of F1 offspring was tested by ERG.

RESULTS

Genetics

One hundred twenty-three mice derived from a cross of cpfl3/cpfl3 and CAST/EiJ were phenotyped and genotyped. Genetic analysis revealed that the functional loss with the cones is due to a mutation in mouse chromosome 3, closely linked to D3Mit286 (Fig. 2). This location suggests that the corresponding human homolog is located on chromosome 1p13, the same location as the human GNA12 gene. On sequence analysis, the mutation was identified as a missense mutation in exon 6 of the GNA12 gene. Specifically, in the cpfl3/cpfl3 mice, a single-base

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substitution at position 598 (G→A) in exon 6 was found that changes codon 200 from GAT to AAT (aspartic acid to asparagine; amino acid change Asp200Asn). As the functional loss within the cones is caused by the missense mutation in GNAT2 designated cpfl3, the gene symbol for the cpfl3 mutation has been changed to GNAT2cpfl3. The cpfl3 mutation results in a new MseI site that enabled us to genotype the cpfl3 mutation by PCR-RFLP (polymerase chain reaction and restriction fragment length polymorphism; data not shown). To confirm the presence of the missense codon in the cpfl3 Gnat2 gene, we re-examined 123 DNAs (58 affected and 65 unaffected mice) from our linkage analysis for the MseI RFLP. We amplified a Gnat2-dF/Gnat2-dR 362-bp genomic fragment that contains two MseI sites in the normal allele and three in the cpfl3 allele. Digestion of the PCR-amplified products with MseI from wild-type, homozygous, and heterozygous cpfl3 DNA revealed the predicted RFLP pattern (wild-type: three bands of 257, 85, and 20 bp; homozygous cpfl3: four bands of 156, 101, 85, and 20 bp; heterozygous cpfl3: five bands of 257, 156, 101, 85, and 20 bp). This analysis showed that there was absolute concordance between the cpfl3/cpfl3 phenotype and the missense mutation. The RFLP pattern thus provides a tool for verification of the presence or absence of the cpfl3 allele in genetic analysis.

Other strains of mice at TJL have been found with the Gnat2 mutation. Because we screen with electroretinography, other mice originally were found with cone abnormalities. Complementation tests in the following stocks or strains: stock Tg(Fos-lacZ)34Efu/J 004623; stock Tg(Trp53A135V)L3Ber/J 002746, SENCARB/PtJ 002,747, and SENCARC/PtJ 002448 were positive, and genotyping these stocks or strains by PCR-RFLP (polymerase chain reaction and restriction fragment length polymorphism; data not shown). To confirm the presence of the missense codon in the cpfl3 Gnat2 gene, we re-examined 123 DNAs (58 affected and 65 unaffected mice) from our linkage analysis for the MseI RFLP. We amplified a Gnat2-dF/Gnat2-dR 362-bp genomic fragment that contains two MseI sites in the normal allele and three in the cpfl3 allele. Digestion of the PCR-amplified products with MseI from wild-type, homozygous, and heterozygous cpfl3 DNA revealed the predicted RFLP pattern (wild-type: three bands of 257, 85, and 20 bp; homozygous cpfl3: four bands of 156, 101, 85, and 20 bp; heterozygous cpfl3: five bands of 257, 156, 101, 85, and 20 bp). This analysis showed that there was absolute concordance between the cpfl3/cpfl3 phenotype and the missense mutation. The RFLP pattern thus provides a tool for verification of the presence or absence of the cpfl3 allele in genetic analysis.

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**Fundus Photography**

Photographs of the fundi of ALS/LtJ- Gnat2cpfl3 homozygotes were taken from birth to 8 months of age and two are shown in Figure 2 at 2 and 8 months of age (Fig. 2). The only noticeable findings were dilated retinal vessels at age 8 months, which is common in early retinal degenerations in mice.

**ERG Phenotype**

Mice homozygous for Gnat2cpfl3 were examined with ERG at age 4 weeks. The initial results showed normal rod-mediated responses and abnormal photopic responses, which were approximately 25% of normal and were extinguished by 9 months (Fig. 3). Scotopic responses showed some diminution with time but were near normal at age 9 months.

**Histopathology**

Toluidine blue–stained sections from mutant retinas at ages 6 and 15 weeks revealed the normal 8 to 10 rows of rod nuclei (Fig. 4), consistent with the normal scotopic ERGs. At 27 weeks, the outer segments of the mutant Gnat2cpfl3 homozygotes were vacuolated (Fig. 4C). Immunofluorescence with anti-cone α-transducin revealed reduced labeling of cone outer segments in mutant retinas by age 4 weeks (Fig. 5) compared with wild-type retina. PNA staining revealed a normal number of cone outer segments in the oldest Gnat2cpfl3 retinas examined (Fig. 5). Thus, cone outer segment structure was retained even as cone α-transducin levels appeared reduced and the photopic ERG were extinguished.

**DISCUSSION**

Although cone dystrophy has been studied in a mouse model,20 this is the first naturally occurring mouse model specifically for achromatopsia—in this case resulting from mutations in Gnat2. This model is based on a spontaneously occurring G→A mutation in exon 6 of Gnat2, which results in a missense mutation of the α-subunit of the protein transducin, thereby preventing photoreceptor hyperpolarization in the phototrans
duction cascade, causing the cone function loss in cpl3/cpl3 mice. The carboxyl terminus of GNAAT2 miscoding (aspartic acid to asparagine; amino acid change Asp200Asn) may eliminate important functional domains of α-transducin, which have been shown to interact with rhodopsin and phosphodiesterase γ-subunits,\textsuperscript{21,22} and this loss of cone transducin function was documented in this study by lack of cone function on ERG. The cpl3 mutation in mouse chromosome 3 has significant homology to human chromosome 1, region p13, in which the GNAT2 gene is located. In humans, seven mutations have been documented in the GNAT2 gene, including small insertions and deletions,\textsuperscript{10} a nonsense mutation, a larger recombination, and frameshift mutations.\textsuperscript{2} This heterogeneity is also found in mutations causing achromatopsia in CNGA3 and CNGB3. In particular, CNGA3 mutations can cause complete, incomplete, or progressive phenotypes. Achromatopsia has been defined as complete at birth and nonprogressive,\textsuperscript{10} although the senior author (JRH) has observed a progressive loss of photopic ERG in some cases diagnosed in early childhood. Eksandh et al.\textsuperscript{13} noted slight remaining cone responses in younger patients with achromatopsia and midperipheral pigmentary degenerations in the oldest patient examined. This finding indicates that some progressive retinal dysfunction may occur in patients with achromatopsia. This novel mouse mutant provides a model for future experimental treatments as well as more detailed examination of disease mechanisms. As cones initially appear to retain structural integrity in this model, at least by PNA staining, this disease may be amenable to gene therapy or similar molecular interventions.

References