

A Novel Homozygous Nonsense Mutation in *CABP4* Causes Congenital Cone–Rod Synaptic Disorder

Karin W. Littink,^{1,2} Maria M. van Genderen,³ Rob W. J. Collin,^{2,4} Susanne Roosing,^{2,4} Arjan P. M. de Brouwer,^{2,4} Frans C. C. Riemsdag,³ Hanka Venselaar,^{4,5} Alberta A. H. J. Thiadens,⁶ Carel B. Hoyng,⁷ Klaus Rohrschneider,⁸ Anneke I. den Hollander,^{2,4,7} Frans P. M. Cremers,^{2,4} and L. Ingeborgh van den Born¹

PURPOSE. The purpose of this study was to identify the causative gene defect in two siblings with an uncharacterized cone–rod dysfunction and to describe the clinical characteristics.

METHODS. Genome-wide homozygosity mapping, with a 250K SNP-array followed by a search for candidate genes, was performed. The patients underwent ophthalmic examination, including elaborate electroretinography.

RESULTS. In a Dutch sib pair, a shared 9-Mb homozygous region was found on 11q13.1-q13.5 that encompasses the *CABP4* gene, previously implicated in autosomal recessive incomplete congenital stationary night blindness (CSNB2) in two small families. A novel homozygous p.Arg216X mutation in *CABP4* was detected in the sib pair. Quantitative RT-PCR on RNA isolated from patient lymphoblast cells showed no nonsense-mediated degradation of mutant *CABP4* mRNA. Clinically, patients presented with reduced visual acuity, photophobia, and abnormal color vision, but they did not experience night blindness. Electroretinograms showed electronegative mixed rod–

cone responses and severely reduced cone responses, as in CSNB2. Isolated rod responses, however, were (sub)normal.

CONCLUSIONS. A novel homozygous nonsense mutation in *CABP4* in two siblings resulted in a phenotype with severely reduced cone function and only negligibly reduced rod function on electroretinography and psychophysical testing. Since these patients and two of three previously described patients do not experience night blindness, the name CSNB2 is confusing for patients as well as clinicians. Therefore, the authors propose to name the phenotype congenital cone–rod synaptic disorder. (*Invest Ophthalmol Vis Sci.* 2009;50:2344–2350) DOI:10.1167/iovs.08-2553

CABP4 (Calcium-binding protein 4) has recently been described in association with autosomal recessive incomplete congenital stationary night blindness (CSNB2).¹ CSNB is a non-progressive retinal disorder characterized by impaired night vision, myopia or hyperopia, nystagmus, and reduced visual acuity, with a wide intra- and interfamilial variability. All modes of Mendelian inheritance have been described for this disorder. The genes associated with CSNB encode different components of the phototransduction cascade or proteins involved in signaling from photoreceptor to the adjacent bipolar cells.^{1–11} Most patients with CSNB (X-linked and autosomal recessive) have a characteristic electronegative electroretinogram (ERG) (Schubert-Bornschein type)—that is, a near-normal a-wave and a substantially reduced b-wave—on testing under scotopic conditions.¹² The X-linked type of CSNB was further subdivided by Miyake et al.¹² into complete CSNB (CSNB1) and incomplete CSNB (CSNB2), based on differences in electrophysiology. In CSNB1 (OMIM 310500; Online Mendelian Inheritance in Man; <http://www.ncbi.nlm.nih.gov/Omim/> provided in the public domain by the National Center for Biotechnology Information, Bethesda, MD), no residual rod function is detected. In CSNB2 (OMIM 300071) the rod contribution to the scotopic b-wave is reduced but recordable. At a 30-Hz flicker stimulation, amplitudes are overall decreased, but they increase in time with a characteristic double-peak appearance (wave separation phenomenon).¹³ Miyake et al.¹² suggested distinct pathogenic mechanisms for the different forms of CSNB, which was confirmed by the identification of the molecular causes of CSNB. For CSNB1 mutations in *NYX* (MIM 300278)⁷ were identified in patients with the X-linked form and mutations in *GRM6* (MIM 604096)⁹ in patients with the autosomal recessive form. Patients with mutations in *GRM6* and *NYX* can be distinguished clinically by a characteristic pattern at a 15-Hz flicker stimulation.¹⁴ X-linked CSNB2 is caused by mutations in *CACNA1F* (MIM 300110),⁶ which encodes the L-type voltage-dependent calcium channel 1.4 (Ca_v1.4).¹⁵ In two small families with a CSNB2-like phenotype and an autosomal recessive mode of inheritance, Zeitz et al.¹ recently identified mutations in the *CABP4* gene (MIM 608965), which encodes the calcium binding protein CaBP4. CaBP4 colocalizes and interacts with Ca_v1.4 in both cone and

From ¹The Rotterdam Eye Hospital, Rotterdam, The Netherlands; the Departments of ²Human Genetics and ⁷Ophthalmology, Radboud University Nijmegen Medical Centre, Nijmegen, The Netherlands; the ³Bartiméus Institute for the Visually Impaired, Zeist, The Netherlands; the ⁴Nijmegen Centre for Molecular Life Sciences, and the ⁵Centre for Molecular and Biomolecular Informatics, Radboud University Nijmegen, Nijmegen, The Netherlands; the ⁶Department of Ophthalmology, Erasmus Medical Center, Rotterdam, The Netherlands; and the ⁸Department of Ophthalmology, University of Heidelberg, Heidelberg, Germany.

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Corresponding author: L.Ingeborgh van den Born, The Rotterdam Eye Hospital, PO Box 70030, 3000 LM Rotterdam, The Netherlands; born@eyehospital.nl.

TABLE 1. Primers Used for Molecular Studies of CABP4

Procedure Gene and Exon	Sequence (5'→3')		Product Size (bp)
	Forward	Reverse	
Direct DNA sequencing			
CABP4 promoter	GCCAGCAGGAAGAGGC	GACCCCAAATGGACTACTACC	377
CABP4 exon 1	GGTCTGAAAGCCAAGG	GGTGAGCTGAGCCCAAGG	502
CABP4 exons 2-3	AGGGGATGAAGGAGGAAGG	CCACTAGCACCCCGATGG	426
CABP4 exon 4	TTTCTTCTAGGTGCAGAGC	GCTGAGACCTGAGTGAGAGG	299
CABP4 exons 5-6	AGCTGGCTGAGGCTGAGG	CTGCTGGGTCTCCATCTCC	554
QPCR			
CABP4 exons 3-4	AGGTCTCGCAGCACATCAAG	CTCAGCTTTGGGCCTATCAG	81
GUSB	AGAGTGGTGTGAGGATTGG	CCCTCATGCTTAGGGTGTG	80
FTSJ1	CAACTCTTCCAAGGCGTGAC	ATCTTGTGGCTCAGCACCTG	80
Procedure Gene and Exon	Sequence (5'→3')		
ARMS primers			
Wild-type forward	5'-TGGGGGTGCGAGAGCTGCGCATCGCCTTAC-3'		
Mutant forward	5'-TGGGGGTGCGAGAGCTGCGCATCGCCTTAT-3'		
Reverse	5'-TAGCTGGTAGTGATCTGAACCATCTCTGA-3'		

Primers were designed using Primer3 software (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi, provided in the public domain by the Whitehead Institute, Massachusetts Institute of Technology, Cambridge, MA).¹⁹

rod photoreceptor synaptic terminals, thereby regulating the calcium influx in the photoreceptor. Patients carrying mutations in *CACNA1F* and *CABP4* show a comparable phenotype. A true genotype-phenotype correlation for *CABP4* has not been established yet, since only three patients have been described.¹

In this article, we present two siblings, carrying a novel homozygous nonsense mutation in *CABP4*, with an ERG suggestive of CSNB2, but with nearly normal rod function and no night blindness. We show similarities between this phenotype and other disorders influencing photoreceptor synaptic calcium channels and propose to add this disorder to a novel spectrum of calcium channelopathies.

SUBJECTS AND METHODS

Patients

Two affected siblings were clinically and genetically examined. Blood samples were obtained from patients and their parents. Informed consents signed by the parents were obtained according to the tenets of the Declaration of Helsinki and the protocol was approved by the ethics committees of The Rotterdam Eye Hospital and the Radboud University Nijmegen Medical Centre. Clinical assessment included best corrected visual acuity, refractive error, slit lamp examination, funduscopy, kinetic Goldmann perimetry (targets V-4e and I-4e), color vision tests (Ishihara Test for Color Blindness, American Optical Hardy-Rand-Rittler Test (AO-HRR), Farnsworth Panel D15 Test) and dark adaptometry (Goldmann-Weekers dark adaptometer). Electroretinograms (ERGs) were recorded according to a previously described protocol.¹² For the standard ISCEV ERG measurements,¹⁶ Xenon tube flashes (duration <10 μs) were delivered in a Ganzfeld (Color Dome; Diagnosys, Littleton, MA). The 15-Hz protocol was recorded intermixed with the standard ISCEV ERG at the appropriate intensities, using LED flashes of 4 ms duration.

Homozygosity Mapping

Total genomic DNA was extracted from EDTA-treated blood samples by using standard procedures.¹⁷ DNA samples for SNP analysis were genotyped for 262,000 SNPs (GeneChip Mapping 250K Nsp array; Affymetrix, Santa Clara, CA). Array experiments were performed ac-

ording to protocols provided by the manufacturer. The 250K SNP genotypes were analyzed with the software package CNAG,¹⁸ and chromosomal segments were accepted as homozygous if the loss-of-heterozygosity (LOH) score was ≥15, which corresponds with an area of >200 SNPs.

Sequence Analysis

All six coding exons, a noncoding exon and the intron/exon boundaries of the *CABP4* gene (NM_145200), were amplified by polymerase chain reaction (PCR). Genomic PCR was performed in 50 μL volumes containing 100 ng genomic DNA, 0.2 mM of each primer (Table 1), 2 mM MgCl₂, 1 mM dNTPs, PCR buffer provided by the manufacturer, and 5 U *Taq* polymerase (Invitrogen, Breda, The Netherlands). PCR reactions were performed as follows: 92°C (3 minutes); cycles with a denaturation at 95°C (30 seconds), an annealing temperature of 68°C (3 cycles), 66°C (3 cycles), 64°C (3 cycles), 62°C (31 cycles; 30 seconds) and an elongation at 72°C (45 seconds); and a final extension at 72°C (5 minutes). PCR products were purified (Qia Quick Gel Extraction Kit; Qiagen, Venlo, The Netherlands) according to the manufacturer's protocol and analyzed in sense and anti-sense directions with dye termination chemistry (BigDye Terminator, ver. 3 on a 3730 or 2100 DNA analyzer; Applied Biosystems, Inc., Foster City, CA). The control panel included 300 alleles from ethnically matched unrelated unaffected individuals and were screened for the c.646C>T mutation, detected in this study, using the Amplification-Refractory Mutation System (ARMS)²⁰ (Table 1).

Furthermore, 71 patients with cone-rod dystrophy and 14 with cone dystrophy were screened for mutations in *CABP4*. DNA of 79 of these patients had been screened before for known *ABCA4* mutations with the *ABCA4* arrayed-primer extension microarray (Asper Ophthalmics, Tartu, Estonia).²¹ In 62 patients, known *ABCA4* mutations were excluded, whereas in 17 patients, one mutation in *ABCA4* was detected.

Cell Culture

Human B-lymphocytes were immortalized by transformation with the Epstein-Barr virus according to established procedures.²² Epstein-Barr Virus transformed lymphoblastoid cell lines (EBV-LCLs) of the patients and controls were grown to a density of 0.7 million cells per milliliter RPMI 1640 medium (Invitrogen-Gibco, Breda, The Netherlands) con-

TABLE 2. Clinical Characteristics of Two Patients with a Homozygous p.Arg216X Mutation in the *CABP4* Gene

Patient	Sex	Age (y)	Visual Acuity		N	Refractive Error*		Color Vision†	Goldmann Perimetry (V4e)	DA (log units)
			OD	OS		OD	OS			
II-1	M	12	20/200	20/200	Pos	+5.0	+5.5	Severely abnormal (deutan)	Normal	0.5
II-2	F	10	20/200	20/400	Pos	+4.5	+4.5	Severely abnormal (deutan)	Normal	0.75

N, nystagmus; Pos, positive; DA, dark adapted final threshold elevation.

* Spherical equivalent in diopters.

† Ishihara, AO-HRR, D-15 Test.

taining 10% (vol/vol) fetal calf serum (Sigma-Aldrich, Zwijndrecht, The Netherlands), 1% penicillin-streptomycin (Invitrogen-Gibco), and 1% cell culturing medium (GlutaMAX; Invitrogen-Gibco). Thirty-five million cells were harvested by centrifugation at 200g for 5 minutes at room temperature and resuspended in 500 μ L 8 mM Na₂HPO₄, 2 mM KH₂PO₄, 137 mM NaCl, and 2.7 mM KCl (pH 7.2; PBS). Cell pellets were subsequently stored at -80° C until RNA isolation.

Quantitative PCR Analysis

Total RNA was isolated from EBV-LCLs according to the manufacturer's protocol (RNeasy minikit; Qiagen). cDNA was synthesized from 2.0 μ g of total RNA using random primed hexamers (GE Healthcare, Hoevelaken, The Netherlands) and M-MLV reverse transcriptase (Invitrogen) in a total volume of 85 μ L, according to the manufacturer's protocol. Primer pairs (Table 1) were validated by serial cDNA dilutions, synthesized from Universal Human Reference RNA (Stratagene, La Jolla, CA) in 5 \times to 80 \times dilutions, in triplicate. The primer pairs were 100% efficient, in that the amount of DNA was doubled in each cycle. The PCR conditions were 95 $^{\circ}$ C (3 minutes) and cycles of 95 $^{\circ}$ C (15 seconds) and 60 $^{\circ}$ C (30 seconds) in a 25- μ L reaction mix containing 5 μ L of cDNA, supermix (IQ SYBR Green; Bio-Rad, Hercules, CA), and 0.3 μ M of each primer. For the actual qPCR experiment, cDNA was diluted twice to make sure that the threshold cycles (C_t) were within the range of the dilution curve. qPCR reactions were performed on a thermocycler (7500 fast real-time PCR system; Applied Biosystems) and quantification was performed using the $\Delta\Delta C_t$ method.^{23,24} *GUSB* was used as a reference gene to normalize expression levels of *CABP4*, as this gene is stably expressed in EBV-LCLs.²⁵ RNA of a patient with a p.Q66X mutation in *FTSJ1*, known to result in nonsense-mediated decay (NMD), was used as a positive control for the occurrence of NMD.²⁶ The qPCR experiment was repeated for both patients on RNA isolated from two independently grown EBV-LCLs to confirm the results of the first qPCR experiment.

Molecular Modeling

Since no crystal structure for CaBP4 was known, we performed homology modeling to predict the effect of the genomic mutation. The C-terminal residues of CaBP4 were modeled on a structure of calmodulin (PDB identifier 1a29, 44% identity).²⁷ Modeling of the N-terminal residues of CaBP4 was not possible as the calmodulin template contains no structural information for these residues. Homology modeling was performed with the WHAT IF Web Interface.²⁸ The effect of the genomic mutation on the three-dimensional structure of the protein was analyzed with YASARA NOVA.²⁹

RESULTS

Clinical Features

A brother and sister, 12 (patient II-1) and 10 (patient II-2) years of age, respectively, presented with a history of decreased visual acuity and nystagmus since early childhood. The clinical

characteristics of the patients are shown in Table 2. Both reported photophobia, but did not experience night blindness. Visual acuities had not changed over the past 6 years. Slit lamp examinations were unremarkable and funduscopy showed no abnormalities, except for a mild granular aspect of the peripheral retinal pigment epithelium in patient II-1. Dark-adaptation curves were biphasic with a slightly elevated final threshold. The standard ISCEV ERG measurements¹⁶ of both patients are shown in Figure 1. The amplitude of the rod isolated (scotopic) responses were normal in patient II-1 (124 μ V) and were 2 SD below the mean in patient II-2 (46 μ V; normal >45 μ V).³⁰ In both children, the mixed rod-cone responses had absent cone a-waves, and an electronegative configuration with absent b-waves. Cone responses were severely reduced and 30-Hz photopic flicker responses showed the double-peak waveform characteristic of CSNB2. Rod ERG responses to a 15-Hz flicker stimulation are shown in Figure 2. The measurements showed intact, slow, sensitive rod pathway responses, but no minimum ERG response or 180 $^{\circ}$ phase shift, indicating absent or severely abnormal fast insensitive rod pathway responses.

Genetic Analysis

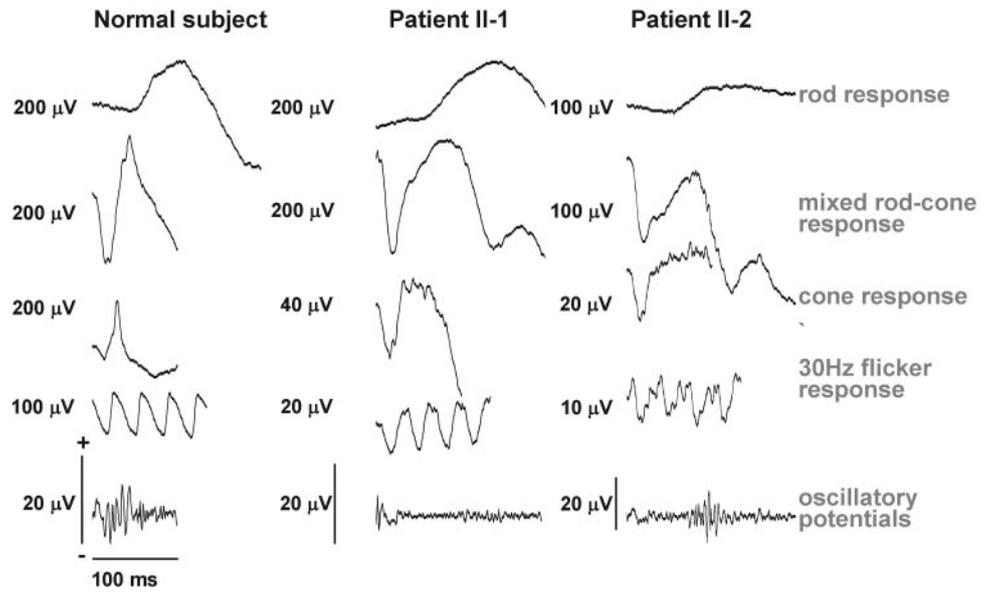
Genome-wide homozygosity mapping revealed two homozygous regions; a 9-Mb area on 11q13.1-q13.5 and a 4-Mb area on 6p22.1. The largest area comprised *CABP4*, a gene previously associated with congenital stationary night blindness.^{1,15} Sequence analysis of *CABP4* revealed a homozygous c.646C>T substitution in exon 4 in both patients, replacing an arginine residue at position 216 by a stop codon (p.Arg216X). In both parents this change was identified heterozygously (Fig. 3). The mutation was not found in 300 alleles of ethnically matched control individuals.

Jalkanen et al.³² showed that a splice-site mutation in the *CACNA1F* gene, the causative gene for X-linked CSNB2, resulted in cone-rod dystrophy. *Cabp4*^{-/-} mice show a progressive retinal phenotype. Therefore, Zeitz et al.¹ hypothesized that mutations in *CABP4* could also lead to cone-rod dystrophy. Sequence analysis of all coding exons of *CABP4* was performed on 85 patients affected with cone or cone-rod dystrophy. No mutations were found in these patients, however, indicating that mutations in *CABP4* are not a frequent cause of cone or cone-rod dystrophy.

Expression Analysis

Since the premature termination codon is localized more than 55 nucleotides upstream of the last exon-exon boundary (Fig. 4), in theory the c.646C>T mutation should result in NMD.³³⁻³⁵ Considering the absence of NMD as the null hypothesis, comparison of normalized expression levels of *CABP4* of the two patients with four control samples showed a nonsignificant 1.58-fold increase ($P = 0.58$, Student's *t*-test;

FIGURE 1. ISCEV standard ERG of a normal subject and patients II-1 and II-2. The amplitude of the rod response of patient II-1 was 124 μV and of patient II-2, 46 μV ; normal is $>45 \mu\text{V}$.³⁰ In the mixed rod-cone responses, the a-wave in the normal subject contains two distinct peaks, one at 19 ms, supposedly the cone peak, and a later one at 24 ms, supposedly the rod peak. In both patients only one peak at 24 ms was distinguished. Therefore these mixed responses featured an absent cone a-wave, with a normal rod a-wave. The b-wave remained too small, resulting in a negative wave shape. Under photopic conditions, the b-wave amplitude was severely reduced but with normal implicit time in both patients. Photopic 30-Hz flicker stimulus revealed decreased amplitude with double peak waveform and normal implicit time. There were no recordable oscillatory potentials under scotopic conditions.



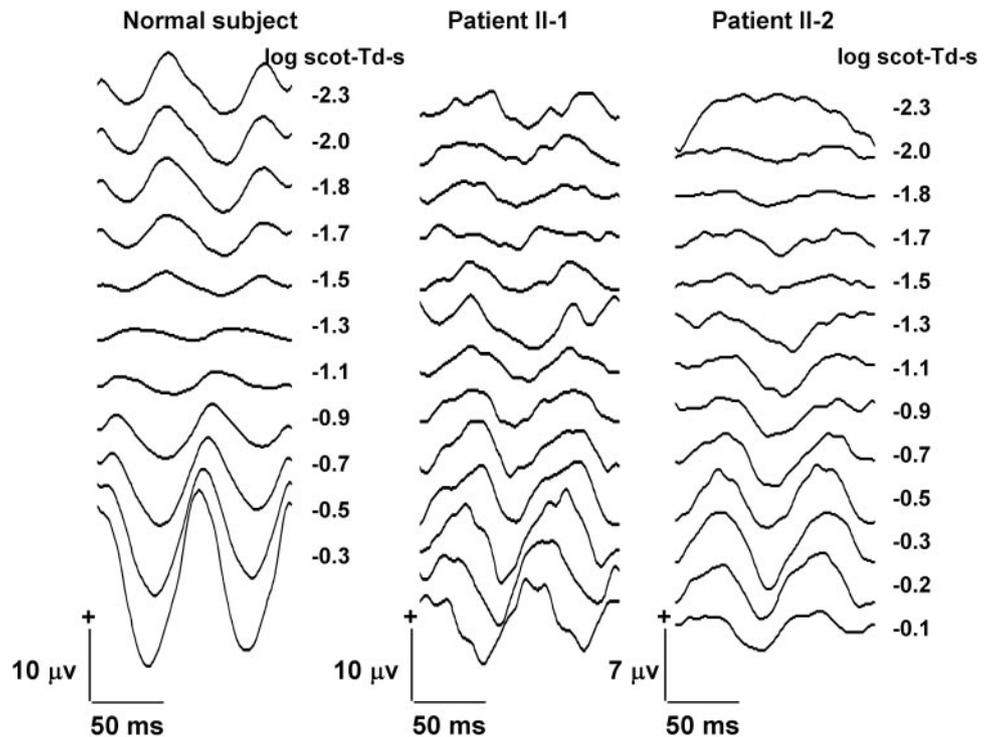
unequal sample size, equal variance). Repetition of the experiment with RNA from independently grown lymphoblast cell lines confirmed these results and showed that NMD of *CABP4* mRNA of patients carrying a homozygous p.Arg216X mutation does not occur. Comparison of normalized levels of *FTSJ1* in the cDNA of a patient with a p.Q66X mutation in *FTSJ1* to four control samples showed a 5.7-fold decrease in *FTSJ1* transcript in the patient ($P = 0.021$), showing that this experiment efficiently demonstrated NMD.

Molecular Modeling and Structural Analysis

Since the premature stop codon does not seem to result in nonsense-mediated degradation of the mutant *CABP4* mRNA, it is likely that a truncated CaBP4 protein of 216 amino acids is present. We predicted the characteristics of this truncated

protein by constituting a molecular model, using the crystal structure of calmodulin (Fig. 5). The modeled domain of CaBP4 contains important negatively charged residues that can bind Ca^{2+} . The location of these residues results in a typical helix-loop-helix structure, also known as an EF hand. The calmodulin template contains four of such EF hands, but because of the absence of an important negatively charged residue in the second EF hand the wild-type CaBP4 has only three functional calcium-binding EF hands.¹⁵ The nonsense mutation described in this study deletes the functional EF hands 3 and 4 (Fig. 5B). For calmodulin, it is known that all four EF hands must be present to fulfill its function.³⁶ Because of the functional and structural overlap between calmodulin and CaBP4,¹⁵ we conclude that the mutant CaBP4, lacking two functional EF hands, is not able to fulfill its physiological function.

FIGURE 2. Rod ERG responses to 15-Hz flicker stimulation obtained from a normal subject and patients II-1 and II-2. Stimulus intensity was $-2.3 \log \text{scot-td-s}$, increasing in steps of approximately 0.25 $\log \text{scot-td-s}$ up to 0.4 $\log \text{scot-td-s}$. In the normal subject, the 15-Hz scotopic flicker ERG showed a minimum response at approximately $-1.0 \log \text{scot-td-s}$, caused by destructive interference of the slow, sensitive (between -2.3 and $-1.3 \log \text{scot-td-s}$) and fast, insensitive (above $-0.9 \log \text{scot-td-s}$) rod pathway signals.³¹ In both patients, the amplitudes of the slow, sensitive rod pathway signals were within normal limits. The fast, insensitive rod pathway signals appeared absent or severely subnormal, because no minimum response and phase shift was recorded. Furthermore, from $-0.5 \log \text{scot-td-s}$ up the amplitudes of the signals were subnormal.



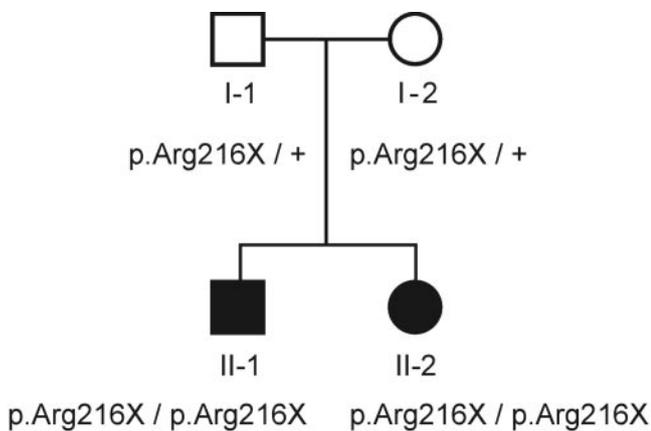


FIGURE 3. Pedigree of a Dutch family with two siblings affected with congenital synaptic cone-rod disorder, caused by a homozygous p.Arg216X mutation. *Black symbols*: affected individuals.

DISCUSSION

Using a genome-wide homozygosity mapping approach, we detected a novel homozygous nonsense mutation (p.Arg216X) in *CABP4* in two siblings with a remarkable cone-dominated dysfunction. Patients presented with considerably reduced visual acuity at a young age, nystagmus, photophobia, and severely abnormal color vision. Mutations in *CABP4* have been associated with autosomal recessive incomplete congenital stationary night blindness (CSNB2). In two siblings with diagnosed CSNB2, but without night blindness, Zeitz et al.¹ discovered a homozygous C-terminal frameshift mutation (p.Glu267fsX91) in the last protein-coding exon, effectively replacing the last 9 amino acids with 91 aberrant amino acids. In a third, isolated patients with CSNB2 compound heterozygous (p.Glu267fsX91/p.Arg124Cys) mutations were found.

Cabp4^{-/-} mice carrying a homozygous null allele show a CSNB2-like phenotype. These mice showed a disturbed transmission of signals from rods and cones to bipolar cells with a more severely disturbed rod than cone function.¹⁵ In contrast to findings in the *Cabp4*^{-/-} mice, our patients with p.Arg216X mutations displayed a prominent cone dysfunction. The presence of this effect was deduced from clinical presentations as well as from ERG responses, which showed an absent cone a-wave in the mixed response and severely reduced amplitude in cone and 30-Hz flicker responses. The rods seemed to function nearly normal: scotopic ERG responses were normal to subnormal, dark-adapted thresholds were only minimally elevated, and patients did not experience night blindness. Another indication that cones were more affected than rods was seen in the 15-Hz flicker stimulation. The fast insensitive rod pathway uses the rod-cone gap junctions and the cone

terminal synapse to transmit its signal to the bipolar cells, whereas the slow sensitive rod pathway transmits its signal directly to the rod bipolar cells.³⁷ Therefore, the combination of a normal, slow, sensitive rod pathway response to 15-Hz flicker stimulation with an abnormal, fast, insensitive rod pathway response suggests an abnormal function of the cones and/or cone terminal synapses. While Morgans et al.³⁸ suggest that residual rod function in patients carrying mutations in *CACNA1F* is maintained because rods signal through the rod-cone gap junction, our electrophysiological data suggest that rod function in patients carrying *CABP4* gene mutations is to some extent reduced because of this pathway.

In the two patients with the frameshift mutation described by Zeitz et al.,¹ no 15-Hz scotopic measurements, dark adaptation, and color vision tests were performed, and no differentiation was made between the cone and rod a-wave at the mixed rod-cone ERG response. Therefore, we do not know to what extent the phenotypes of these patients are comparable to ours. The third patient in Zeitz et al.¹ did complain of night blindness and had a mildly elevated threshold on dark adaptation (1 log unit). Comparison of all five patients described so far suggests that homozygous protein-truncating mutations (nonsense or frameshift) leads to a more severe phenotype, with a reduced visual acuity at a young age (20/100 around the age of 10 years), whereas the patient with a compound heterozygous frameshift/missense mutation showed a relatively preserved visual acuity (20/30 at age 15).¹

The phenotypical similarities in patients carrying protein-truncating mutations do not correspond with the different findings in mRNA expression levels found in Zeitz et al.¹ and in our study. Quantitative PCR analysis of the previously described mutations revealed a 30% to 40% residual amount of *CABP4* transcript in both a homozygous and a compound heterozygous patient,¹ despite the fact that the protein-truncating mutation resides in the last exon and therefore is theoretically not susceptible to nonsense mediated mRNA decay.³³⁻³⁵ In view of the significant amounts of *CABP4* mRNA in both patients, these mutations could not be conclusively classified as null mutations. The mutation described in our study results in the truncation of the 59 (22%) most C-terminal amino acids. Based on the location of the p.Arg216X mutation in exon 4, the transcribed mRNA should theoretically be degraded through nonsense-mediated mRNA decay.³³⁻³⁵ Surprisingly, no difference in expression levels of *CABP4* was found in cDNA of our patients compared with cDNA of control individuals, indicating that NMD does not occur and suggesting the presence of *CABP4* transcripts. To explain why different amounts of *CABP4* mRNA can result in comparable retinal phenotypes, we hypothesize that the differentially truncated CaBP4 proteins both are degraded, or present in a nonfunctional form. Genotype-phenotype correlations based on EBV-LCL mRNA quantification, however, should be interpreted with caution, because *CABP4* mRNA stability may be different in peripheral blood

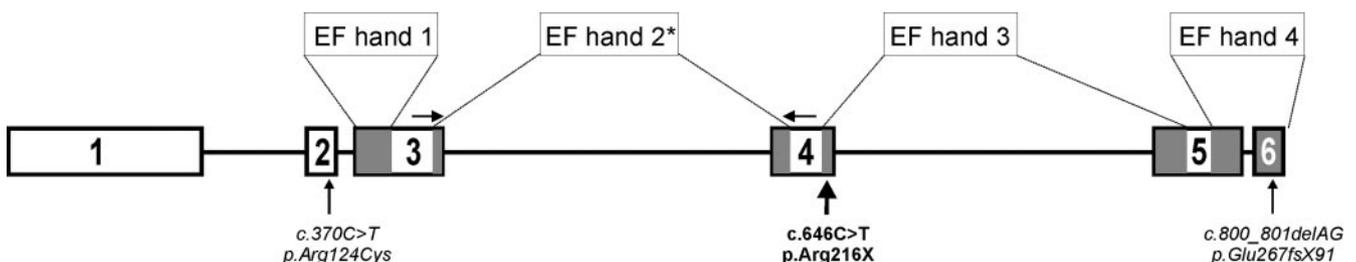


FIGURE 4. The *CABP4* gene. *Boxes*: exons; *lines*: introns; *gray boxes*: parts of the gene that encode the EF hands, the calcium-binding elements in CaBP4; *arrows*: the location of primers used for quantitative PCR analysis. In bold is the mutation described in this article and in italic are the previously identified mutations in *CABP4*.¹ *EF hand 2 is not functional.

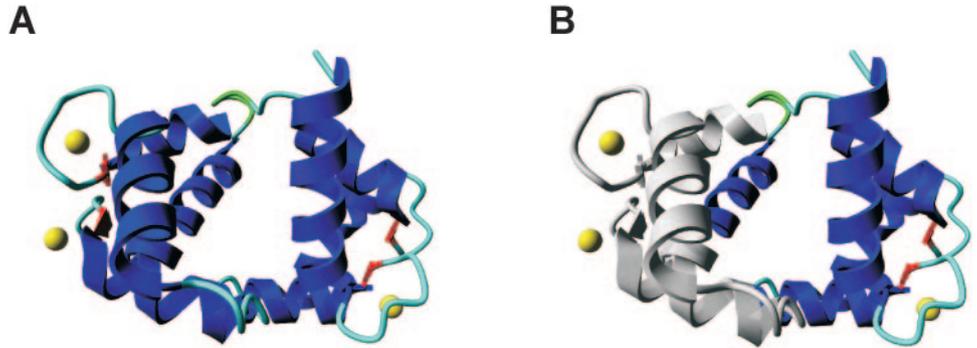


FIGURE 5. CaBP4 protein model. **(A)** Ribbon model of the calcium binding domains in normal CaBP4. **(B)** Model showing the deleted calcium binding domains (gray). Yellow balls: calcium atoms.

cells versus retinal cells, and because *CABP4* mRNA levels in EBV-LCLs are quite low.

CaBP4 is expressed in the photoreceptor synaptic terminals, both in rods and cones, where it colocalizes and interacts with the α_1 -subunit of the L-type voltage-dependent calcium channels $Ca_v1.3$ ^{39–41} and $Ca_v1.4$.¹⁵ Although $Ca_v1.4$ channels are localized in the synaptic terminal of both cone and rod photoreceptors, $Ca_v1.3$ channels are only localized in the synaptic terminal of the cones.³⁸ On binding with these L-type voltage-dependent calcium channels, CaBP4 increases Ca^{2+} influx into the synapse, which increases the amount of neurotransmitter release.¹⁵ The effect of CaBP4 on $Ca_v1.3$ and $Ca_v1.4$ channels, resulting in the maintenance of calcium influx through the channel, is achieved in different ways. In $Ca_v1.3$ channels CaBP4 inhibits a mechanism called calcium-dependent inactivation, a negative feedback mechanism activated by calmodulin that rapidly inactivates calcium channels when intracellular calcium concentrations are elevated.^{40,42} $Ca_v1.4$ channels are not subject to this mechanism, but it has been suggested that CaBP4 increases calcium influx through $Ca_v1.4$ channels by shifting the channels to a hyperpolarized voltage.¹⁵ Although CaBP4 interacts differently with the $Ca_v1.3$ and $Ca_v1.4$ channels and distribution of $Ca_v1.3$ and $Ca_v1.4$ channels differs among the synaptic terminals of rods and cones, it remains speculative why dysfunctional human CaBP4 has a stronger effect on cone function than on rod function. In our young patients with short follow-up no definite conclusions about progression can be drawn. However, a progressive course of the disease was suggested by the fact that all three patients described by Zeitz et al.¹ had slowly progressive visual loss. Furthermore, more pronounced changes in the outer plexiform layer were seen in 6- to 8-months-old *Cabp4*^{-/-} mice than in 2-month-old mice. The presence of both stationary and progressive phenotypes caused by mutations in one gene has been described for mutations in *CACNA1F*, causing both X-linked CSNB2 and progressive cone-rod dystrophy.³² The absence of *CABP4* mutations in 85 patients from The Netherlands affected with cone or cone-rod dystrophy suggests that *CABP4* mutations are not a major cause for these progressive retinal dystrophies. Of interest, a slowly progressive form of autosomal recessive cone dystrophy has been described, with a rather similar, but milder phenotype than patients carrying mutations in *CABP4*. This disorder is caused by nonsense mutations in the *CACNA2D4* gene, which encodes the $\alpha_2\delta$ -subunit of L-type voltage-dependent calcium channels. These patients presented with increasing photophobia and mildly decreasing visual acuity starting around age 30, and no night blindness. Their ERG showed mildly reduced isolated rod responses, electronegative mixed rod-cone responses, markedly diminished cone responses and the wave separation phenomenon at 30-Hz flicker stimulation.⁴³ Thus, genes involved in the process of calcium influx in the synaptic terminal seem to lead to a spectrum of phenotypes affecting

predominantly cones (*CACNA2D4* and *CABP4*)^{1,43} or both rods and cones (*CACNA1F*)^{6,11} in either a stationary or slowly progressive course.

In summary, we describe a novel homozygous nonsense mutation in *CABP4* in two siblings with a phenotype in which cones are predominantly affected. Based on the electronegative mixed rod-cone responses on ERG, the disorder has previously been classified as a form of CSNB2. However, four of five patients described with mutations in *CABP4* do not experience night blindness, and a stationary course of the disease is not completely evident. Furthermore, the clinical characteristics are more in agreement with a form of cone dystrophy, like the phenotype associated with mutations in *CACNA2D4*.⁴³ Moreover, the dysfunctional protein is localized within the photoreceptor synaptic terminal. Therefore, we propose to rename this phenotype congenital cone-rod synaptic disorder.

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References

1. Zeitz C, Kloeckener-Gruissem B, Forster U, et al. Mutations in *CABP4*, the gene encoding the Ca²⁺-binding protein 4, cause autosomal recessive night blindness. *Am J Hum Genet.* 2006; 79(4):657–667.
2. Carr RE. Congenital stationary nightblindness. *Trans Am Ophthalmol Soc.* 1974;72:448–487.
3. Dryja TP, Berson EL, Rao VR, Oprian DD. Heterozygous missense mutation in the rhodopsin gene as a cause of congenital stationary night blindness. *Nat Genet.* 1993;4(3):280–283.
4. Gal A, Orth U, Baehr W, Schwinger E, Rosenberg T. Heterozygous missense mutation in the rod cGMP phosphodiesterase beta-subunit gene in autosomal dominant stationary night blindness. *Nat Genet.* 1994;7(4):551.
5. Dryja TP, Hahn LB, Reboul T, Arnaud B. Missense mutation in the gene encoding the alpha subunit of rod transducin in the Nougaret form of congenital stationary night blindness. *Nat Genet.* 1996; 13(3):358–360.
6. Bech-Hansen NT, Naylor MJ, Maybaum TA, et al. Loss-of-function mutations in a calcium-channel alpha1-subunit gene in Xp11.23 cause incomplete X-linked congenital stationary night blindness. *Nat Genet.* 1998;19(3):264–267.
7. Bech-Hansen NT, Naylor MJ, Maybaum TA, et al. Mutations in *NYX*, encoding the leucine-rich proteoglycan nyctalopin, cause X-linked complete congenital stationary night blindness. *Nat Genet.* 2000; 26(3):319–323.
8. Yamamoto S, Sippel KC, Berson EL, Dryja TP. Defects in the rhodopsin kinase gene in the Oguchi form of stationary night blindness. *Nat Genet.* 1997;15(2):175–178.
9. Dryja TP, McGee TL, Berson EL, et al. Night blindness and abnormal cone electroretinogram ON responses in patients with muta-

- tions in the GRM6 gene encoding mGluR6. *Proc Natl Acad Sci U S A*. 2005;102(13):4884-4889.
10. Fuchs S, Nakazawa M, Maw M, Tamai M, Oguchi Y, Gal A. A homozygous 1-base pair deletion in the arrestin gene is a frequent cause of Oguchi disease in Japanese. *Nat Genet*. 1995;10(3):360-362.
 11. Strom TM, Nyakatura G, Apfelstedt-Sylla E, et al. An L-type calcium-channel gene mutated in incomplete X-linked congenital stationary night blindness. *Nat Genet*. 1998;19(3):260-263.
 12. Miyake Y, Yagasaki K, Horiguchi M, Kawase Y, Kanda T. Congenital stationary night blindness with negative electroretinogram: a new classification. *Arch Ophthalmol*. 1986;104(7):1013-1020.
 13. Miyake Y, Horiguchi M, Ota I, Shiroyama N. Characteristic ERG-flicker anomaly in incomplete congenital stationary night blindness. *Invest Ophthalmol Vis Sci*. 1987;28(11):1816-1823.
 14. Zeitz C, van Genderen M, Neidhardt J, et al. Mutations in GRM6 cause autosomal recessive congenital stationary night blindness with a distinctive scotopic 15-Hz flicker electroretinogram. *Invest Ophthalmol Vis Sci*. 2005;46(11):4328-4335.
 15. Haeseleer F, Imanishi Y, Maeda T, et al. Essential role of Ca²⁺-binding protein 4, a Cav1.4 channel regulator, in photoreceptor synaptic function. *Nat Neurosci*. 2004;7(10):1079-1087.
 16. Marmor MF, Holder GE, Seeliger MW, Yamamoto S. Standard for clinical electroretinography (2004 update). *Doc Ophthalmol*. 2004;108(2):107-114.
 17. Miller SA, Dykes DD, Polesky HF. A simple salting out procedure for extracting DNA from human nucleated cells. *Nucleic Acids Res*. 1988;16(3):1215.
 18. Nannya Y, Sanada M, Nakazaki K, et al. A robust algorithm for copy number detection using high-density oligonucleotide single nucleotide polymorphism genotyping arrays. *Cancer Res*. 2005;65(14):6071-6079.
 19. Rozen S, Skaletsky H. Primer3 on the WWW for general users and for biologist programmers. *Methods Mol Biol*. 2000;132:365-386.
 20. Little S. Amplification-refractory mutation system (ARMS) analysis of point mutations. *Curr Protoc Hum Genet*. 2001;Chapter 9:Unit 9.8.
 21. Jaakson K, Zernant J, Kulm M, et al. Genotyping microarray (gene chip) for the ABCR (ABCA4) gene. *Hum Mutat*. 2003;22(5):395-403.
 22. Wall FE, Henkel RD, Stern MP, Jenson HB, Moyer MP. An efficient method for routine Epstein-Barr virus immortalization of human B lymphocytes. *In Vitro Cell Dev Biol Anim*. 1995;31(2):156-159.
 23. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. *Methods*. 2001;25(4):402-408.
 24. Pfaffl MW. A new mathematical model for relative quantification in real-time RT-PCR. *Nucleic Acids Res*. 2001;29(9):e45.
 25. de Brouwer AP, van Bokhoven H, Kremer H. Comparison of 12 reference genes for normalization of gene expression levels in Epstein-Barr virus-transformed lymphoblastoid cell lines and fibroblasts. *Mol Diagn Ther*. 2006;10(3):197-204.
 26. Freude K, Hoffmann K, Jensen LR, et al. Mutations in the FTSJ1 gene coding for a novel S-adenosylmethionine-binding protein cause nonsyndromic X-linked mental retardation. *Am J Hum Genet*. 2004;75(2):305-309.
 27. Haeseleer F, Sokal I, Verlinde CL, et al. Five members of a novel Ca²⁺-binding protein (CABP) subfamily with similarity to calmodulin. *J Biol Chem*. 2000;275(2):1247-1260.
 28. Vriend G. WHAT IF: a molecular modeling and drug design program. *J Mol Graph*. 1990;8(1):52-6, 29.
 29. Krieger E, Koraimann G, Vriend G. Increasing the precision of comparative models with YASARA NOVA: a self-parameterizing force field. *Proteins*. 2002;47(3):393-402.
 30. van Genderen M, Riemsdag F, Jorritsma F, Hoeben F, Meire F, Stilma J. The key role of electrophysiology in the diagnosis of visually impaired children. *Acta Ophthalmol Scand*. 2006;84(6):799-806.
 31. Scholl HP, Langrova H, Weber BH, Zrenner E, Apfelstedt-Sylla E. Clinical electrophysiology of two rod pathways: normative values and clinical application. *Graefes Arch Clin Exp Ophthalmol*. 2001;239(2):71-80.
 32. Jalkanen R, Mantyjarvi M, Tobias R, et al. X linked cone-rod dystrophy, CORDX3, is caused by a mutation in the CACNA1F gene. *J Med Genet*. 2006;43(8):699-704.
 33. Nagy E, Maquat LE. A rule for termination-codon position within intron-containing genes: when nonsense affects RNA abundance. *Trends Biochem Sci*. 1998;23(6):198-199.
 34. Cheng J, Belgrader P, Zhou X, Maquat LE. Introns are cis effectors of the nonsense-codon-mediated reduction in nuclear mRNA abundance. *Mol Cell Biol*. 1994;14(9):6317-6325.
 35. Zhang J, Sun X, Qian Y, Maquat LE. Intron function in the nonsense-mediated decay of beta-globin mRNA: indications that pre-mRNA splicing in the nucleus can influence mRNA translation in the cytoplasm. *RNA*. 1998;4(7):801-815.
 36. Forsén S, Vogel HJ, Drakenberg T. Biophysical studies of calmodulin. In: Chung E, ed. *Calcium and Cell Function*. Vol. 6. San Diego, CA: Academic Press; 1986;6:113-157.
 37. Wassle H. Parallel processing in the mammalian retina. *Nat Rev Neurosci*. 2004;5(10):747-757.
 38. Morgans CW, Bayley PR, Oesch NW, Ren G, Akileswaran L, Taylor WR. Photoreceptor calcium channels: insight from night blindness. *Vis Neurosci*. 2005;22(5):561-568.
 39. Yang PS, Alseikhan BA, Hiel H, et al. Switching of Ca²⁺-dependent inactivation of Ca (v) 1.3 channels by calcium binding proteins of auditory hair cells. *J Neurosci*. 2006;26(42):10677-10689.
 40. Cui G, Meyer AC, Calin-Jageman I, et al. Ca²⁺-binding proteins tune Ca²⁺-feedback to Cav1.3 channels in mouse auditory hair cells. *J Physiol*. 2007;585:791-803.
 41. Lee A, Jimenez A, Cui G, Haeseleer F. Phosphorylation of the Ca²⁺-binding protein CaBP4 by protein kinase C zeta in photoreceptors. *J Neurosci*. 2007;27(46):12743-12754.
 42. Budde T, Meuth S, Pape HC. Calcium-dependent inactivation of neuronal calcium channels. *Nat Rev Neurosci*. 2002;3(11):873-883.
 43. Wycisk KA, Zeitz C, Feil S, et al. Mutation in the auxiliary calcium-channel subunit CACNA2D4 causes autosomal recessive cone dystrophy. *Am J Hum Genet*. 2006;79(5):973-977.