LETTER TO JMG

X linked cone-rod dystrophy, CORDX3, is caused by a mutation in the *CACNA1F* gene

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Background: X linked cone-rod dystrophy (CORDX) is a recessive retinal disease characterised by progressive dysfunction of photoreceptors. It is genetically heterogeneous, showing linkage to three X chromosomal loci. CORDX1 is caused by mutations in the *RPGR* gene (Xp21.1), CORDX2 is located on Xq27.2-28, and we recently localised CORDX3 to Xp11.4-q13.1. We aimed to identify the causative gene behind the CORDX3 phenotype.

Methods: All 48 exons of the *CACNA1F* gene were screened for mutations by DNA sequencing. RNA from cultured lymphoblasts and peripheral blood activated T lymphocytes was analysed by RT-PCR and sequencing.

Results: A novel *CACNA1F* mutation, IVS28-1 GCGTC>TGG, in the splice acceptor site of intron 28 was identified. Messenger RNA studies indicated that the identified mutation leads to altered splicing of the *CACNA1F* transcript. Aberrant splice variants are predicted to result in premature termination and deletions of the encoded protein, $Ca_v1.4$ α_1 subunit.

Conclusion: *CACNA1F* mutations cause the retinal disorder, incomplete congenital stationary night blindness (CSNB2), although mutations have also been detected in patients with divergent diagnoses. Our results indicate that yet another phenotype, CORDX3, is caused by a mutation in *CACNA1F*. Clinically, CORDX3 shares some features with CSNB2 but is distinguishable from CSNB2 in that it is progressive, can begin in adulthood, has no nystagmus or hyperopic refraction, has only low grade astigmatism, and in dark adaptation lacks cone threshold and has small or no elevation of rod threshold. Considering all features, CORDX3 is more similar to other X chromosomal cone-rod dystrophies than to CSNB2.

X linked cone-rod dystrophy (CORDX) is a progressive retinal disease primarily showing cone photoreceptor dysfunction. The disease is characterised by diminished visual acuity, photophobia, myopia, central scotomas in visual fields, impaired colour vision, and disturbed cone or cone-rod responses in electroretinogram (ERG).[1–6] Fundus findings vary from normal or subtle granularity of macula, to bull's eye maculopathy and central geographic atrophy of retinal pigment epithelium. In some patients tapetal-like retinal sheen has also been observed.[1 2 7] The disease begins usually in the first two decades of life and progresses gradually; however, there is intrafamilial variation with respect to age of onset and severity of symptoms.[1 6] CORDX is genetically heterogeneous and three gene loci have been identified to date. *CORDX1* (MIM 304020), located in Xp21.1, is caused by mutations in the ORF15 of the *RPGR* gene, which is also known to be the causative gene for RP3 type retinitis

pigmentosa[8–10] and atrophic macular degeneration.[11] *CORDX2* (MIM 300085) is localised to the long arm of the X chromosome (Xq27.2–28), but the causative gene is still unknown.[12] We recently mapped the third X linked cone-rod dystrophy locus, *CORDX3* (MIM 300476), to Xp11.4–q13.1.[13]

CORDX has some similarities with X linked congenital stationary night blindness (CSNBX). CSNBX is considered to be a non-progressive retinal disorder characterised by a negative ERG, that is, b wave amplitude is smaller than the a wave in a mixed rod-cone response.[14] CSNBX is both clinically and genetically heterogeneous. The major clinical features are variable and may include life long impairment of night vision, myopia, nystagmus, strabismus, and reduced visual acuity; however, there is a wide intra- and interfamilial variation in the symptoms.[15–17] According to the ERG findings, CSNBX can be subdivided into a complete type (type 1, CSNB1) and an incomplete type (type 2, CSNB2).[18–20] The causative genes of CSNBX have recently been identified: CSNB1 (MIM 310500, Xp11.4) is caused by mutations in *NYX*,[21 22] and CSNB2 (MIM 300071, Xp11.23) results from mutations in the calcium channel α_1 subunit gene, *CACNA1F*.[23 24]

The *CACNA1F* gene (MIM 300110) consists of 48 exons spanning a region of 28 kb in Xp11.23.[23 24] The *CACNA1F* protein product, $Ca_v1.4/\alpha_{1F}$, shows strong homology to α_1 subunits of the voltage dependent L-type calcium channels (VDCCs).[23 24] VDCCs are hetero-oligomeric complexes composed of pore forming α_1 subunit accompanied by modulating subunits $\alpha_2\delta$ and β, and variably by γ.[25] In neuroretina, three different VDCC α_1 subunits have been detected: $Ca_v1.2$ (α_{1C}, encoded by *CACNA1C*), $Ca_v1.3$ (α_{1D}, *CACNA1D*), and $Ca_v1.4$ (α_{1F}, *CACNA1F*).[26 27]

By immunofluorescent staining of rat retina sections, $Ca_v1.4$ has been localised to the outer plexiform layer, and in lesser amounts to the inner plexiform layer and the outer nuclear layer.[26] In the outer plexiform layer, $Ca_v1.4$ localises to rod and possibly to cone active zones, implicating these channels in the release of glutamate from photoreceptor synaptic terminals. The biophysical properties of $Ca_v1.4$ channels make them especially suitable for mediating tonic neurotransmitter release in sensory cells.[28 29] Besides its retinal expression,[23 24] *CACNA1F* is also expressed in bone marrow, thymus, adrenal gland, skeletal muscle,[29] and T cells,[30] suggesting a wider role of $Ca_v1.4$ in human physiology.

As *CACNA1F* maps within the Xp11.4-q13.1 candidate gene region of CORDX3,[13] we sequenced this gene in members of a large Finnish family with cone-rod dystrophy, CORDX3.

METHODS
Subjects
Blood samples were collected from family members of a large Finnish CORDX3 family.[13] A complete pedigree and clinical studies of the family members have been published earlier.[6] The research followed the tenets of the Declaration of Helsinki. Informed consent was obtained from all participants in accordance with the requirements of the University of Kuopio Ethics Committee. DNA samples from 50 unrelated Canadian females and 100 Finnish male blood donors were used as normal controls.

DNA analysis
All 48 exons of the *CACNA1F* gene were PCR amplified from genomic DNA using published primer sequences.[31] PCR products were run on agarose gels, gel purified, and then sequenced with either a forward or a reverse primer. *Fnu*4HI (New England Biolabs, Beverly, MA) restriction endonuclease site analysis was used to study the segregation of the identified sequence alteration in the CORDX3 family and normal controls.

RNA analysis
The effect of the mutation on *CACNA1F* mRNA was studied by RT-PCR and cDNA sequencing. RNA was extracted from lymphoblastoid cell lines of two CORDX3 patients and one unrelated control using the RNeasy Mini Kit (Qiagen, Hilden, Germany). Complementary DNA was synthesised using M-MLV (H-) RT enzyme and random 6-mer primers (Promega, Madison WI) according to manufacturer's instructions. Exons surrounding the mutation were amplified from cDNA with forward primers located in exons 27 and 28, and reverse primers in exons 30, 31, and 34 (primer sequences and PCR conditions are available upon request). As these amplifications yielded multiple products, three different approaches were used to separate different sized PCR products for sequencing. First, amplified fragments were separated in 1–2% MetaPhor agarose (BioWhittaker Molecular Applications, Rockland, ME), excised, and column purified with the QIAquick Gel Extraction Kit (Qiagen). Second, amplified fragments were separated in a 6% polyacrylamide gel, after which silver stained[32] bands were excised and dissolved in 10 mM Tris-HCl pH 8.5 overnight on a shaker. Extracted bands were re-amplified and column purified using the QIAquick PCR Purification Kit (Qiagen). Third, amplified PCR fragments were cloned using the TOPO TA Cloning Kit (Invitrogen, Carlsbad, CA), and plasmids were extracted using the Plasmid Mini Kit (Qiagen). Purified fragments and plasmids were sequenced using the ABI PRISM BigDye Terminator v3.1 Cycle Sequencing Kit and the ABI310 Genetic Analyzer (Applied Biosystems, Foster City, CA).

Peripheral activated T lymphocytes were isolated from 15 ml of blood from one affected male, one carrier female, and one unrelated control, and cultured using the method of Kotturi and co-workers,[30] briefly described here. The separated peripheral blood mononuclear cells (PBMCs) were washed in PBS and resuspended in RPMI, 10% fetal calf serum, 2 mM glutamine, 20 mM HEPES, and 1 mM sodium pyruvate, followed by 24 h stimulation with plate-bound anti-CD3 antibody. The PBMCs were washed and resuspended in RPMI supplemented with 5 ng/µl recombinant human interleukin-2 for 14 days. Reverse transcription analysis was performed on total RNA extracted from activated peripheral blood T cells on days 11–14 using Trizol Reagent (Invitrogen, Burlington, ON). Isolated RNA was treated with the MessageClean Kit (GenHunter, Nashville, TN) to remove contaminating DNA, and then used as the template for first strand cDNA synthesis using the

Omniscript RT Kit (Qiagen). A touchdown PCR protocol was used in RT-PCR with sense primer (exon 28 TACCGTGTGGGATCTCAGTGT) and anti-sense primer (exon 33 CGGATCCCTTCCCCTTACT). The resulting PCR products were separated on 1.5% agarose gels. Respective bands were excised, purified using the Gel Purification Kit (Qiagen), and sequenced with sense and anti-sense primers, as described in the previous paragraph.

RESULTS
Mutation analysis
DNA sequence analysis of *CACNA1F* in a patient from our CORDX3 family revealed a novel mutation, IVS28-1 GCGTC>TGG, in the splice acceptor site of intron 28 (fig 1). This *CACNA1F* mutation co-segregated completely with the disease phenotype in the CORDX3 family (seven affected males, 10 carrier females, 33 non-affected family members) but was not observed in 200 control chromosomes.

RNA studies in lymphoblastoid cells from CORDX3 patients revealed no normal transcripts but several aberrant *CACNA1F* splice variants. Using either an exon 27 or 28 forward primer and exon 30 or 31 reverse primer, five different splice variants were detected in patient samples (fig 2A,B), which were not observed in a control sample. With exon 28 forward primer and exon 34 reverse primer two additional splice variants were observed, namely the skipping of exon 32 and skipping of exons 31 and 32. Both were also identified in a control sample. Analysis of RNA from activated T cells of an affected male and a carrier female identified two of the aberrant splice variants (d and f, fig 2B), while the skipping of exon 32 was observed in RNA from both the patient and a control sample.

Predicted consequences of the identified *CACNA1F* splice site mutation on the Ca$_v$1.4 protein are illustrated in fig 2C. Two of the observed abnormal splice variants (b and f) contain different premature stop codons in the cytoplasmic
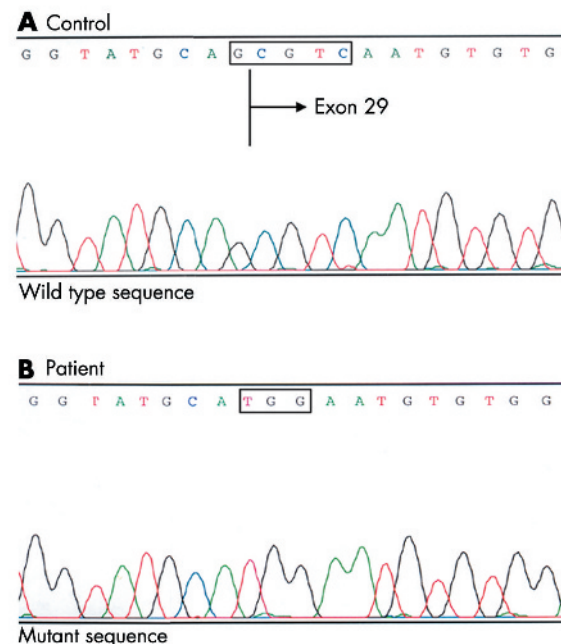

Figure 1 Electropherogram of the sense strand of an amplified genomic DNA fragment of *CACNA1F* from a CORDX3 patient. The change, IVS28-1 GCGTC>TGG in *CACNA1F*, was present in the DNA of the patient (B) and absent in the normal control DNA (A).
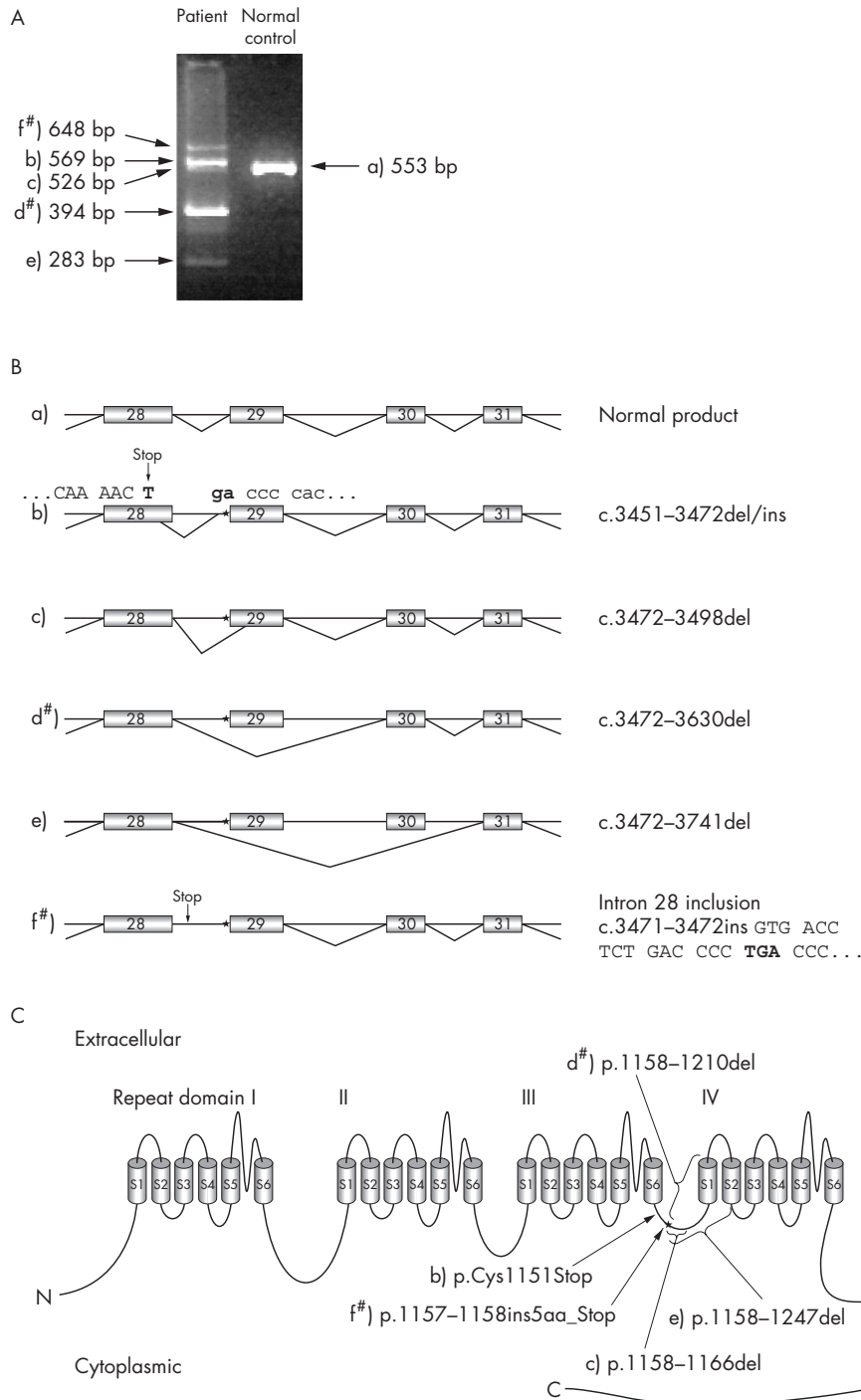
Figure 2 Analysis of transcripts involving the exon 28–31 region of *CACNA1F*. (A) RT-PCR analysis of lymphoblast cells from a CORDX3 patient and unrelated control. (B) Normal splicing product (a), and five different outcomes of the *CACNA1F* splice site mutation, IVS28-1 GCGTC>TGG, in CORDX3 patients: b) use of alternative splice donor site within exon 28 and alternate splice acceptor in intron 28, c) use of canonic intron 28 donor splice site and alternative acceptor splice site in exon 29, d) skipping of exon 29, e) skipping of exons 29 and 30, and f) no splicing, in which case intron 28 is retained in mRNA. The splice variants that were also seen in activated T cells are indicated by #. An asterisk denotes the location of the mutation. (C) The putative membrane topology of the human L-type calcium channel Ca$_v$1.4 α_1 subunit encoded by *CACNA1F*. The protein is composed of four repeat domains (I–IV), each containing six transmembrane segments (S1–S6), and connecting intra- and extracellular loops. The predicted consequences of the identified splice mutation, IVS28-1 GCGTC>TGG, on the protein are shown. Mutation numbering is according to NCBI nucleotide and protein sequences, NM_005183 and NP_005174, respectively.

linker region connecting the repeat domains III and IV, which result in truncation of the Ca$_v$1.4 protein. The other three aberrant transcript variants (c, d, and e) predict variably sized deletions in the domain III–IV linker region and the IVS1–S2 transmembrane region.

DISCUSSION

In this study, we identified the causative gene for X linked cone-rod dystrophy, CORDX3. The novel *CACNA1F* gene mutation, IVS28-1 GCGTC>TGG, destroys the splice acceptor site of intron 28 leading to altered splicing of the *CACNA1F*

Table 1 Clinical characteristics of X chromosomal cone-rod dystrophies and CSNB2

	CORDX3	CSNB2	X chromosomal cone-rod dystrophy (CORDX1, CORDX2)
	Mäntyjärvi et al[6]	Pearce et al[16] Tremblay et al[18] Bech-Hansen et al[23] Boycott et al[15] Nakamura et al[41] Langrova et al[49] Allen et al[19] Jacobi et al[40]	Pinckers and Timmerman[3] Jacobson et al[2] Kellner and Foerster[50] Meire et al[4] Hong et al[5] Bergen and Pinckers[12] Brown et al[1]
1. Onset	As child, adult	Congenital	As child, adult
2. Progression	VA, refraction, VF, colour defect	Stationary	VA, refraction, VF, colour defect
3. VA (range)	20/300–20/40	20/400–20/25	20/800–20/15
4. Refraction (range)	−1 to −24 D	+8 to −18.25 D, mostly myopia	+3.25 to −20 D, mostly myopia
5. Astigmatism >1.5 D (eyes)	0/20	137/266, up to 5.5 D	9/32, up to 3 D
6. Congenital nystagmus (patients)	0/10	67/139	Rare
7. Ocular fundus	Normal, myopic changes	Normal, hypopigmentation, no foveal reflex, tilted disc, pale disc, myopic changes	Normal, macular atrophy, ''bull's eye'', hypopigmentation, myopic changes
8. Colour vision	Normal, protan	Normal, tritan, non-specific defect	Tritan, deutan, protan, achromatopsia
9. VF	Normal, central scotoma, central reduced sensitivity, concentric constriction	Normal, central scotoma	Normal, central scotoma, paracentral scotoma, reduced central, paracentral sensitivity
10. Dark adaptation	Cone threshold missing or elevated, rod threshold normal or elevated <1 log unit	Cone threshold elevated, rod threshold elevated 0.5–3 log units	Cone threshold missing or elevated, rod threshold normal or elevated 1 log unit
11. ERG	Negative ERG, 30 Hz flicker decreased	Negative ERG, 30 Hz flicker abnormal/decreased with double-peaked wave	Scotopic a and b wave decreased, photopic b wave decreased, 30 Hz flicker decreased, negative ERG possible

ERG, electroretinogram; D, diopter; VA, visual acuity; VF, visual field.

transcript. We detected five aberrant splice variants in lymphoblast RNA from CORDX3 patients. These variants are predicted to cause variably sized deletions of the $Ca_v1.4$ protein or truncation of the C-terminal part of the protein (fig 2) and are thought to underlie the pathophysiology in the patients from this Finnish family with CORDX3. A recent study of transient expression of *CACNA1F* mutants in human embryonic kidney cells demonstrated that truncation of the cytoplasmic C terminus of $Ca_v1.4$ leads to absence of the protein, indicating that the C-terminal tail has an important role in protein processing and targeting.[33] Moreover, some missense mutations have been shown to cause notable changes in channel gating, or to even completely prevent channel function.[33][34] Based on these data, it is highly likely that the splice site mutation we observed in the Finnish CORDX3 patients results in total absence or significantly altered function of the $Ca_v1.4$ channel.

Boycott and co-workers[31] previously described alternative splicing for the exons 1, 2, and 9 of the *CACNA1F* gene. In this study we also identified mutation independent splice variants, in which exon 32 or exons 31 and 32 were excised. Exon 32 codes for a short sequence, NGGHLGE, constituting part of the loop between the domain IVS3 and S4 segments. Similar splice variants, where a short exon encoding part of this loop is spliced out, have also been described for other L-type calcium channels, $Ca_v1.1$, $Ca_v1.2$, and $Ca_v1.3$.[35–38] The splice variant missing both exons 31 and 32 leads to a deletion of the IVS3 transmembrane segment and part of the IVS3–S4 linker region of the protein, suggesting that the membrane topology of the C-terminal part of the protein is altered. A similar splice variant has been identified for the $Ca_v1.3$ channel as well.[36] Moreover, extensive splice variation has been detected within exons 31–34 that encode the IVS3–S4 region of the $Ca_v1.2$ protein.[39] In the light of these observations, it seems that alternative splicing of L-type calcium channels is a common phenomenon and results in increased channel variability, although the functional significance of such variation is not yet understood.

Over 60 unique *CACNA1F* mutations, including 10 in splice acceptor and donor sites, have been described to date. Most of the mutations have been identified in patients with a CSNB2 phenotype.[23][24][31][40–44] However, some of the patients carrying a *CACNA1F* mutation have overlapping but sometimes divergent diagnoses: AIED-like phenotype,[42][45] CSNB2 with atypical retinal atrophy and visual field defects,[46] retinal and optic disc atrophy with progressive decline of visual function,[47] and severe CSNB2-like phenotype associated with female carrier symptoms and intellectual disability.[48] The present study indicates that yet another phenotype, CORDX3, is also caused by a mutation in the *CACNA1F* gene.

Clinically, CORDX3 and CSNB2 have some features in common, such as the range of visual acuities, myopic refraction, and the ERG abnormalities (table 1). In some features CORDX3 is different from CSNB2. The onset of CORDX3 varies from childhood (3 years) to adulthood (33 years), and the disease is progressive (visual acuity, refraction, colour vision and visual field), whereas CSNB2 is considered to be stationary and severe cases are usually observed early in life. Congenital nystagmus, hyperopic refraction, and astigmatism >1.5 D are not found in CORDX3 patients but are not infrequent in CSNB2. Ocular fundus changes, other than myopic changes, are not seen in CORDX3 patients, while both retinal and optic disk changes have been reported in several CSNB2 patients. In dark adaptation, CORDX3 patients have elevated or missing cone threshold and normal or only slightly elevated rod threshold.

In CSNB2, the cone threshold is elevated but not missing, and the rod threshold is variably elevated, from 1 to 3 log units. CORDX3 has many clinical features similar to other X chromosomal cone-rod dystrophies (table 1), but lacks their tendency to hyperopia and astigmatism >1.5 D and has different ocular fundus changes.

The phenotype described in a Maori family with a *CACNA1F* mutation shares several features with CSNB2, but is considered a distinct clinical entity due to the severity of the phenotype and the presence of intellectual impairment in several male patients.[48] Moreover, all carrier females in this family had clinical and ERG abnormalities. Patients with a *CACNA1F* mutation and retinal and optic disk atrophy described by Nakamura *et al*[47] also have some similarities with CSNB2. However, their phenotype included the distinctive features of retinal atrophy with attenuated vessels, optic disc atrophy, and progressive decline of visual function. A family described as having AIED-like disease[45] has upon re-evaluation of the clinical features been rediagnosed as having CSNB2.[42]

Mutations in *CACNA1F* are evidently present in patients with different clinical diagnoses[45–48] including (as we show here) cone-rod dystrophy. Invariably, the phenotypic features in these conditions overlap those of CSNB2, the disorder in which *CACNA1F* mutations were originally identified.[23 24] The observation that mutations in one gene can lead to different phenotypes is not uncommon among retinal diseases, as is seen, for example, in the case of the *RPGR* gene[8–11] and peripherin/*RDS* gene (see Retina International's Mutation Databases: http://www.retina-international.org/sci-news/mutation.htm). The differences observed in the phenotypes resulting from *CACNA1F* mutations may reflect alterations in Ca$_v$1.4 channel function,[34] together with the influence of modifying genes from the different genetic backgrounds of the patients, as previously suggested.[15] The recent analysis of a *Cacna1f* knockout mouse has implicated a defect in the formation or maintenance of the synapses between photoreceptors and second order neurons of the retina as being central to the pathophysiology in this mouse model of CSNB2.[51] Further genetic dissection of the molecular biology of synapse formation may assist us in understanding the clinical variability associated with mutations in *CACNA1F*.

In summary, our findings contribute to a better understanding of the phenotypic variability of retinal disorders and the underlying genetic defects. Such information is of potential help in both clinical and molecular genetic diagnostics, and the genetic counselling of patients and families affected by these eye diseases.

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ELECTRONIC-DATABASE INFORMATION

Retina International's Mutation Databases can be found at http://www.retina-international.org/sci-news/mutation.htm

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