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Thirty distinct *CACNA1F* mutations in 33 families with incomplete type of XLCSNB and *Cacna1f* expression profiling in mouse retina

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X-linked CSNB patients may exhibit myopia, nystagmus, strabismus and ERG abnormalities of the Schubert-Bornschein type. We recently identified the retina-specific L-type calcium channel $\alpha 1$ subunit gene *CACNA1F* localised to the Xp11.23 region, which is mutated in families showing the incomplete type (CSNB2). Here, we report comprehensive mutation analyses in the 48 *CACNA1F* exons in 36 families, most of them from Germany. All families were initially diagnosed as having the incomplete type of CSNB, except for two which have been designated as Åland Island eye disease (ÅIED)-like. Out of 33 families, a total of 30 different mutations were identified, of which 24 appear to be unique for the German population. The mutations, 20 of which are published here for the first time, were found to be equally distributed over the entire gene sequence. No mutation could be found in a classic ÅIED family previously shown to map to the CSNB2 interval. *Cacna1f* expression in photoreceptor-negative mice strains indicate that the gene is expressed in the outer nuclear, the inner nuclear, and the ganglion cell layer. Such a distribution points to the central role of calcium regulation in the interaction of retinal cells that mediate signal transmission. *European Journal of Human Genetics* (2002) 10, 449–456. doi:10.1038/sj.ejhg.5200828

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Introduction

X-linked congenital stationary night blindness (XLCSNB; OMIM 310500) is a non-progressive eye disorder exhibiting life-long night vision impairment and variably reduced day vision. Patients with XLCSNB may also suffer from myopia, hyperopia, nystagmus and strabismus. In XLCSNB, the electroretinogram (ERG) is of the Schubert-Bornschein type

thereby allowing for a differentiation into two subtypes, namely the incomplete (ie CSNB2) and the complete phenotype (ie CSNB1).^{1,2} While in CSNB1 patients no post-receptor rod-mediated function is measurable with ERG, some activity is retained in the incomplete subtype. In contrast, cone function is more impaired in patients with the incomplete type, as can be seen in scotopic 30 Hertz flicker responses and dark adaptation tests.^{3–6}

We and others have recently isolated the genes mutated in CSNB1^{7,8} and CSNB2 families.^{9,10} The *CACNA1F* gene, mutated in CSNB2 families, comprises 48 exons and codes for a protein which consists of 1966 amino acids showing high homology

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to α 1-subunits of L-type calcium channels. Its expression is restricted to the retina and initial mutation analyses revealed loss of function as well as missense mutations.⁹

So far, mutation spectra have been published only for mixed populations^{10,11} or few Japanese families.¹² In order to establish a mutation spectrum for the German population and to further support the strong association of the *CACNA1F* gene with *CSNB2*, we completed analysis in 10 families partially characterised previously⁹ and included another 24 *CSNB2* families to the study. Additionally, we analysed the entire coding region of *CACNA1F* in two patients published with an \dot{A} IED-related phenotype, and in the classic \dot{A} IED family.^{13,14} To further understand the molecular pathogenesis of the mutated *CACNA1F* gene, we initiated a refined analysis of the gene expression in two mouse models displaying photoreceptor degeneration.

Results

The majority of *CSNB2* patients harbour mutations in the *CACNA1F* gene

We have recruited 33 non-related XLCSNB families and one sporadic case, diagnosed with the incomplete type of CSNB, based on ERG data and dark adaptation tests. Recently, we

published 10 of these families showing mutations in the *CACNA1F* gene by analysing 38 of the 48 exons.⁹ After completion of the mutation analyses, we identified a recurrent stop mutation (R82X) and a novel missense mutation (L849P) in families R3 and R6 respectively, in addition to a previously identified missense mutation R508Q. Including the newly recruited families, we have identified one insertion mutation, three deletion mutations, eight nonsense mutations, five alterations affecting conserved residues of splice sites, and 13 distinct missense mutations (Table 1).

In family T9 with four affected males we identified a missense mutation (R1171P) and a stop mutation (R1816X) in *CACNA1F*. Recently we also found a predicted missense mutation (V36F) in the *RPGR* gene¹⁵ in this family. The V36F alteration was found to segregate within the family and was not present in 600 controls.¹⁶ All three variants were found in the four affected males but not in controls (Figure 1). A novel missense mutation (C1488R) was identified in family R11 with four males suffering from incomplete CSNB. Sequencing revealed a change at nucleotide position 4462, causing a non-conservative amino acid exchange from cysteine to arginine at amino acid position 1488. The substituted cysteine residue is located in the

Table 1

Family	Base change	Amino acid change	Exon	Type
F40	151del5bp		2	frameshift
S41	220T→C	Cys 74 Arg	2	missense
R3	244C→T	Arg 82 ter ^b	2	stop
R4	244C→T	Arg 82 ter ^b	2	stop
T15	685T→C	Ser 229 Pro	6	missense
T22	781G→A	Gly 261 Arg	6	missense
R2 ^a	1106G→A	Gly 369 Asp	8	missense
T7	1282C→T	Gln 428 ter	10	stop
T2	1840C→T	Arg 614 ter	14	stop
B27	2225T→G	Phe 742 Cys	17	missense
T24	2387-1 (G→C)		20	splice
R6	2546T→C	Leu 849 Pro	21	missense
T10	2673+3 (G→A)		21	splice
T26	2673+3 (G→A)		21	splice
T17	2674-2,3 (delCA)		22	splice
R14 ^a	2872C→T	Arg 958 ter	24	stop
R26	3019G→A	Gly 1007 Arg	25	missense
R13 ^a	3145C→T	Arg 1049 Trp	27	missense
T13	3203T→C	Leu 1068 Pro	27	missense
R22 ^a	3133insC ^b		27	frameshift
R5	3658del12 bp		30	in frame deletion
R25	3862C→T	Arg 1288 ter ^b	33	stop
T12	3942+2(T→C)		33	splice
T6	3942+2 (T→A)		33	splice
R21 ^a	4042C→T	Gln 1348 ter	35	stop
R15 ^a	4091T→A	Leu 1364 His	35	missense
H15	4101-1(G→C)		36	splice
R11	4462T→C	Cys 1488 Arg	38	missense
R29	4466C→G	Pro 1489 Arg	38	missense
R7	4466C→G	Pro 1489 Arg	38	missense
R23	4490T→C	Leu 1497 Pro	39	missense
R10 ^a	4771A→T	Lys 1591 ter	41	stop
T9	5446C→T	Arg 1816 ter	46	stop

^aFamilies reported previously (Strom *et al*⁹); ^bother known mutations.

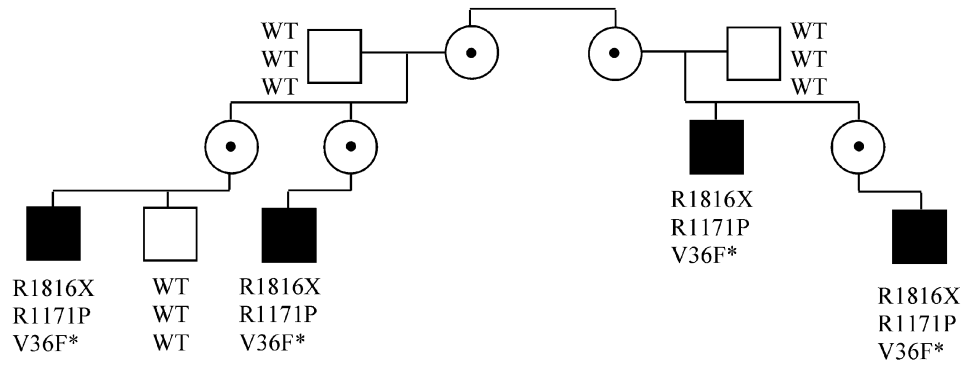


Figure 1 Mutation analysis in CSNB2 family T9. Pedigree of the family with affected males indicated by black boxes and obligate carrier females by a circle with a black dot. All patients and female heterozygotes are carrying both a stop (R1816X) and a missense mutation (R1171P) in *CACNA1F*, together with a missense mutation (V36F*) in *RPGR*. These variants were not present in controls (WT).

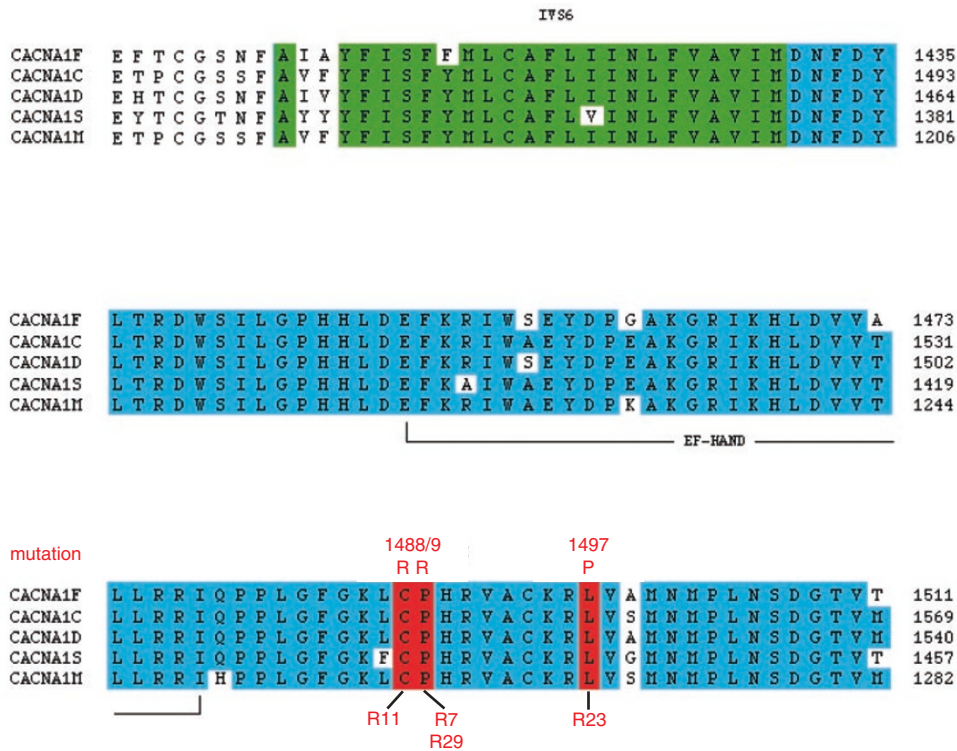


Figure 2 Partial alignment of the *CACNA1F* protein with other human L-type calcium channels. The figure shows the alignment from the respective transmembrane domains IVS6 (green) and the carboxyterminal cytoplasmic domain (blue). Non-shaded alignment positions indicate amino acids that are divergent from the overall *CACNA1* consensus of the illustrated region. Conserved positions where three different missense mutations reside (in family R11 with C1488R, families R29 and R7 with P1489R, and family R23 with L1497P) are highlighted by red-shaded boxes. The putatively affected EF-hand motif is put into brackets.

cytoplasmic domain of the protein, similar to the two other novel missense mutations P1489R and L1497P (Table 1). These mutations may alter the calcium ion-binding site which is formed by the adjacent EF hand motif¹⁷ (Figure 2), although consequences for the interaction with other proteins cannot be excluded.

Other non-conservative amino acid changes were found at position 229 in family T15, at position 261 in family T22 (both exon 6), and at amino acid position 1068 (family T13). Mutations S229P and G261R reside in the amino-terminal and carboxy-terminal intracellular loop, respectively, located between transmembrane segments ISS and

IS6. Mutation 3203T>C in family T13 results in a non-conservative leucine to proline change at peptide position 1068. This region belongs to a highly conserved domain in the center of the intracellular loop connecting *CACNA1F* transmembrane segments IIIS5 and IIIS6 that both carry dihydropyridine interaction residues. Remarkable conservation can be observed between the related L-type calcium channels *CACNA1D*, *CACNA1C* and *CACNA1S* in the region from amino acid position 1058–1085. None of these missense mutations segregating in the corresponding families were found in 120 controls, and with the exception of L849P, affect amino acid residues which are highly conserved between the human and mouse α 1-subunits of L-type calcium channels.⁹

In addition, we detected six distinct splice site mutations in seven unrelated families (Table 1). In all cases, the consensus splice donor or acceptor sites were affected and the variant was segregating with the disease. As the gene is not expressed in leukocytes, the expected consequences on the RNA splicing behaviour could not be determined.

A mutation in the *CACNA1F* gene was also found in a Finnish family (F40) with a phenotype diagnosed as being ÅIED-like.¹⁸ The PCR product, which generated an SSCP mobility shift which segregated in the family (Figure 3a), was sequenced and revealed a 5 bp deletion in exon 2 of the gene (Figure 3b), predicting a truncation of the protein 65 amino acid residues downstream, resulting in a newly created stop codon TGA at nucleotide position 350. In addition, a missense mutation was found in a single sporadic case (S41) diagnosed with an ÅIED-related phenotype based on a hypopigmented fundus, pale optic discs, strabismus and congenital nystagmus. The T-to-C transition at nucleotide position 220, causing C74R, was not identified in 120 control chromosomes; however, the mutation occurred in the patient's mother. Notably, the affected cysteine residue is located in the highly conserved amino-terminal intracellular loop.

No mutations were found in families T19 and T20 as well as an isolated CSNB2 case (data not shown). These patients were also analysed by complete sequencing of *CACNA1F*.

Mutation screening in the original Åland Island eye disease family

Åland Island eye disease has been mapped to the pericentromeric region of the X chromosome overlapping the region for CSNB2.^{14,19} Several ophthalmological findings in this family such as negative scotopic and abnormal photopic waveforms of the electroretinogram, and the defective dark adaptometry²⁰ suggested allelic heterogeneity to incomplete XLCSNB.¹⁴ Analysis of all 48 exons of the *CACNA1F* gene by complete DNA sequencing revealed no aberration in an affected male.¹⁴ However, intronic mutations causing eg exon skipping cannot be assessed as retinal tissue was not available from affected individuals.

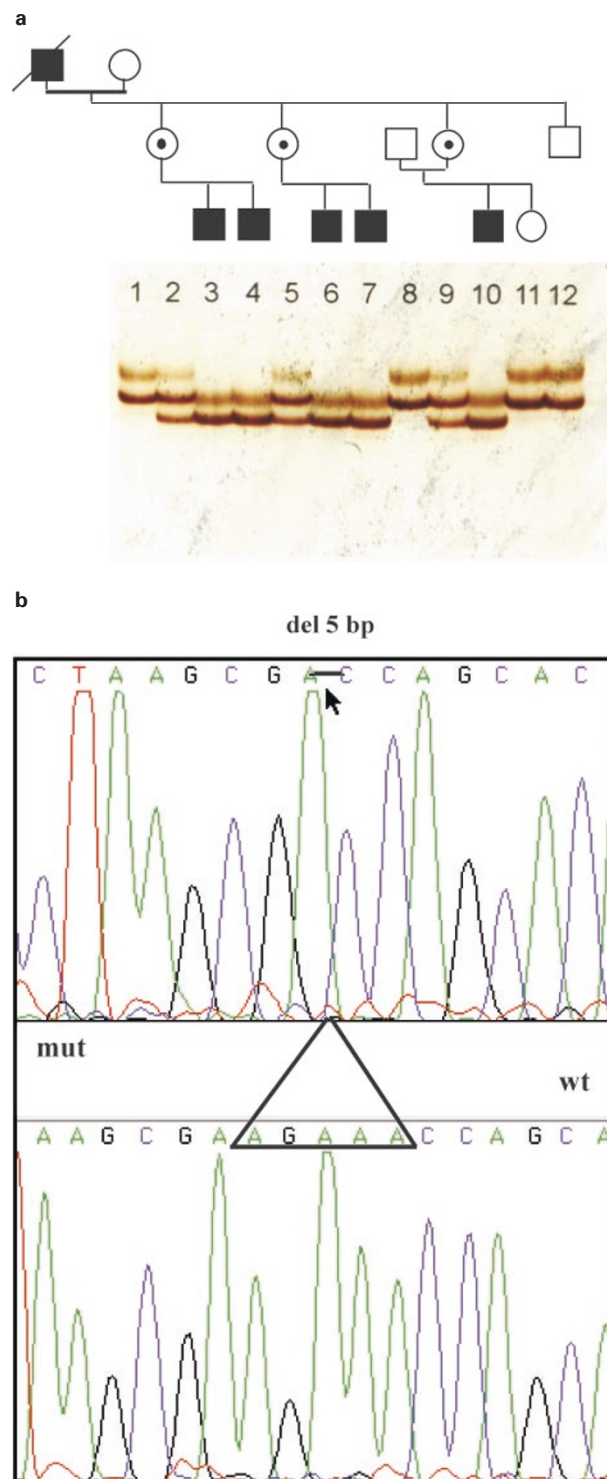


Figure 3 Family F40 from Southern Finland with an ÅIED-like phenotype.¹⁸ (a) Results of the *CACNA1F* exon 2 SSCP analysis (bottom panel) are shown in relation to the pedigree members (top panel). (b) Mutation analysis via direct sequencing of PCR products reveals a 5-bp deletion (Δ -AGAAA) in exon 2 of *CACNA1F* (top panel). For comparison, the bottom panel displays the wild type sequence of an unaffected proband.

Expression of the *CACNA1F* gene is not restricted to photoreceptor cells

Initial RNA *in situ* hybridisation experiments using mouse eye sections were done with a human *CACNA1F* probe.⁹ The data showed that transcriptional activity is primarily present within the photoreceptor cells as well as in other retinal cell types originating from the inner nuclear and the ganglion cell layer. We have now repeated the *in situ* hybridisation experiments with a riboprobe encoding the orthologous gene in mice, termed *Cacna1f*. This has confirmed expression in the outer part of the nuclear layer, the inner part of the inner nuclear layer, and the ganglion cell layer as well (data not shown).

To further refine the expression analysis of the *Cacna1f* gene, we performed RT-PCR experiments in mouse strains with photoreceptor degeneration. In particular, we used the mouse strain C57BL6J/*rd* le gus-s^h which shows retinal degeneration (*rd*) and is characterised by the loss of photoreceptors,^{21,22} and the Swiss Webster (SW) mouse strain, formerly associated with congenital cataract.^{23,24} A semi-quantitative RT-PCR experiment measuring opsin expression present in the outer segments and the outer nuclear layer of the photoreceptors²⁵ was performed in the mutant mouse strains and a healthy control animal. Consistent with the complete loss of photoreceptors in both mutant mice strains, no opsin expression could be detected in the retinal tissues obtained from the homozygous *rd* mouse as well as the SW mouse (Figure 4, row 2). In contrast, apparent normal expression of the murine *Cacna1f* gene could be demonstrated in the SW mouse, and, at a reduced level, in the *rd* mouse. Overall, the expression pattern of *CACNA1F/Cacna1f* appears to be identical to the tyrosine kinase *Jak1* (Figure 4, row 1 and 3 respectively). This gene is known to be expressed in the inner parts of the nuclear layer and the retinal ganglion cell layer,²⁶ with the *Jak1* protein being part of known signal transduction pathways.²⁶

Discussion

Genotype-phenotype correlation between *CACNA1F* mutations and CSNB2

Thirty-three families or patients participating in this study were diagnosed as suffering from the incomplete CSNB phenotype after ophthalmological examinations at the University Eye Hospitals in Tübingen, Regensburg, Paris and Basel. One family was referred from Belgium. In brief, all patients displaying an essentially normal fundus were tested for the presence of a Schubert-Bornschein type ERG that is characterised by a normal a-wave with an extremely reduced b-wave in the maximal response of the ISCEV standard protocol. Such a 'negative ERG' together with impaired cone function, as can be seen in scotopic 30 Hertz flicker responses and dark adaptation tests, was noted in all patients and allowed discrimination between CSNB1 and CSNB2 families.

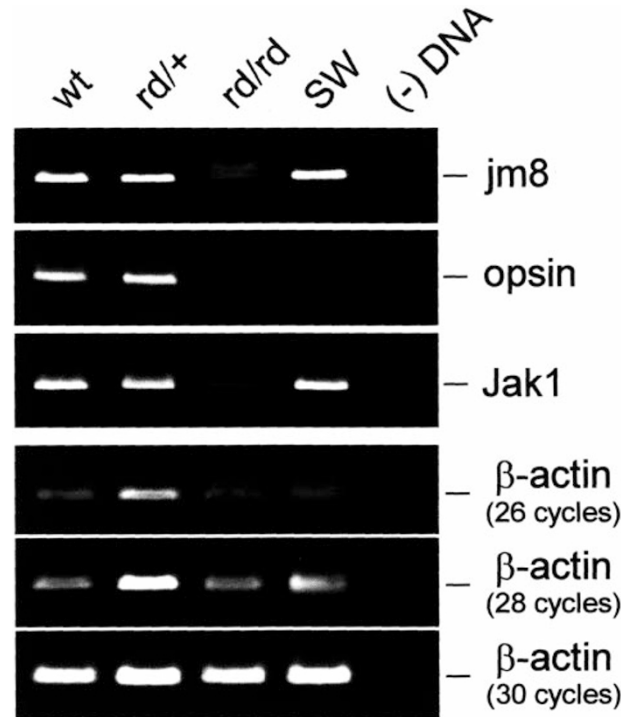


Figure 4 Semi-quantitative RT-PCR expression studies in mouse strains (wt: wild type, *rd/+*, *rd/rd*, and SW). Note that the expression profile of *Cacna1f* (here *jm8*) is identical to that of *Jak1* (panels 1 and 3 respectively) and thus suggests expression of *Cacna1f* in retinal cell types other than the photoreceptors. The housekeeping gene *β-actin* was used as a reference template employing different numbers of PCR cycles to monitor retro-transcription fidelity and to trigger for amplification efficiencies (panels 4–6).

We identified disease-associated mutations in the *CACNA1F* gene in 31 out of the 34 unrelated patients with the incomplete type of XLCSNB. Additionally, one family and a single sporadic case diagnosed previously as ÅIED-related were also shown to carry mutations in this gene. It is interesting to note that reevaluation of the literature indicated full compatibility of the patients' ophthalmological phenotypes with CSNB2. Family F40, showing a frameshift mutation in the *CACNA1F* gene (Figure 3, Table 1) has previously been diagnosed, due to the observation of a slightly reduced a-wave, with an ÅIED-related phenotype.¹⁸ Other clinical data, however, were more compatible with features known from CSNB2. Specifically, the b-wave in the mixed response for the white stimulus was greatly suppressed rather than being normal as observed in ÅIED.¹³ Similarly, mainly due to a hypopigmented fundus, a sporadic patient (S41, Table 1) that had previously been suspected to display an ÅIED-related phenotype, was more suggestive of CSNB2.

Intriguing is the spectrum of alterations found in family T9. As genes like rhodopsin and PDEB are both associated

with retinitis pigmentosa and can both cause congenital stationary night blindness,^{27,28} we decided to screen the *RPGR* gene¹⁵ for mutations in XLCSNB families that showed no recombination with the *RP3* locus. Only in the T9 family could we detect a variant (V36F, Figure 1) that could not be found in 600 control samples tested, and therefore was thought to cause the allelic disease CSNB.¹⁶ However, subsequent screening of the *CACNA1F* gene in this family revealed first the predicted missense alteration R1171P, not found in 120 control samples tested, and later, the novel disease-causing stop mutation R1816X. What makes these alterations so interesting, is that the ophthalmological phenotype of the affected males from this family display no clinical features of retinitis pigmentosa. Furthermore, these patients do not exhibit a more severe course of incomplete CSNB than males with single mutations in the *CACNA1F* gene. Thus we conclude, that the two missense mutations, one in *RPGR* and the other in *CACNA1F*, are rare polymorphisms instead of mutations afflicting protein function.

Careful analysis of the ERG and dark adaptation data published for two patients of the classic ÅIED family¹³ shows that all features of incomplete XLCSNB are present, ie attenuated rod b-waves, and a 'negative' ERG waveform to bright flashes (a large a-wave followed by a small b-wave), absent photopic flicker potentials, and a moderately elevated dark adaptation with the final threshold being at least 1 log unit higher than normal. The associated hypopigmentation of the fundus might be explained by the high myopia and/or the blond phenotype. Therefore, we speculate that the still lacking mutation in this family may be located intronically or may reside in the promotor region. The same assumption can be made for the three unequivocally CSNB2 typed patients for whom the mutations have yet to be revealed.

Comparison of *CACNA1F* mutation spectra

Family F40 is a Finnish family and S41 is a sporadic patient from Switzerland. The family B27 from Belgium shows the novel mutation F742C which segregates within the family. In addition, family B15 exhibiting a novel acceptor splice site mutation, has been sent from Paris. In 29 out of 32 German CSNB2 patients, 27 distinct mutations, 24 of them unique, were identified. These include nine stop or frameshift mutations that cause truncated α 1-subunits. In addition, although the deleterious character of the five different splice site mutations could not be demonstrated in the CSNB2 patients, all of them are predicted to cause aberrantly spliced RNAs and truncated proteins. Twelve mutations were of the missense type, with only P1489R occurring twice, and one mutation causes an *in-frame* deletion of four amino acids. All of the affected amino acids, except for L849P, are highly conserved between the four known α 1-subunits of L-type calcium channels.

Of the 30 distinct mutations identified in our study cohort, 20 are described for the first time. Two stop mutations (R82X and R1288X) have also been identified in other populations¹¹ and might be caused by the frequent C-to-T transition²⁹ rather than by a founder mutation. From eight mutations published recently,⁹ the mutation 3133insC has been found in Mennonite families which have been emigrated since 1683 from Germany, Netherlands, Poland and Russia to North America.^{11,30}

No overlap is found between our mutation spectrum and the mutation pattern in five Japanese families carrying the incomplete type of CSNB.¹² In this study, one missense, one nonsense, one splice site mutation, a single nucleotide insertion and a del/ins combination could be detected. The latter has also been identified by Boycott *et al.*¹¹ as families from Japan had been included in this study.

Congenital stationary night blindness and channelopathies

L-type calcium channels localised in photoreceptors of non-mammalian vertebrates have been shown to be involved in the regulation of glutamate release which increases during dark adaptation and decreases after hyperpolarisation.³¹ A reduced release of glutamate, caused by a diminished influx of calcium ions, might change the sensitivity of dark adaptation. The rod signalling is further transmitted to the bipolar cells, which also have been shown to express L-type calcium channels and thus participate in the regulation of glutamate release.³² The glutamate subsequently binds to glutamate receptors of rod amacrine cells, which in turn stimulate the bipolar cells in a feedback loop. Our *in situ* hybridisation and expression studies using photoreceptorless mouse strains provide further evidence that the *CACNA1F/Cacna1f* gene is not exclusively expressed in photoreceptors but in other retinal cell types as well. These data are in agreement with the findings of others.^{10,33–35} Moreover, the expression pattern overlaps that of the *Jak1* transcript, which has been shown to be expressed in the inner part of the inner nuclear layer and the retinal ganglion cell layer by means of *in situ* hybridisation and immuno-histochemistry.²⁶

Materials and methods

Patients and families

XLCSNB patients were referred by the ophthalmological departments at the Universities in Tübingen (T, H), Regensburg (R), Paris (B) and Basel (S) because of reduced visual acuity, nystagmus and strabismus, with only a minority of patients complaining of poor night vision. Every patient had a general ophthalmologic examination, including best-corrected visual acuity at distance, orthoptic evaluation, slit-lamp biomicroscopy, funduscopy, and Ganzfeld ERG according to the ISCEV standard. Additionally, Gold-

mann perimetry, dark-adapted whole-field ERG, and light-adapted ERG was performed. Most patients were also tested using color vision evaluation, kinetic or static perimetry, and dark adaptometry as described.^{5,6,9}

All family members had normal peripheral visual fields. Visual acuities ranged from 20/30 to 20/400, with elevation of rod dark adaptation final thresholds from 0.3 to about 2.5 log units. Dark-adapted whole-field ERGs showed attenuated rod b-waves, and a 'negative' ERG waveform to bright flashes, ie a large a-wave followed by a small b-wave, the peak of which was below the ERG baseline. In the light-adapted ERG, there was a moderate to marked decrease in cone amplitude and a delay of peak times; 30 Hertz flicker stimuli evoked a characteristic double-peak response in most patients. All affected patients were found to have CSNB of the incomplete type, as previously described in some of the families^{5,6,9} and X-linked inheritance was determined in part by pedigree analysis, with affected males occurring in two or more generations with transmitting asymptomatic females.

All investigations followed the tenets of the Declaration of Helsinki, and informed consent and full institutional review board approval were obtained.

Genomic PCR amplifications and SSCP analysis

Genomic DNA was isolated from peripheral EDTA blood by standard techniques. *CACNA1F* exons were amplified with intronic primers (published with our permission in Boycott *et al.*¹¹). Amplified fragments from 48 exons (Acc. nos. AF235097 and XM_010200) were analysed by SSCP using Hydrolink gels (AT Biochem.) at 20°C with and without glycerol as described.¹⁵ Staining and detection was performed with VistraGreenTM and the FluorImager (Molecular Dynamics), respectively. Variant bands were reamplified and used for direct sequencing with both the sense and antisense primer using cycle sequencing on automated DNA sequencers (Applied Biosystems).

In situ hybridisation on tissue sections and RT-PCR experiments

These experiments were essentially carried out as described by Strom and coworkers.⁹ Instead of using a human *CACNA1F* probe (jm8), we designed the corresponding mouse probe by sequencing cosmids isolated with the jm8 probe from a human cosmid library (RZPD Berlin). Primers used for the amplification of jm8Mus were 5'GGG TTT TCA TGT TCA CTA TCC CG-3' (jm8Mus-c44F1) and 5'-CAT CTC CCA GGT CCT CTT CAT CA-3' (jm8Mus-c48R1; T_A of 60°C).

The reverse transcribed (RT-PCR) reactions were performed in a volume of 25 µl, adding 1-5 mM MgCl₂. Each reaction contained 1 µl of first strand cDNA (total RNA from mouse eye) as template and 15 pmol each primer. PCR conditions were the following: 94°C for 5 min, 94°C for 30 s, specific T_A of each primer pair for

30 s, 30 s at 72°C, and a final extension at 72°C for 5 min. Each PCR was performed for 30 cycles. For the β-actin control expression, the reaction products were harvested at 26, 28 and 30 cycles to perform a semi-quantitative analysis of the amount of first strand cDNAs present in the various samples analysed. The oligonucleotides used were designated XAHR20 (5'-ACC CAC ACT GTG CCC ATC TA-3') and XAHR17 5'-CGG AAC CGC TCA TTG CC-3') (T_A of 58°C; Acc. no.: X03765). For testing opsin expression we applied the primer pair opsinF (5'-TTC ACC ACC ACC CTC TAC AC-3') and opsinR (5'-GTT GAG GGT GGT CTT GGT GG-3') at a T_A of 58°C (Acc. no.: M55171). Finally, simultaneous expression of the protein kinase acting in signal transduction pathways was accomplished with oligonucleotides *Jak1F* (5'-ATG TCC ACC CAA CTG TCC TG-3') and *Jak1R* (5'-AAG GGA GAG TGT CAG CCA AG-3') at an annealing temperature of 58°C (Acc. no.: S63728).

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