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Novel mutations in *CACNA1F* and *NYX* in Dutch families with X-linked congenital stationary night blindness

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Purpose: To describe the clinical features and genetic analysis of eight X-linked congenital stationary night blindness (XLCSNB) Dutch patients.

Methods: Electroretinogram (ERG) measurements were assessed in Dutch patients. Molecular genetic testing by denaturing high performance liquid chromatography (DHPLC), single stranded conformation polymorphism (SSCP) analysis, and direct sequencing of the *CACNA1F* and *NYX* genes were performed in the patients possessing a negative Schubert Bornschein ERG.

Results: Molecular genetic testing of *CACNA1F* and *NYX* revealed three novel and two known *CACNA1F* sequence variants as well as two novel sequence alterations in the *NYX* gene. While one of the *CACNA1F* sequence variants (5756G>A, R1919H) has been previously described as a common polymorphism in Japanese families, we did not find this transition in 100 European control alleles.

Conclusions: In a pool of eight diagnosed XLCSNB patients, five showed a sequence variation in the *CACNA1F* and two in the *NYX* gene. In only one of the eight patients no sequence alteration could be detected. This might be explained by a mutation in other, as yet unidentified coding or regulatory sequences of *NYX* or *CACNA1F* or additional genes.

Congenital stationary night blindness (CSNB) is a nonprogressive retinal disorder characterized by a negative-type ERG in which the amplitude of the b-wave is smaller than that of the a-wave [1] even though the fundus of CSNB patients is essentially normal. Nystagmus, strabismus, myopia, and hyperopia sometimes accompany the other symptoms [2]. The disease is transmitted in an autosomal recessive, autosomal dominant or X-linked mode of inheritance. Several groups have shown genetic heterogeneity of the X-linked form of congenital stationary night blindness (XLCSNB) by identification of two different loci on chromosome X (CSNB1 and CSNB2) [3]. In addition, based on the electroretinogram (ERG), XLCSNB can be divided clinically into two subtypes [4] namely the incomplete type (OMIM 300071) and the complete type (OMIM 310500). While in patients with the incomplete type still some post-receptor rod mediated function is measurable by ERG, no activity is retained in the complete subtype. However, cone function is more impaired in the incomplete type, as revealed by scotopic 30 hertz flicker responses after dark adaptation tests [5-7].

CACNA1F, a gene that maps to Xp11.23, encodes the α -1F subunit of an L-type calcium channel and was identified as the gene mutated in patients with the incomplete form of XLCSNB [8,9]. *CACNA1F* consists of 48 exons and codes for a protein with 1966 amino acids. Originally it was believed

that its expression is restricted to the retina, but recently it was found that it is widely distributed outside the retina, suggesting a broader role in human physiology [8-10].

By positional cloning, we and others have isolated a gene in Xp11.4, *NYX* (nyctalopin), that is mutated in CSNB1 patients [11,12]. It consists of three exons and encodes a 481 amino acid polypeptide of the leucine rich repeat family (LRR). Recently, we isolated the mouse ortholog of *NYX* [13] and showed that the human and mouse nyctalopin are extracellular, membrane bound proteins [14].

Although previous studies have linked mutations in *CACNA1F* and *NYX* to the incomplete and complete forms of XLCSNB, respectively, recent genotype-phenotype comparisons showed no correlation [15]. Noticeably, the frequency of the types of mutations is different in both genes. To our knowledge, 73 *CACNA1F* mutations have been identified in patients with XLCSNB; 51% are nonsense mutations, 32% are missense mutations, 8% are frameshift mutations, 8% are in-frame deletions or insertions, and 1% represent splice site mutations [8,9,15-20]. Of the 53 *NYX* mutations reported so far, 51% of them account for missense mutations, 32% for in-frame insertions or deletions, 7% for frameshift mutations, 4% for large deletions, only 4% for nonsense mutations, and 2% for splice site mutations [11,12,15,16].

In this study, we screened eight unrelated Dutch XLCSNB patients for mutations in the *CACNA1F* and/or *NYX* genes.

METHODS

Patients: Eight unrelated patients were diagnosed with XLCSNB at the University Medical Center of Nijmegen by

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different tests, notably analysis of the pedigree, electroretinography (ERG), and electro-oculography (EOG).

For ERG tests, a scleral contact lens equipped with measuring electrodes was inserted. A reference electrode was placed on the forehead and the patient was earthed by means of two earclip electrodes. Two 40 W incandescent lamps were applied to furnish a Ganzfeld adaptation and a Xenon flash was used for stimulation (flash luminance was 6.85 cd/m² photopic and 0.8 cd/m² scotopic, respectively). Measurements were recorded during photopic and scotopic (after a 12 min dark adaptation) circumstances. Recorded electric potentials were plotted against flash intensity and the voltage of the response was determined for both eyes as the average between the measurement at 0.2 and 0.4 joules for photopic circumstances and the response after 12 min dark adaptation for the scotopic measurement. The lower limit of normality with this technique was 10 μ V for the photopic b-wave and 150 μ V for the scotopic b-wave with a white stimulus [21]. EOG was performed by placing electrodes on the skin of the lateral and medial canthi. After pre-adaptation at 100 Lux, the sphere was darkened and the EOG was recorded every 2 min during 12 min. This was followed by a recording every min at 2500 Lux during another 15 min. Subsequently, the ratio between the dark trough and the light peak was determined (Arden ratio). Details of these techniques were described previously [21].

Mutation screening: Genomic DNA was isolated from EBV transformed lymphoblastoid cells by standard techniques. *CACNA1F* exons were amplified with intronic primers [19]. PCR amplified fragments from 48 exons of the *CACNA1F* gene were first examined either with denaturing high performance liquid chromatography (DHPLC) or single stranded conformation polymorphism (SSCP) analysis [22,23]. Probes resulting in different elution profiles or band shifts were sequenced on an automated DNA sequencer (ABI PRISM 3100,

Applied Biosystems, Rotkreuz, Switzerland).

NYX exons were amplified with intronic primers as follows: exon 1 and exon 2 within one fragment with HotStarTaq DNA Polymerase and Q-solution (Qiagen, Hombrechtikon, Switzerland), 1.5 mM MgCl₂ at 56 °C annealing temperature with a forward and a reverse primer (5'-CCG GGG ATA AAG CCG ATT GG-3' and 5'-GAA GCT CTG TGG CTT CCA CC-3'). Additional nested primers for the sequencing of exon 1 and exon 2 of *NYX* were: 5'-CTT AGC CC AAC ACC AGG GTC-3' and for the sequencing of exon 3: 5'-CCT CAC CAC GTC CAG TCC-3', and 5'-GTC TCC ATC GAC CTG GAC C-3'.

Exon 3 was amplified as three fragments (A, B, C) with different forward and reverse primer combinations. Fragment A (primers 5'-TTC TCC TCC TTC CCG ACT C-3' and 5'-CGG CAG GCT GCT AGG TCT-3') was amplified using with HotStarTaq DNA Polymerase and Q-solution (Qiagen), 2.5 mM MgCl₂ at 58 °C annealing temperature. Fragments B (primers 5'-GAC CTG CGC TAC CTG CAC-3' and 5'-CGG AGC CCT CCA GT-3') and C (primers 5'-GCT CAA CGA CAA CCT GCT G-3' and 5'-ACA AAC ACA CTC AAG CCC AG-3') of exon 3 were both amplified with HotStarTaq Polymerase and Q-solution (Qiagen), 1.5 mM MgCl₂ at 58 °C and 60 °C annealing temperature.

RESULTS & DISCUSSION

Eight patients were diagnosed with CSNB Schubert-Bornschein type by ERG and EOG (Table 1). One of them (2490) showed a clear X-linked mode of inheritance. When the patients were originally examined, they were not classified as complete or incomplete forms of XLCSNB. A genotype-phenotype correlation in British XLCSNB families revealed that a discrimination between the two forms can be made by analyzing the scotopic oscillatory potentials, the 30

TABLE 1. CLINICAL DATA OF THE DESCRIBED PATIENTS

Patient	Diagnosis	Age at exam	Visual acuity		Scotopic ERG RE/LE		Photopic ERG RE/LE		Electro-oculography (lp/dt)		Dark adaptation	Nystagmus
			RE	LE	a-wave [μ V]	b-wave [μ V]	a-wave [μ V]	b-wave [μ V]	RE	LE		
2417	CSNB, ablation LE	27	0.8	0.3	30/ 25	60/70	0/100	60/70	1.75	1.94	-	Yes
2436	CSNB	34	0.3	0.3	90/ 80	20/25	60/ 50	35/40	-	-	Affected	Yes
2445	CSNB, strabismus	19	0.4	0.4	50/ 70	50/75	35/ 55	60/70	-	-	Affected	Yes
2446	CSNB	11	0.2	0.2	130/ 70	50/60	75/ 50	30/20	-	-	Normal	Yes
2454	CSNB	11	0.2	0.2	15/ 15	50/60	45/ 45	50/50	2.24	2.2	Affected	Yes (L>R)
2477	CSNB, tilted disc	36	0.3	0.3	110/100	5/20	100/ 85	10/10	1.26	1.38	Affected	Yes
2490	CSNB	66			20/ 20	90/90	50/ 50	30/40	-	-	-	Unknown
2496	CSNB, strabismus	8	0.4	0.4	60/NM	40/NM	75/ 65	55/55	-	-	-	Yes

The clinical data of eight Dutch patients shows typical findings for patients with XLCSNB. Right eye (RE), left eye (LE), electroretinogram (ERG), light peak/dark trough (lp/dt), and not measured (NM) are abbreviated in the table. The retina of the left eye was ablated in patient 2417.

Hz flicker ERG, and the OFF response [15]. However, these data were not available for the patients described here. In future studies these ERG measurements always should be performed, providing a genotype-phenotype correlation. This would allow preselecting patients associated with one or the other form, which would make the mutation analysis in both genes less time and cost consuming.

Mutation analysis in *CACNA1F* in XLCSNB patients revealed three novel and two known sequence variants in five independent families (Table 2). None of the mutations were detected in 100 control chromosomes from European individuals. The novel C to T nonsense mutation at position 2038 in exon 15 is predicted to lead to a version of the protein that is shortened by 1286 amino acids. The C to T nonsense mutation at position 2650 in exon 21 likewise causes a protein truncation and was previously identified in another family [11]. The two novel *CACNA1F* mutations G to T at position 3761 and C to A at position 3853 lead to the amino acid substitutions S1254I and R1285S, respectively. The known missense mutation G to A at position 5756 has been previously identified in 21 control alleles in the Japanese population [17]. Thus, we conclude that this latter sequence variant is most likely non-causative and that the polymorphism occurs more frequently in the Japanese population than in Europeans. This is an assumption that must be verified in the future. However, computational analysis by means of exonic splicing enhancer prediction software (ESE version 1.1) provided evidence that the G to A mutation at position 5756 is located in an ESE site of exon 48 and thus may affect normal splicing (data not shown). Further experimental studies are necessary to confirm this prediction.

Protein sequence alignments of *CACNA1F* have shown homology to the L-type calcium channel α -1 subunit. The α -1 subunits function as the pore and voltage sensor in ion selective L-type calcium channels [24-26]. The novel stop mutation found in exon 15 is situated in the extracellular loop in the second domain (II), thereby deleting about two thirds of the protein, including part of domain II, the entirety of domains III and IV, the EF-hand motif, and the cytoplasmic C-terminus. The stop mutation in exon 21 occurs in the extracel-

ular loop of the third domain (III), thereby deleting part of domain III, the entirety of domain IV, the EF-hand motif, and the cytoplasmic C-terminus. The novel missense mutations detected in exons 31 and 33 affect the potential transmembrane segments S3 and S4 of repeat IV of the *CACNA1F* protein. Although the consequences of the mutations on channel function have not been investigated, it appears most likely that the incomplete type of CSNB is associated with loss of function mutations. Presumably they decrease the calcium influx and tonic glutamate release in darkness with consequent relative depolarization of bipolar cells [27], leading to the typical reduced ERG b-wave found in patients with the incomplete type of CSNB.

Mutation screening in *NYX* revealed two novel missense mutations in exon 3, in two different families, that were not detected in 100 control alleles (Table 3). Nyctalopin was shown to be an LRR cell surface protein attached extracellularly to the membrane [14]. The P57T exchange in patient 2436 occurs in the N-terminal cysteine LRR and the A64E amino acid substitution in patient 2454 in the first typical LRR in patient 2454. Both residues are conserved in human and mouse nyctalopin [13]. Previous studies have shown that mutations in the *RP2* gene can disrupt the correct subcellular localization, presumably leading to the phenotype diagnosed in patients with retinitis pigmentosa (RP2) [28]. However, different constructs containing disease associated mutations found in *NYX* have not shown any effect on the cellular localization in comparison to wild type constructs [14]. Thus, the function of this protein remains to be elucidated. Many LRR proteins have been found to be involved in protein-protein interactions [29]. Consequently, the mutated variants may disrupt these processes leading to congenital stationary night blindness.

In this study, we detected in seven of eight unrelated CSNB diagnosed patients sequence variants, five of which were novel. Our results confirm previous findings that most pathogenic sequence alterations in *CACNA1F* are nonsense mutations, while missense mutations are predominant in *NYX*. Furthermore, one alteration was already known to be a polymorphism in Japanese families. In one patient (2445), we could rule out the presence of *NYX* and *CACNA1F* mutations. In this case, other, not yet identified coding or regulatory sequences of *NYX* and *CACNA1F* or additional genes may be mutated. Additionally, we cannot exclude that this patient has a different mode of inheritance of CSNB.

TABLE 2. SEQUENCE VARIANTS IN THE *CACNA1F* GENE

Patient	Exon	Mutation	Protein	Reference
2446	15	2038C>T	R680X	
2477	21	2650C>T	R884X	[8,19]
2417	31	3761G>T	S1254I	
2490	33	3853C>A	R1285S	
2496	48	5756G>A	R1919H	[17]

Three novel mutations in the *CACNA1F* gene. R884X has been previously found by Bech-Hansen et al. [8] in one patient and by Strom et al. [9] in two unrelated patients. R1919H has been previously found in one patient and described as a polymorphism in 21 of 100 control alleles [17]. The nucleotide positions in the mutation column are based on the start codon of the *CACNA1F* gene (AJ006216).

TABLE 3. SEQUENCE VARIANTS IN THE *NYX* GENE

Patient	Exon	Mutation	Protein
2436	3	169C>A	P57T
2454	3	191C>A	A64E

Two novel mutations in the *NYX* gene. The nucleotide positions in the mutation column are based on the start codon in the *NYX* (gene originally named *CLRP*; AJ278865).

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