

Slow and Fast Rod ERG Pathways in Patients with X-Linked Complete Stationary Night Blindness Carrying Mutations in the NYX Gene

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PURPOSE. To study the slow and fast rod signals of the scotopic 15-Hz flicker ERG in patients carrying mutations in the *NYX* gene, which has been recently identified as the cause of the complete form of congenital stationary night blindness, CSNB1.

METHODS. Twenty eyes of 11 patients with CSNB1 who had nondetectable standard ERG rod b-waves were involved in the study. Scotopic ERG response amplitudes and phases to flicker intensities ranging from -3.37 to -0.57 log scotopic trolands \cdot sec (scot td \cdot sec) were measured at a flicker frequency of 15 Hz. ERG signals to flicker intensities between -3.37 and -1.97 and between -1.17 and -0.57 log scot td \cdot sec were considered to represent primarily the slow and fast rod ERG pathway, respectively. Additionally, standard ERGs were performed. Twenty-two normal volunteers served as control subjects.

RESULTS. For the slow rod ERG pathway, all patients exhibited ERG signals that were indistinguishable from noise. Accordingly, there was no systematic phase behavior for the slow rod signals. For the fast rod ERG pathway, the signals were significantly above noise, but they were significantly reduced in amplitude and advanced in phase.

CONCLUSIONS. There is evidence that the slow and the fast rod ERG signals can be attributed to the rod bipolar-AII cell pathway and the rod-cone-coupling pathway, respectively. The current study provides evidence to suggest that a defective *NYX* gene product (nyctalopin) prevents detectable signal transmission through ON rod bipolar cells, but there is a residual transmission through rod-cone gap junctions in CSNB1, possibly through the OFF cone pathway. (*Invest Ophthalmol Vis Sci.* 2001;42:2728-2736)

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Two subtypes of congenital stationary night blindness (CSNB) have been mapped to different loci on the X-chromosome.¹ In both genetic subtypes of X-linked CSNB, the ERG shows essentially normal rod a-waves and greatly diminished b-waves, generally referred to as the Schubert-Bornschein type of CSNB² and indicative of a defect in signal transmission to second-order neurons.³⁻⁷ The two forms can be distinguished by means of the standard ERG alone: The X-linked complete form of CSNB (CSNB1) has been associated with absent rod and subnormal cone b-wave amplitudes, whereas X-linked CSNB, displaying the incomplete phenotype (CSNB2), exhibits some rod function, but both the rod and the cone ERG signals are considerably reduced in amplitude.^{4,7}

The gene responsible for CSNB2 has been identified and shown to encode a retina-specific L-type voltage-gated calcium channel α -subunit, CACNA1F.^{8,9} The ERG findings in CSNB2 could be most simply explained if CACNA1F is considered to mediate voltage-dependent glutamate release from photoreceptor synaptic terminals, a process known to depend on L-type calcium channels.^{10,11}

The CSNB1 locus has been mapped to a 5-centimorgan (cM) interval in Xp11.4.^{1,12-14} Very recently, the gene that is responsible for CSNB1 has been identified and designated *NYX*. It encodes a glycosylphosphatidylinositol (GPI)-anchored, extracellular, 481-amino-acid protein nyctalopin, which possibly is involved in cell-cell contacts in the retina.^{15,16} Its effective functional role in CSNB1, however, cannot be deduced from the molecular genetic findings so far. To further characterize the abnormalities of retinal function associated with mutations in nyctalopin, we examined patients with CSNB1 who displayed a mutated *NYX* gene, by means of the scotopic 15-Hz flicker ERG.¹⁷⁻²¹ In the normal observer, the response versus intensity function of the scotopic 15-Hz flicker ERG has two limbs (reflecting activity of slow and fast ERG rod signals); these two ERG signals have been attributed to two different retinal rod pathways: the rod bipolar-AII cell pathway and the rod-cone-coupling pathway.^{17,18,20} Our findings provide the first evidence of differential alterations of rod signal transmission in patients with mutations in the *NYX* gene and also provide evidence to suggest a residual signal transmission through rod-cone gap junctions in CSNB1.

METHODS

Patients and Normal Subjects

Twenty eyes of 11 patients with complete CSNB of the Schubert-Bornschein type (median age, 22 years; range, 12-46 years; data of one eye in patient 5 were excluded for technical reasons; patient 7 had an anophthalmus in one eye due to trauma) were included in the study. A detailed history (including family history) and an ophthalmic routine examination including visual acuity and Ganzfeld electroretinography according to the International Society for Clinical Electrophysiology of Vision (ISCEV) standard²² were performed and provided the basis for the clinical diagnosis (visual acuity was measured as visus on a quasi-

logarithmic ordinal scale; for use in regression models it was ranked 1–10 and treated as quasicontinuous). A subset of the patients with CSNB have been included in a previous study.⁷

Twenty-two eyes of 22 normal subjects (median age, 29.5 years; range, 19–58 years) served as a control. Detailed ERG data on this control group have been published previously.²¹ Both, the scotopic flicker ERG and the scotopic standard ERG were recorded from these 22 normal subjects. The normative values for the photopic standard ERG were obtained from another group of normal subjects. We therefore excluded these ERG data from statistical analysis.

Informed consent was obtained from all subjects after explanation of the purpose and possible consequences of the study. The study was conducted in accordance with the tenets of the Declaration of Helsinki and with the approval of our institutional ethics committee in human experimentation.

ERG Stimulation, Recording, and Procedure

The apparatus, the stimulation, and the procedure of the ERG measurements have been reported recently²¹ and are very similar to those used in previous studies.^{18–20} Briefly, we used a Ganzfeld stimulator and data acquisition system (Universal Testing and Analysis System—Electrophysiology 2000 [UTAS-E 2000]; LKC Technologies, Inc., Gaithersburg, MD). Stimulus and recording conditions were in accordance with the ISCEV standard.²² The subjects, positioned with the aid of a headrest, viewed the center of a Ganzfeld bowl. The bowl was homogeneously illuminated by white flashes repeated at a frequency of 15 Hz produced by a xenon discharge lamp (flash duration $\sim 10 \mu\text{sec}$; correlated color temperature $\sim 6000 \text{ K}$; see Table 1 [2.4.4] in Ref. 23). The flicker yielded by this device was full-field. To avoid stray light, we masked all sites of light leakage by black tape. In addition, the subjects were surrounded by a black curtain so that accidental light or stray light (e.g., arising from the computer monitor) had no influence on the Ganzfeld illumination and the ERG recording. Each flash was triggered by the computer (LKC Technologies, Inc.), which was also used to store and analyze the ERG recordings. Maximal intensity was 1.43 log scot td \cdot sec. To attenuate the flash, neutral density (ND) filters (Wratten; Eastman Kodak, Rochester, NY) mounted in a filter wheel were inserted. The maximum attenuation was -4.8 log units ND and the step size was 0.2 log units ND. We continued the measurements up to a retinal illuminance of -0.57 log scot td \cdot sec, which is well below the cone threshold in the Ganzfeld ERG (approximately 0.75 log scot td \cdot sec).²⁴

Each subject had been dark adapted for 30 minutes. In the control subjects, one eye was dilated with a mydriatic (0.5% tropicamide); in the patients, tropicamide (0.5%) and phenylephrine (5%) were used. Pupil diameters were determined before ERG recordings; there was no difference in pupil diameter between the two subject groups. DTL fiber electrodes were positioned on the conjunctiva directly beneath the cornea and attached at the nasal and lateral canthus. Reference electrodes (Ag-AgCl) were placed over both temporal bones, and a ground electrode was placed on the forehead. The ERG responses to the periodic flashes were recorded and stored on computer (LKC Technologies, Inc.). To avoid the effects of the rapid changes of gain control mechanisms in the rod system that accompany the onset of flickering lights, we discarded the responses to the flashes presented during the first 5 seconds. The signals were band-pass filtered (1–70 Hz), and averaged 50 to 100 times on-line. The noise level was determined by recording an ERG signal with the xenon discharge lamp covered by black cardboard (similar to a procedure described elsewhere).²⁵

Data Analysis

To determine the periodicity of the ERG responses, we computed a fast Fourier transform (FFT) on the sampled data.^{26,27} As a result, we found that all responses were dominated by the fundamental component.²¹ Therefore, we identified the ERG response amplitude and phase as the amplitude and phase of this fundamental component. In

a previous study in normal subjects, we found that the flicker null (and the large phase shift of approximately 180°) occurred between intensities of -1.77 and -1.37 log scot td \cdot sec (three intensity levels).²¹ Therefore, the ERG signals at flicker intensities between -3.37 and -1.97 log scot td \cdot sec (eight intensity levels) were considered to be dominated by the slow rod ERG signals and ERG signals between -1.17 and -0.57 log scot td \cdot sec (four intensity levels) by the fast rod ERG signals.

Determination of CSNB1 Haplogroups and Mutation Analysis in the NYX Gene

For haplotype analyses in patients, 10 established microsatellite loci (DXS556, DXS8042, DXS1368, DXS574, DXS993, DXS8012, DXS1207, DXS1201, DXS8085, and DXS228) located within Xp11.4 were analyzed. The locus order was confirmed by physical mapping of the markers to the Xp11 subregional panels and yeast artificial chromosomes. One of the primers of each pair of oligonucleotides was fluorescence-labeled at the 5' terminus with FAM, HEX, or TET. A touchdown protocol for PCR amplification (GeneAmp 9600 PCR cyclor; PE Biosystems, Foster City, CA) was accomplished with an initial denaturation of 5 minutes at 94°C; 5 cycles at 94°C for 15 seconds, 60°C for 30 seconds, and 72°C for 30 seconds; 5 cycles at 94°C for 15 seconds, 57°C for 30 seconds, and 72°C for 30 seconds; and 25 cycles at 94°C for 15 seconds, 52°C for 15 seconds, and 72°C for 30 seconds; and a final extension at 72°C for 5 minutes. The PCR products were pooled and an internal length standard (Prism Genescan-500; PE Biosystems) was added. The pooled products were separated using 6% denaturing polyacrylamide gels on an automated DNA sequencer (model ABI 373A, equipped with Genescan software; PE Biosystems).

The procedure for identifying mutations in the NYX gene has been described in detail.¹⁵ Briefly, blood was collected from patients with CSNB1 and control subjects, and DNA was extracted from white blood cells according to standard procedures.²⁸ Five overlapping segments covering the entire NYX coding region were amplified by means of polymerase chain reaction (PCR). The PCR products were purified by agarose gel-extraction (QiaQuick; Qiagen, Chatsworth, CA), cycle sequenced with dye-terminator chemistry (BigDye; PE Biosystems), and analyzed on a DNA sequencer (model ABI 377; PE Biosystems). Cosegregation analysis of mutations and their exclusion in control subjects was performed by restriction fragment length polymorphism (RFLP), single-strand conformational polymorphism (SSCP), Southern hybridization, or direct sequence analysis.

Statistical Analysis

Data were analyzed by computer (JMP ver. 4.0.2 software; SAS Institute, Inc., Cary, NC). Results with $P < 0.05$ were considered statistically significant. The amplitudes and implicit times of the slow and fast rod ERG signals for the patients with CSNB1 and the normal subjects were compared by a multivariate analysis of covariance (MANCOVA) with a factor eye, because the CSNB1 group comprised data from two eyes that are not independent (for descriptive statistics such as the median and the percentiles, we included the mean of the right and left eye of each patient).²⁹ The influence of age on the ERG data was thereby corrected. For the standard ERG parameters, a similar analysis of variance (ANOVA) with patients as a random factor was used. Furthermore, we calculated canonical correlations between the amplitude and phase data and the refraction error and visual acuity.

RESULTS

Clinical Characteristics

A summary of the findings in all 11 patients with CSNB1 is given in Table 1. Reduction in visual acuity was variable (median: 0.5; range: 0.1–0.8). The considerable asymmetry in visual acuity between eyes in some of the patients (patients 1, 4, 7, 8, and 9) was due to amblyopia caused by congenital squint.

TABLE 1. Characteristics of the Patients with CSNB1

Patient	Age (y)	Visual Acuity		Refractive Error	
		OD	OS	OD	OS
1*	18	20/50	20/32	-3.5 sph -1.0 cyl A 95°	-2.75 sph -0.5 cyl A 95°
2**	15	20/32	20/25	-7.25 sph -1.25 cyl A 104°	-7.25 sph -1.25 cyl A 78°
3**	17	20/32	20/25	-10.75 sph -1.0 cyl A 93°	-9.5 sph -0.75 cyl A 93°
4**	22	20/32	20/25	-13.5 sph -0.5 cyl A 104°	-12.5 sph -1.0 cyl A 99°
5	12	20/50	20/50	-9.75 sph -0.5 cyl A 112°	-10.25 sph -1.25 cyl A 44°
6*	27		20/32		-26.0 sph
7	14	20/64	20/200	-4.5 sph -1.0 cyl A 88°	-4.75 sph -1.0 cyl A 53°
8	35	20/64	20/40	-7.5 sph -1.75 cyl A 9°	-7.5 sph -2.0 cyl A 142°
9***	33	20/40	20/40	-1.75 sph -3.5 cyl A 120°	-2.75 sph -4.75 cyl A 70°
10***	32	20/40	20/200	-8.0 sph -3.75 cyl A 90°	-7.0 sph -1.25 cyl A 80°
11*	46	20/32	20/32	-11.5 sph -0.75 cyl A 85°	-11.0 sph -0.75 cyl A 90°

*, **, ***: patients belonging to the same families. sph, sphere; cyl, cylinders; A, axis.

Patient 6 had an anophthalmus due to trauma in the right eye. All patients were myopic (median: -8.75 diopters [D]; range: -3.5 to -26 D spherical equivalent). Their ages were significantly lower than that of the normal subjects ($P = 0.02$, unpaired t -test).

Haplogroup Determination and NYX Mutation Analysis

Five different NYX mutations were identified in the patient collective. We determined six different haplogroups, thus indicating that mutation C35X occurred independently in two families (patients 8 and patients 9 and 10). The data are summarized in Table 2.

We were not able to correlate a respective genotype out of a unique haplogroup with neither a specific phenotype nor a distinct feature. Moreover, extensive intrafamilial variability in clinical parameters and features is apparent in all members belonging to the same family. Considerable intrafamilial variability within families segregating the same disease-related mutation is known in other Mendelian genetic disorders, such as retinitis pigmentosa with rhodopsin gene mutations.³⁰

Standard ERG

No standard rod ERG b-waves were detectable in any of the patients with CSNB1. Additionally, in 10 of 21 eyes, the a-wave amplitude was below the 5th percentile of the normal subjects. The b-wave amplitudes of the maximal response were considerably below the 5th percentile of normal subjects in all patients. The b- to a-wave ratio was below unity in every patient (Table 3).

TABLE 2. NYX Mutations in Patients with CSNB1

Patient	Codon	Nucleotide Position	Predicted Protein Change
1*	1-20	Del exons 1 and 2	Unknown
2**	243-246	727-738del 12 bp	AELP243-246del
3**	243-246	727-738del 12 bp	AELP243-246del
4**	243-246	727-738del 12 bp	AELP243-246del
5	307	920T → C (CTG → CCG)	L307P
6*	1-20	Del exons 1 and 2	Unknown
7	101	301-303del TCA	I101del
8	35	105C → A (TGC → TGA)	C35X
9***	35	105C → A (TGC → TGA)	C35X
10***	35	105C → A (TGC → TGA)	C35X
11*	1-20	Del exons 1 and 2	Unknown

The single-letter code is used to denote mutations. *, see Table 1.

The amplitude of the photopic b-wave and of the photopic 30-Hz flicker ERG were below the 5th percentile of normal subjects in 11 and 8 of 21 eyes, respectively. The implicit times for these ERG responses were not prolonged in any of the patients (Table 3).

The amplitudes and implicit times of the standard flash ERG were statistically analyzed with an ANOVA with factors "disease" and "eye" nested under subject and disease. For the scotopic a-wave amplitude to the maximal flash, the ANOVA revealed a significantly lower mean for the patients with CSNB1 ($t = 3.2$; $P = 0.003$). The scotopic b-wave to the maximal flash was significantly lower in amplitude ($t = 312$; $P < 0.0001$), but also significantly shorter in implicit time ($t = 38$; $P < 0.0001$). A subsequent Bonferroni-Holm procedure to correct for multiple comparisons (multiple $\alpha = 0.05$) revealed that all these measures exhibited significant differences between the subject groups.

Slow Rod ERG Signals

Figure 1 displays the original ERG signals to visual stimulation of the 15-Hz flicker at scotopic conditions for a normal subject (Fig. 1A, left) and a patient (patient 8; Fig 1B, right). For the normal subject, the ERG signals increased slightly with increasing flicker from -3.37 to -2.97 log scot td · sec and then decreased thereafter. At flicker intensities between -2.17 and -1.77 log scot td · sec, there was a minimum ERG response. To higher flicker intensities (from -1.37 to -0.97 log scot td · sec), the ERG signals rapidly increased in amplitude again and were considerably advanced in phase (Fig. 1, arrow). In the patient, however, the ERG signals of the slow rod pathway (flicker intensity between -3.37 and -1.97 log scot td · sec) were indistinguishable from noise, whereas there was a definite ERG response to flicker intensities above -1.17 log scot td · sec.

The ERG signals were Fourier analyzed and the amplitude and phase of the fundamental component were determined. We estimated the signal reduction within the two rod ERG pathways for each patient with CSNB1 separately.²¹ The relative percentage proportion of ERG amplitude for the slow and fast rod ERG signals (average of the right and left eyes) was compared with the mean of the normal subjects.²¹ Consequently, we obtained a global estimate of the amplitude reduction for each ERG rod pathway for each patient with CSNB1. We found that the slow rod ERG signals were below the noise level in every patient.

In Figure 2, the noise level and the median and 5th and 95th percentiles of both subject groups are displayed. In the patients with CSNB1, the ERG signals of the slow rod pathway

TABLE 3. Standard ERG of the Patients with CSNB1

Patient	b-Wave		a-Wave		Maximum Response: b-wave				Ratio of b- to a-wave				Photopic b-Wave				30-Hz Flicker					
	Amplitude		Amplitude		Amplitude		Implicit Time		b- to a-wave		Amplitude		Implicit Time		Amplitude		Implicit Time		Amplitude		Implicit Time	
	OD	OS	OD	OS	OD	OS	OD	OS	OD	OS	OD	OS	OD	OS	OD	OS	OD	OS	OD	OS	OD	OS
1	ND	ND	88.41	96.95	43.9	51.22	32.5	32.5	0.5	0.53	47.56	46.34	27.9	27.6	27.8	23.2	29.8	29.5	29.8	29.5	29.8	29.5
2	ND	ND	251.8	201.8	81.71	66.46	32	32	0.32	0.33	132.7	102	28.5	28.5	54.9	54.4	29	28.7	29	28.7	29	28.7
3	ND	ND	145.1	199.4	54.88	81.1	31.5	31.5	0.38	0.41	51.22	53.9	28.2	28.5	45.6	48.5	27.8	27.9	27.8	27.9	27.8	27.9
4	ND	ND	103.7	100.6	44.51	53.66	33	33.5	0.43	0.53	64.63	65.37	30	30.3	38.5	35.1	31.2	31.4	31.2	31.4	31.2	31.4
5	ND	ND	193.9	201.2	98.17	88.41	29.5	33	0.51	0.44	124.9	115.1	27.3	27.6	61	61	27.1	27.1	27.1	27.1	27.1	27.1
6	—	ND	—	146.3	—	94.51	37.5	37.5	0.65	0.65	—	64.88	—	31.4	—	44.1	—	31.3	—	31.3	—	31.3
7	ND	ND	282.3	316.5	132.3	126.2	29	30	0.47	0.4	108.5	122.7	29.4	29.7	64.9	81.5	29.3	29.5	29.3	29.5	29.3	29.5
8	ND	ND	214	226.2	89.63	103.7	31.5	32	0.42	0.46	115.4	136.1	30.3	30.3	85.9	89	30.9	30.5	30.9	30.5	30.9	30.5
9	ND	ND	151.8	159.2	70.12	70.73	34	34.5	0.46	0.44	115.9	122.4	30	30	64.1	64.6	30.7	31.4	30.7	31.4	30.7	31.4
10	ND	ND	122	126.2	54.27	65.85	31.5	33	0.45	0.52	38.78	34.88	29.1	28.8	26.3	31.2	29.2	30.7	29.2	30.7	29.2	30.7
11	ND	ND	220.7	218.3	135.4	136.6	36	36	0.61	0.63	62.2	80.49	30.4	30.2	54.4	62	31.9	31.5	31.9	31.5	31.9	31.5
Median:			172.3		74.1		32.3		0.48		71.3		29.6		54.7		30.0		30.0		30.0	
5% percentile			97.4		48.3		30.4		0.36		41.9		27.6		27.1		27.5		27.5		27.5	
95% percentile			263.1		132.6		36.8		0.64		122.9		30.9		80.3		31.5		31.5		31.5	
Normal Median:			268.3		438.4		43.3		1.89		150.5		30.4		74.0		29.4		29.4		29.4	
Normal 5% percentile			184.2		317.7		33.0		1.55		97.0		27.7		47.0		27.2		27.2		27.2	
Normal 95% percentile			435.4		537.2		47.0		2.39		223.0		32.8		112.0		32.2		32.2		32.2	

Results of the ERG, according to the ISCEV standard of the individual patients with CSNB1. Amplitudes are in microvolts and implicit times are in milliseconds. For the scotopic standard ERG, the bottom lines provide the median and the 5th and 95th percentiles of the patients and normal subjects. ND, not detectable.

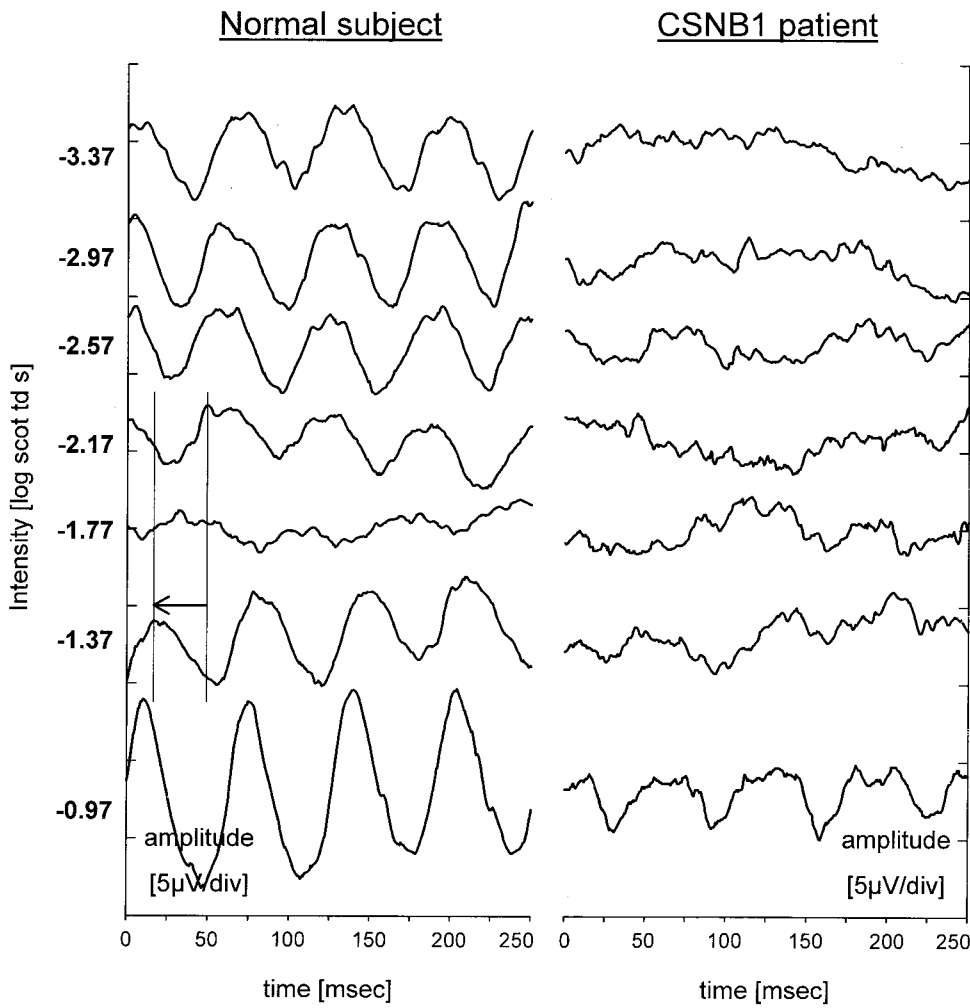


FIGURE 1. Original tracings of the rod ERG responses to 15-Hz flicker stimulation obtained from a normal subject (*left*) and a patient with CSNB1 (patient 8, *right*). Traces showing 250 msec of the ERG signal are displayed. On the *ordinate*, the flicker intensity is given at which the ERG response was obtained. Step size is 0.4 log units ND attenuation of the maximal intensity, beginning with the lowest intensity of -3.37 log scot $\text{td} \cdot \text{sec}$ (*top*). One division indicates $5 \mu\text{V}$. In the normal subjects, two features can be observed: First, the amplitude increases somewhat with increasing flicker intensity and then decreases to a minimum at -1.77 log scot $\text{td} \cdot \text{sec}$ with a rapid increase of the ERG response amplitude thereafter. Second, with increasing flicker intensity, there is a shift of the timing of the ERG signal toward larger phases (corresponding to shorter implicit times; *arrow*). In the patient with CSNB1, however, there were no ERG signals distinguishable from noise at the lower flicker intensities. At the higher flicker intensities, an ERG signal became apparent, which was, however, reduced in amplitude.

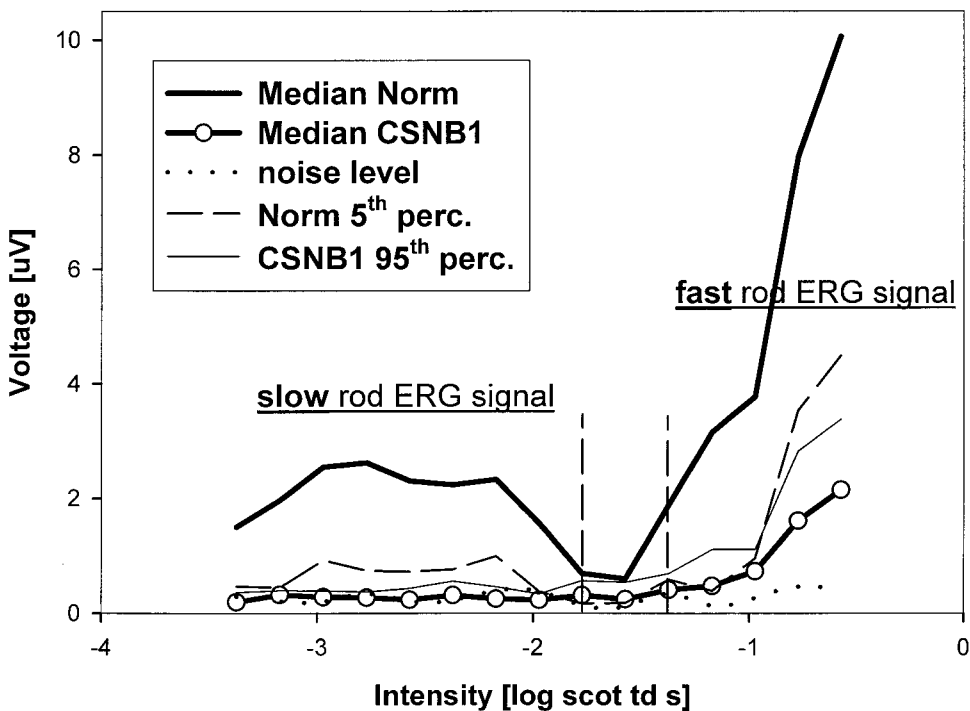


FIGURE 2. Rod ERG amplitudes to 15-Hz flicker stimulation with increasing flicker intensity for the normal subjects and the patients with CSNB1. For the slow pathway, the ERG signals were at the noise level for the patients with CSNB1 and there was virtually no overlap between the two subject groups. For the fast rod ERG signals, the patients with CSNB1 exhibited ERG signals that were considerably above the noise level. The ERG amplitudes, however, were considerably reduced.

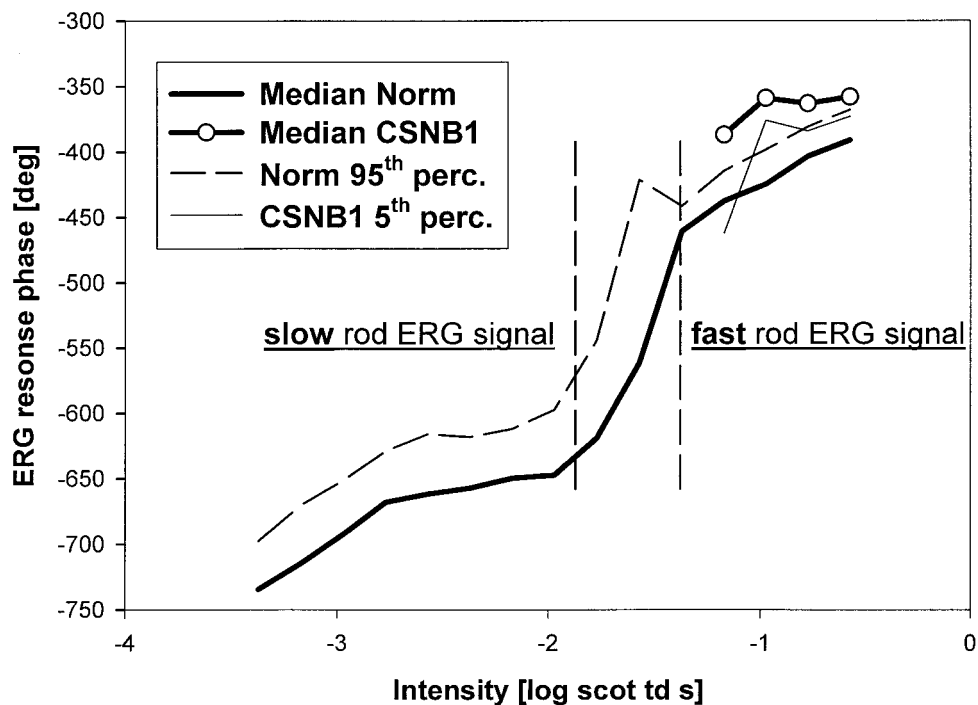


FIGURE 3. Rod ERG phases to 15-Hz flicker stimulation with increasing flicker intensity for the normal subjects and the patients with CSNB1. Because the ERG signals were indistinguishable from noise for the slow pathway, there was simultaneously no proper alignment of ERG response phases. Accordingly, the phase data for the slow rod ERG signals are omitted. For the fast rod ERG signals, however, there was a proper alignment of adjacent phase data. However, these ERG response phases were significantly increased (advanced in phase) in the patients with CSNB1.

were all indistinguishable from noise. There was virtually no overlap between the two subject groups for the slow ERG rod signal.

We nevertheless analyzed the amplitudes of the slow ERG rod signals (eight intensity levels) using a MANCOVA (to correct for the influence of age). Because the residuals of amplitudes for both rod ERG pathways did not follow a normal distribution, we converted the amplitudes into their logarithms, which had normal distributed residuals. The MANCOVA revealed significantly lower amplitudes in the patients with CSNB1 (F test, exact F = 190; $P < 0.0001$).

The ERG phases served as a second proof that there was no ERG response at the lower flicker intensities. In Figure 3, the median and the 5th and 95th percentiles of the normal subjects and the patients with CSNB1 are displayed. However, it was not possible to define an ERG response phase for the slow rod signals, because there was no proper alignment of the phase behavior between adjacent ERG responses, whereas this was the case for all normal subjects: In these subjects, there was a gradual increase in ERG response phase (phase advance) with increasing flicker intensity (see Fig. 3). In the patients with CSNB1, the ERG phases of the slow pathway were therefore omitted in Figure 3.

Fast Rod ERG Signals

As displayed in Figure 1, there was an ERG signal detectable in patient 8 for flicker intensities above -1.17 log scot td \cdot sec. This was true for all patients with CSNB1. In the patients as a group, the ERG amplitudes of the fast ERG rod signal were significantly above noise (Fig. 2). However, they were considerably lower than normal. Individual patients exhibited ERG signals only slightly above noise level (patients 3, 10, and 11). The calculation of the relative percentage proportion of the normal mean ERG amplitude for the fast rod ERG signals revealed that the amplitudes were reduced to values between 5% and 34% (median: 18%).

A MANCOVA of logarithms of fast rod ERG amplitudes (at four intensity levels) revealed significantly lower amplitudes in the patients with CSNB1 (F = 64; $P < 0.0001$). There was no

significant influence of age (F = 3.6; $P = 0.07$). There was next to no canonical correlation with refractive error ($r = 0.09$; $n = 20$) or visual acuity ($r = 0.03$; $n = 20$).

The ERG phases of the fast rod ERG signal showed a proper alignment between adjacent data points, which served as proof that there was an actual ERG response, even in the patients with ERG responses near noise level. As for the normal subjects, the ERG response phase increased with increasing flicker intensity (Fig. 3). Compared with the normal subjects, however, the level of all ERG phases of the fast rod ERG signals was increased (advanced), in the patients with CSNB1. A MANCOVA of fast rod ERG phases revealed that this phase advance was significant (F = 52; $P < 0.0001$). Age did not influence the ERG phases significantly (F = 1.3; $P = 0.26$). There was little canonical correlation with refractive error ($r = 0.06$; $n = 20$) and visual acuity ($r = 0.13$; $n = 20$).

DISCUSSION

We studied the slow and fast rod signals of the scotopic 15-Hz flicker ERG in patients with CSNB1 who carried mutations in the NYX gene. The slow rod ERG signals were indistinguishable from noise. The fast rod ERG signals, however, were significantly above noise, but they were significantly reduced in amplitude and advanced in phase.

The general group characteristics were in good agreement with those reported in the literature for CSNB1.^{4,7} Many of our patients with CSNB1 had moderate or high myopic refractive errors. None of them was hyperopic. The variable decrease in visual acuity was also on the order of magnitude reported in recent studies.

In accordance with our previous study,⁷ all patients with CSNB1 exhibited a negative ERG, as reported first by Schubert and Bornschein²—that is, the amplitude of the a-wave was larger than that of the b-wave for the maximal response. As suggested by Miyake et al.,⁴ two different groups of X-linked CSNB with such a negative ERG can be differentiated on the basis of the standard scotopic ERG alone—namely, a complete form (CSNB1) with no rod function (rod b-wave) and an in-

complete form (CSNB2) with residual rod function.⁴ Accordingly, in none of our patients with CSNB1 was the rod ERG b-wave detectable. Miyake et al. further reported that the a-wave amplitude was within normal limits for most of their patients in both subgroups; however, they did not provide data to show whether there is a significant decrease in amplitude in patients with X-linked CSNB, as a group. In 10 of 21 eyes in our patients with CSNB1, however, the a-wave was subnormal and in the patients as a group, there was a significant amplitude decrease. It therefore may be oversimplified to state that patients with CSNB of the Schubert-Bornschein type have grossly normal a-waves. In accordance with Miyake et al., in the current study a subset of the patients with CSNB1 exhibited decreased amplitudes of both the photopic b-wave and the 30-Hz flicker ERG; the implicit times were all normal for the two ERG responses.

The standard ERG does not allow differentiation of distinct rod pathways in normal or affected retinae. However, anatomic and physiological studies of the mammalian retina have revealed the existence of separate rod pathways. Rods are thought to synapse with a single type of bipolar cell, the rod ON bipolar cell.³¹⁻³³ This cell, in turn, contacts the AII amacrine cell at a sign-preserving glutamate synapse.³⁴⁻³⁸ Signals from the AII cell then infiltrate the main cone circuitry by exciting ON cone bipolar cells and inhibiting OFF cone bipolar cells.^{35,38-40} Thereafter, ON bipolar cells excite ON ganglion cells, and OFF bipolar cells excite OFF ganglion cells. A second pathway (the rod-cone-coupling pathway) infiltrates the ON and OFF cone bipolar circuitry at the earliest possible stage, through gap junction contacts, which allows electrical synaptic transmission.^{36,41-43} Through these gap junctions, signal flow involves ON and OFF cone bipolar cells and thereafter ON and OFF ganglion cells.^{36,42,44,45} A number of studies have provided evidence that the slow and fast rod ERG signals revealed in the human scotopic 15-Hz flicker ERG represent electrophysiological signals that are driven by these two separate rod pathways.^{17,18,20} It cannot be ruled out, however, that a direct rod-to-cone OFF bipolar cell pathway that has been recently described in the wild-type mouse⁴⁶ may be involved in generating the scotopic 15-Hz flicker ERG. However, it is presently unclear whether this third rod pathway is common to all mammalian retinae.⁴⁷

Similarities between the response versus intensity functions of the various components of the single flash ERG—the scotopic threshold response (STR), the DC component, and the rod b-wave on the one hand and the slow and fast rod ERG signals on the other hand—suggest that the slow ERG signals, the STR, and the DC component reflect activity in one common pathway and the fast rod signal and the single-flash rod ERG b-wave in another.¹⁸ Moreover, in the clinical routine, the rod b-wave is measured with flash intensities at which we obtained the fast rod signals ($-0.97 \log \text{scot td} \cdot \text{sec}$; cf. Figs. 1, 2, and 3) supporting the assumption that the rod b-wave is driven by the rod-cone-coupling pathway as well.¹⁸ However, the comparison is complicated, because the single-flash ERG and the scotopic 15-Hz flicker ERG are measured under very different conditions: The single-flash ERG is measured with flashes that are separated in time with the expressed purpose of avoiding the effects of light adaptation, whereas the scotopic 15-Hz flicker ERG is measured with prolonged trains of flashes. As a result, light adaptation plays a much greater role in the production of the scotopic 15-Hz flicker ERG than of the single-flash ERG response.¹⁸

There is plenty of evidence that the origin of the rod b-wave is postreceptoral and that it is determined predominantly by activity in the depolarizing (ON) bipolar cells.⁴⁸⁻⁵¹ In analogy, it has been suggested before, that the scotopic 15-Hz flicker ERG reflects electrical activity mainly of rod and cone bipolar

cells, although a contribution of many retinal elements (such as the receptors, the rod-cone gap junctions, or the AII cells) cannot be completely ruled out.¹⁷

CSNB1 was initially thought to be due to defective neuro-transmission from rods to rod ON bipolar cells,^{2,4} but later studies, mainly using long-duration stimuli (eliciting ERG waveforms at light onset and offset), revealed that the defect is general to the retinal ON pathway involving both rod and cone signals.⁵²⁻⁵⁵ Because we found no ERG signal for the lower flicker intensities we suggest that a defective nyctalopin leads to a complete blockage of signal transmission from rods to rod ON bipolar cells. However, there is apparently residual signal transmission through rod-cone gap junctions. Therefore, we provide the first evidence suggestive of postreceptoral rod-derived ERG signals in complete CSNB. Our findings contradict the preliminary observations made by Sharpe and Stockman²⁰ in two patients with CSNB of the Schubert-Bornschein (complete) type. No detectable fast rod ERG signals were shown in these patients, and these findings challenged the model of a rod-cone-coupling pathway. The existence of a residual fast rod ERG signal in our patients with CSNB1, however, provides further support for this model suggested by Sharpe and Stockman.^{17,18,20,56,57}

It is tempting to speculate about the origin of these residual responses that were below 50% of the normal mean amplitude. Possibly, this residual rod signal transmission involves the OFF cone bipolar cells. Reports of several studies have shown that for long flash stimuli, the b-wave is greater than the d-wave.^{55,58} Consistently, the complete loss of signal transmission through ON cone bipolar cells would be consistent with the survival of the fast rod ERG signal but also with its magnitude of amplitude decrease. The timing of the fast rod ERG signals in patients with CSNB1 would also be consistent with rod signal transmission through OFF cone bipolar cells. As discussed previously,²¹ the actual response phases can differ by integer multiples of 360° from the phases obtained from the Fourier analysis. We therefore assimilated the phase data of each subject to the implicit times obtained from the single flash ERG at a comparable flash intensity. Under these conditions, we determined that the ERG response phase for the fast rod signals was approximately 50° increased (i.e., the ERG response was approximately 50° advanced in phase) in the patients with CSNB1. This would correspond to a 9-msec time difference compared with the normal subjects, assuming that a time-delay difference is the cause of the phase difference. (It is very implausible that the ERG response phase should be decreased by 310° corresponding to a time delay of 57 msec). Sieving et al.⁵⁹ reported that the ERG response latency for the photopic OFF pathway is 5- to 9-msec shorter than for the ON pathway, which is on the order of magnitude of the acceleration of the fast rod ERG signals in the patients with CSNB1.

In conclusion, our data favor the hypothesis that in patients with CSNB1 and *NYX* gene mutations there is absent rod signaling through rod ON bipolar cells, but preserved rod signaling through rod-cone gap junctions and OFF cone bipolar cells. However, other mechanisms or retinal sites of alteration in rod function caused by *NYX* mutations cannot be excluded. It has been hypothesized that nyctalopin may play a role in development of retinal circuitries^{15,16} leaving the possibility that there may be abnormal rod pathways in CSNB1 that are normally absent or unimportant.

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