ORIGINAL CONTRIBUTION

Molecular Genetic Reevaluation of the Dutch Hyperekplexia Family

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Objectives: To confirm linkage of the locus of the major form of hyperekplexia to markers on chromosome 5q, to screen for a point mutation in the gene encoding the α1 subunit of the glycine receptor, and to investigate whether the putative "minor" form of hyperekplexia, consisting of an excessive startle response without stiffness, is based on the same genetic defect as the major form.

Design: A survey of various symptoms of hyperekplexia was performed in the Dutch pedigree. Linkage studies were performed for these symptoms.

Setting: Subjects were visited at home, and the genetic study was performed at University Hospital Leiden, (the Netherlands).

Patients: A history was taken from 76 subjects in the pedigree, and neurologic examinations were performed on 61 subjects from four generations of the pedigree.

Main Outcome Measures: The main outcome measures were lod scores for markers on chromosome 5q for the major and minor forms of hyperekplexia and periodic leg movements during sleep. Mutations in the α1 subunit of the glycine receptor were detected by screening the exons with denaturing gradient gel electrophoresis.

Results: Exaggerated startle responses were reported in 44 patients. The major form consisted of stiffness in addition to the excessive startle reaction and occurred in 28 subjects. Sixteen of 44 subjects had startle responses without stiffness, indicating the minor form. Linkage was found between markers CSF1-R, D5S209, and D5S119 and the disease locus for the major form, but not for the minor form. The α1 subunit of the glycine receptor showed a G to A transition mutation in codon 271 for the major form, but not for the minor form.

Conclusions: Linkage and an abnormal glycine receptor were found only in the major form of hyperekplexia. Recognition of a major form is based on additional stiffness. This is therefore the most important diagnostic symptom. The minor form is not a different expression of the same genetic defect and may represent a normal but pronounced startle response.

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HYPEREKPLEXIA, or startle disease, is an autosomal dominant disorder characterized by exaggerated startle reactions to unexpected, particularly auditory, stimuli. Suhren et al[1] described the syndrome in detail in a large Dutch family in 1966. Subsequent reports[2-8] have confirmed its familial occurrence. In the Dutch family, two forms of expression of this disease were recognized. The major form is characterized by excessive startle reactions followed by a short period of generalized stiffness, during which voluntary movements are impossible. Patients remain conscious during the response. The patients with the major form showed an extreme generalized stiffness immediately after birth, which normalizes during the first years of life. The severity of the disorder in adults is determined by the frequency of startle responses; it varies considerably, not only between subjects but also over time.

Besides patients with the major form, patients with a putative "minor" form were described based on excessive startle reactions without stiffness. In the Dutch family, patients with the major form had appeared to pass on the minor form of the disease to their children; in one case, a patient with what seemed the minor form had offspring with the major form.

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See Patients and Methods on next page

PATIENTS AND METHODS

PATIENTS

We reinvestigated the Dutch family with hyperekplexia described by Suhren et al[1] in 1966 and included a new generation (**Figure 1**). A history was obtained from 76 individuals (32 patients and 44 unaffected relatives), and a neurologic examination was performed in 61 individuals (31 patients and 30 unaffected relatives) from four generations. In 15 subjects, no neurologic examination was performed: one patient (patient IV-3) refused, while 14 unaffected family members lived far from our hospital. Blood samples were obtained from 59 of the 61 individuals examined (two patients were young children) and 15 spouses.

The major form of hyperekplexia was defined by a history of exaggerated startle responses to auditive, tactile, or visual stimuli and the presence of stiffness following the startle reaction. In the absence of stiffness, the minor form was diagnosed. In addition, the presence or absence of PLMS was verified, as either noted by the patients themselves or their partners. Patients were also included in this category when they experienced sudden body jerks on falling asleep at least once a week.

GENETIC MARKER ANALYSIS

Genomic DNA was isolated from freshly collected blood, as previously described.[17] Analysis of microsatellite markers was performed with multiplex polymerase chain reactions on all individual DNA samples.[17,18] The microsatellite markers interleukin-9,[19] D5S210, D5S207,[20] CSF1-R,[21] D5S209,[18] D5S119,[20] D5S422,[22] and D5S211[20] were included (**Figure 2**). Reaction products were separated on a 6% denaturating polyacrylamide gel containing 7-mol/L ureum. The gels were fixed, dried, and subjected to autoradiography for 12 to 18 hours, without an intensifying screen. Marker genotypes were subsequently determined for each individual by visual inspection.

LINKAGE ANALYSIS

Statistical analysis was performed by the computer program Linkage package, version 5.04.[23] Hyperekplexia was assumed to be an autosomal dominant disease with complete penetrance. Frequencies of marker alleles were determined in spouses and in the family with hyperekplexia. Linkage analysis was performed on individual sibships and on the complete pedigree; evidence for or against linkage is given as logarithm base 10 of the odds in favor of linkage (lod score). Initially, only the classic form of hyperekplexia was scored in the linkage analysis; persons with the minor form or PLMS were treated as unaffected. The hypothesis of the excessive startle reaction being a minor form of hyperekplexia was investigated by evaluating whether patients with the minor form had inherited the marker alleles that were linked to the disease gene in this family. The possibility of an additional gene on chromosome 5 being responsible for PLMS in this family was investigated separately; for this purpose, the occurrence of excessive startle responses or stiffness was neglected. The frequency of PLMS in the general population was assumed to be approximately 5%.[24]

SCANNING EXONS FOR POINT MUTATIONS BY DGGE AND SEQUENCING OF GLRA1

Polymerase chain reaction primers within intron sequences of the GLRA1 were designed to amplify all exons from genomic DNA of affected individuals and controls (R.S., oral communication, 1994, data not shown). The goal was to amplify the exons from DNA from individuals with hyperekplexia and to use DGGE to scan for DNA sequence alterations unique to the affected individuals.[16] Exons showing an aberrant DGGE pattern were cloned and sequenced.

Polymerase chain reaction products from exon 6 were directly cloned into the pCRII vector with use of a cloning kit (Invitrogen TA, San Diego, Calif).[16] Six indepedent clones were sequenced on an automated sequencing apparatus (Pharmacia ALF, Uppsala, Sweden). Mutations were detected by comparing cloned sequences with the published cDNA sequence.[16]

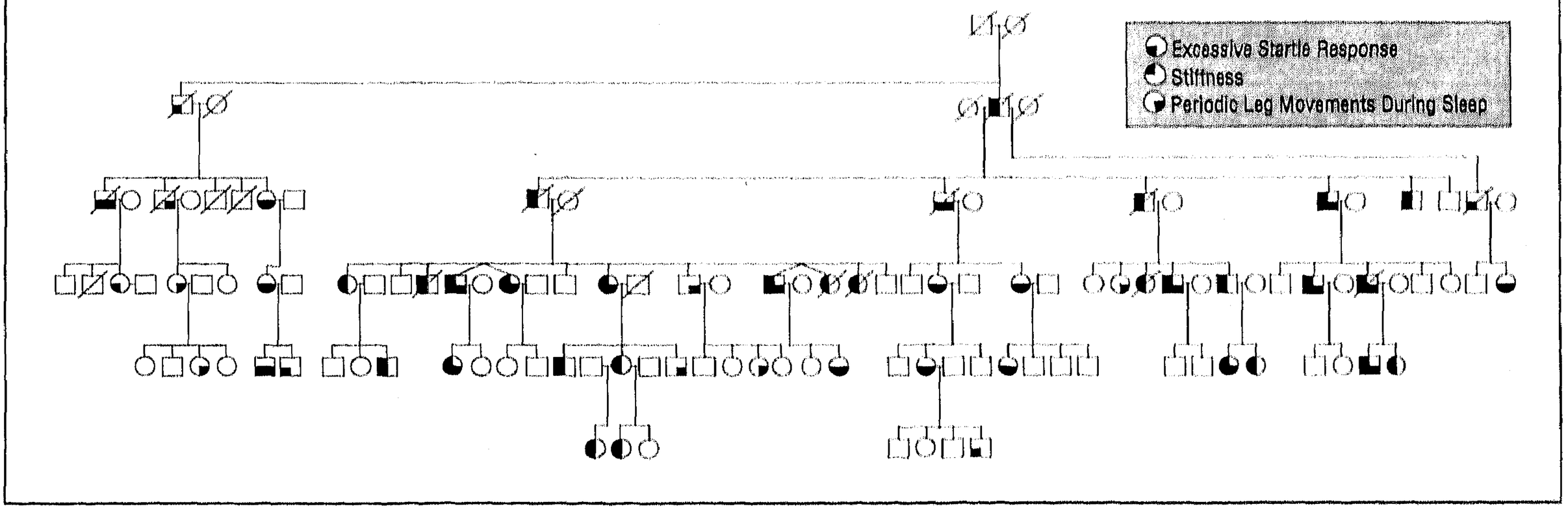

Figure 1. *Hyperekplexia pedigree. The numbers in the pedigree are similar to the numbers of the original pedigree.[1] In the fifth and sixth generations, new subjects are included.*

Accordingly, the two forms were considered to represent variations in expression of the same disease gene. In other families segregating for hyperekplexia, the occasional occurrence of the minor form has been confirmed.[2,8-10]

Autosomal dominant inheritance with nearly complete penetrance and variable expressivity is seen in most pedigrees.[2-5,8,11,12] On the basis of findings obtained in small sibships, some authors suggested an autosomal recessive inheritance pattern or the occurrence of new mutations.[2,4,6,8,13]

Hypnagogic myoclonus and periodic leg movements during sleep (PLMS) are frequently associated with hyperekplexia.[2-6,8,10,11,13-15] The occurrence of PLMS in unaffected sibs has not been signaled.

Recently, Ryan et al[7,12] mapped a hyperekplexia locus on chromosome 5q33-q35 in four families with the major form of hyperekplexia. Further studies identified point mutations in the gene encoding the α1 subunit of the glycine receptor (GLRA1). Two different mutations were found in the same position, resulting in substitution of an uncharged amino acid (leucine or glutamine) for Arg271 in the mature protein.[16] Neither the minor form of hyperekplexia nor the occurrence of PLMS was discussed in those studies.

The first aim of our study was to confirm linkage of the major form of hyperekplexia on chromosome 5q33 in the Dutch hyperekplexia pedigree. Second, we tested whether the family members with the putative minor form had inherited the chromosome with the major hyperekplexia gene defect, as determined by haplotyping. The third aim was to record the occurrence of PLMS in the pedigree in relation to the genetic markers. Finally, we scanned the gene for GLRA1 for point mutations using denaturing gradient gel electrophoresis (DGGE) in patients with the major and minor form, and sequenced exon 6.

RESULTS

Exaggerated startle responses to auditive, tactile, or visual stimuli were found in 44 patients (22 male and 22 female), 32 of whom were alive at the time of investigation. Stiffness in addition to the startle reaction occurred in 28 of the 44 patients, indicating, according to the definition, the major form; 23 of these were known to have had generalized stiffness directly after birth. No reliable information could be obtained concerning the neonatal occurrence of stiffness in the remaining five individuals of generation III (Figure 1). All patients who were known to have had stiffness at birth also suffered from stiffness in relation to the startle reaction. Among 44 patients with excessive startle reactions, 16 had never manifested stiffness either after startle or at birth; these patients had the minor form of hyperekplexia.

The transmission of the major form of hyperekplexia in this family was consistent with autosomal dominant inheritance. The major form was frequently passed on as the major form and four times as the minor form. However, those with the minor form only passed on the minor form, never the major form. Patient III-8 seemed to be one exception to this rule (Figure 1). In 1966, he had reported an excessive startle reaction without stiffness and passed the major form of the disease on to his children. However, based on detailed family history of several close relatives, who reported to have seen him fall several times owing to generalized stiffness in relation to his startle reaction, he is now considered to have the major form.

In **Table 1**, evidence for linkage between each of the chromosome 5 markers and the major form of hyperekplexia is presented as lod scores. Close linkage without recombination was found between markers CSF1-R, D5S209, and D5S119 and the disease locus. For these markers, an identical haplotype was found in all 19 patients with the major form, indicating tight linkage.

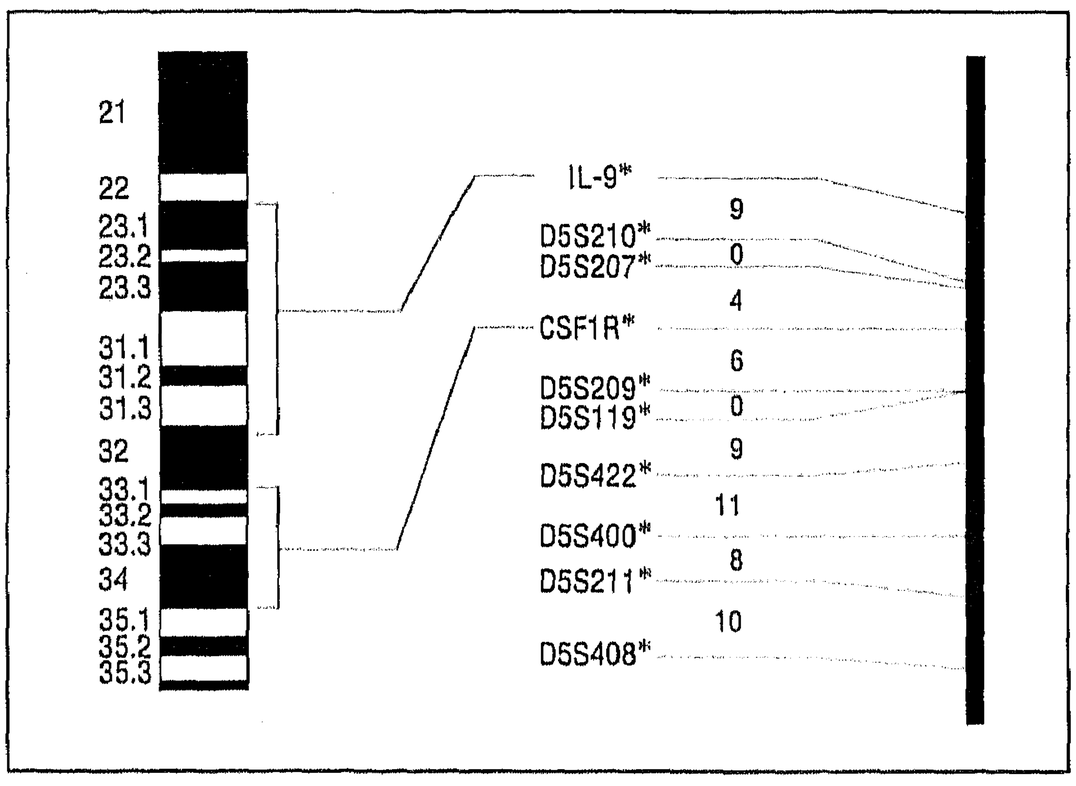

Figure 2. *Sex-averaged map of chromosome 5q21-qter[21,25] and used markers.*

Table 1. The Lod Scores for Markers on Chromosome 5q for the Major Form of Hyperekplexia

Marker	Theta*							Marker Heterozygosity
	0.0	0.01	0.05	0.1	0.2	0.3	0.4	
D5SIL9	−∞	1.708	3.933	4.395	4.007	2.955	1.527	0.62
D5S210	−∞	−6.054	−0.341	1.613	2.688	2.437	1.461	0.75
D5S207	−∞	−3.074	−0.511	0.377	0.868	0.754	0.385	0.69
CSF1-R	6.005	5.890	5.417	4.800	3.480	2.057	0.618	0.86
D5S209	9.894	9.754	9.170	8.391	6.664	4.682	2.409	0.71
D5S119	−∞	8.897	9.035	8.535	6.980	4.975	2.569	0.49
D5S422	−∞	−9.268	−4.796	−3.168	−1.734	−0.965	−0.438	0.84
D5S211	−∞	14.973	−6.864	−3.665	−1.035	−0.061	0.182	0.72

**Theta is the recombination fraction; heterozygosity indicates the allele frequency of the different markers in the population.*

Of the 10 patients with the putative minor form, three (patients III-5, IV-11, and V-66) had a haplotype similar to those patients with the major form, but seven had different haplotypes. This is consistent with what one would expect based on chance alone.

Individuals with PLMS are also shown in Figure 1. The frequency of PLMS in patients with the major form or the minor form of hyperekplexia and the healthy sibs is presented in **Table 2**; PLMS occurs with high frequency in all three groups. Exclusion of tight linkage between the DNA markers and PLMS is evidenced by the highly negative lod scores obtained at small values of the recombination frequency (**Table 3**). Interestingly, marker D5S209 yielded positive lod scores at higher recombination frequencies, consistent with a location of PLMS more than 20 cM away from this marker. However, this tentative location was not confirmed by markers located at some distance of D5S209, such as D5S210 and D5S422.

By screening the exons of GLAR1 with DGGE, a change was found in exon 6 in a patient with the major form of hyperekplexia (patient IV-14). After cloning exon 6 of this patient, sequencing eight clones showed that three had the wild type sequence, while five had a G to A mutation in codon 271, reflecting the heterozygotic state of the patient. A patient (patient IV-11) with the minor form of hyperekplexia showed no change by screening with DGGE, and no further sequencing was performed. No changes were found with DGGE in a patient with PLMS (patient IV-19).

COMMENT

Clinical studies in families with hyperekplexia have reported highly variable expression, including major and minor forms.[1-8,11,13] This variability might in fact reflect different origins for excessive startle responses. This could be investigated only when the cause could be ascertained. As a first step in approaching the pathophysiology, Ryan et al[7] recently reported linkage to the markers CSF1-R, D5S119, D5S209, and D5S379 in four families with an autosomal dominant form of hyperekplexia. In additional studies based on the linkage results, point mutations were found in GLAR1. In the families studied, stiffness at birth and/or in relation to the startle reaction were invariably present and considered the criterion for the disease (major form).

In our family, comprising both the major and the putative minor form of hyperekplexia, we confirmed linkage between the CSF1-R, D5S119, and D5S209 markers and the major form hyperekplexia locus. The same marker alleles that were shared by all patients with the major form were found only in a minority (three of 10) of the patients with the minor form, suggesting that the minor form is not part of the same disease. This hypothesis was confirmed by screening GLRA1 with DGGE. Patients with the major form showed a mutation in exon 6. By sequencing exon 6, a G to A transition in codon 271 was found. Patients with the minor form did not have this mutation. Together, these results exclude the possibility that major and minor forms of hyperekplexia constitute different phenotypes of the same gene defect.

Therefore, the question arises whether the minor form does in fact constitute an integral part of the disease. A common genetic basis for the major and minor forms appeared to be plausible in the original evaluation of this pedigree, as the major form was once transmitted trough a patient with the minor form. However, it has subsequently become clear that this patient, who had not volunteered any information on stiffness following startle response, should be classified as having the major form of the disease based on independent family history information from several close relatives.

In other reports, the number of patients with stiffness at birth and/or in relation to the startle reaction varies. In most large families, all patients had the major form.[3,5,12,14,15,25] However, in four families, both the major and the minor forms occurred. In these families, the major form was passed on as the minor form and vice versa.[2,8-10] It is important to realize that only one or two patients had the major form of hyperekplexia in these families.

While our findings disprove the hypothesis that the familial occurrence of the major and minor forms of hyperekplexia are caused by a single gene defect, one might sug-

Table 2. The Frequency of Periodic Leg Movements During Sleep (PLMS) in Patients With the Major and Minor Forms of Hyperekplexia and in Healthy Sibs

		PLMS		
Diagnosis	No.	Present	Absent	?
Hyperekplexia				
Major form	28	11	11	6
Minor form	16	12	2	2
Healthy sibs	44	9	30	5

Table 3. The lod Scores for Markers on Chromosome 5q for Periodic Leg Movements During Sleep (PLMS)

	Theta*						
Marker	0.0	0.01	0.05	0.1	0.2	0.3	0.4
D5S210	−15.037	−11.981	−7.478	−4.568	−1.599	−0.270	0.169
D5S207	−4.363	−3.477	−2.311	−1.458	−0.389	0.062	0.145
CSF1-R	−4.516	−2.915	−1.159	−0.273	0.305	0.217	−0.068
D5S209	−3.565	−1.518	0.610	1.545	2.022	1.708	0.950
D5S119	−4.511	−3.051	−0.979	0.204	1.154	1.194	0.696
D5S422	−9.552	−8.399	−5.808	−3.974	−1.877	−0.744	−0.150

**Theta is the recombination fraction.*

gest alternatively that apart from the major hyperekplexia gene on the chromosome 5q, another gene elsewhere in the genome is responsible for the minor form of hyperekplexia. The rarity of the disease makes the presence of two mutations in one pedigree very unlikely. However, it remains possible that the occurrence of an excessive startle response as an autosomal dominant or sporadic trait may be much more common than previously believed.[13] Obviously, startle responses are well known by all subjects in these pedigrees, increasing the chance that pronounced but normal startle reactions are considered abnormal in a hyperekplexia pedigree. If so, linkage studies in hyperekplexia will be complicated by the admixture of such "other" startle responses. For a better discrimination between normal and abnormal startle reactions, the normal variation in motor startle reactions should be delineated further through quantitative neurophysiologic investigations.

The occurrence of hypnagogic myoclonus and PLMS in the families with hyperekplexia presents another classification problem. Presumably, PLMS exists in 5% to 6% of the healthy population.[24] We found an occurrence of PLMS of at least 21% in nonhyperekplectic sibs of patients with hyperekplexia. The distribution of PLMS over the pedigree suggests an autosomal dominant inheritance of PLMS, with incomplete penetrance, unrelated to hyperekplexia. We hypothesized that a gene for PLMS might be located in the vicinity of the hyperekplexia locus and that recombination could account for the occurrence of PLMS without excessive startle reactions. This was not confirmed by a linkage study with three markers of the hyperekplexia locus or with the markers tested further away on chromosome 5q.

In conclusion, the present molecular genetic reevaluation of the Dutch family with hyperekplexia clearly demonstrates that only the major form constitutes part of the hyperekplexia phenotype. Stiffness, after the startle reaction and in the first years of life, is therefore the strongest diagnostic criterion, even though the startle responses attract more attention.

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