J Neurol (2002) 249 : 1515 – 1519
DOI 10.1007/s00415-002-0860-8

ORIGINAL COMMUNICATION

A. M. J. M. van den Maagdenberg
E. E. Kors
E. R. Brunt
W. van Paesschen
J. Pascual
D. Ravine
S. Keeling
K. R. J. Vanmolkot
F. L. M. G. Vermeulen
G. M. Terwindt
J. Haan
R. R. Frants
M. D. Ferrari

Episodic ataxia type 2
Three novel truncating mutations and one novel missense mutation in the CACNA1A gene

Received: 20 December 2001
Received in revised form: 15 April 2002
Accepted: 22 April 2002

M. D. Ferrari, MD PhD (✉) · E. E. Kors · G. M. Terwindt · J. Haan
Department of Neurology, K5Q
Leiden University Medical Centre
P. O. Box 9600
2300 RC Leiden, The Netherlands
Tel.: +31–71/5 26 28 95
Fax: +31–71/5 24 82 53
E-Mail: M.D.Ferrari@LUMC.nl

A. M. J. M. van den Maagdenberg · K. R. J. Vanmolkot · F. L. M. G. Vermeulen · R. R. Frants
MGC-Department of Human Genetics
Leiden University Medical Centre
Leiden, The Netherlands

E. R. Brunt
Department of Neurology
Groningen University Hospital
Groningen, The Netherlands

W. van Paesschen
Department of Neurology
University Hospital Gasthuisberg
University of Leuven, Belgium

J. Pascual
Department of Neurology
University Hospital Marques de Valdecilla
Santander, Spain

D. Ravine · S. Keeling
Institute of Medical Genetics
University Hospital of Wales
Cardiff, United Kingdom

Abstract We analysed the CACNA1A gene, located on chromosome 19p13, in three unrelated families and one sporadic case with episodic ataxia type 2 (EA–2). In two of the families and the sporadic patient, novel truncating mutations, which disrupt the reading frame and result in a premature stop of the CACNA1A protein, were identified in exons 14, 16 and 26. In the remaining family, a novel missense mutation (H253Y) was found. Of the twenty two EA–2 mutations identified thus far, including those of the present study, seventeen are truncating mutations and five are missense mutations, all resulting in an EA–2 clinical phenotype.

Key words episodic ataxia type 2 · EA–2 · CACNA1A · calcium channel · mutation analysis

Introduction

Episodic ataxia type 2 (EA–2) is an autosomal dominant disorder characterised by acetazolamide-responsive attacks of cerebellar ataxia, lasting for hours to days. Attacks are often provoked by fatigue, stress, alcohol or exercise. Interictal nystagmus, vertigo and cerebellar atrophy has been reported [1, 2, 20].The EA–2 locus was identified on chromosome 19p13 [12, 16–18], and the causative gene, CACNA1A, encodes a pore-forming subunit of P/Q-type calcium channels [14]. Mutations in this gene cause a diverse spectrum of diseases, including a severe subtype of migraine with aura (familial hemiplegic migraine; FHM), spinocerebellar ataxia type 6 (SCA6), EA–2 and epilepsy [11, 14, 21]. FHM is caused by missense mutations and SCA6 is the result of moderate expansions of a carboxy terminal CAG-repeat encoding a small polyglutamine stretch. Truncating mutations, resulting in premature stops and leading to putative aberrant CACNA1A proteins were identified in EA–2 patients [5, 10, 11, 14]. At present, fourteen different truncating mutations are known [5, 10, 11, 14, 15].

Recently, four different missense mutations in the CACNA1A gene in EA–2 patients were identified [6–9]. The clinical phenotype associated with these EA–2 missense mutations (F1406C, F1491S, R1662H (named R1666H by the authors) and E1757K) is indistinguishable from the phenotype associated with the truncating mutations. So, no simple correlation between the type of

mutation and the phenotype in the CACNA1A gene exists.

As a range of truncating mutations along the CACNA1A gene is associated with an EA–2 phenotype, it was initially suggested that the disease may occur as a result of haplo-insufficiency [14]. Two truncation mutations (R1549X and R1279X) have been analysed and revealed a complete loss of channel function, supporting the hypothesis of haploinsufficency as the plausible cause of EA–2 [9, 19]. The same effect on calcium channel function was observed for missense mutations F1491S and F1406C [8, 9]. In contrast, CACNA1A protein harbouring the AY1593/1594D mutation was shown to be able to form calcium channels in *Xenopus laevis* oocytes but with pronounced loss of function [19]. Another mechanism, namely a dominant negative effect, has been observed recently with the R1820X truncation [11]. Here, we report on the finding of three novel truncating and one missense mutation in four EA–2 families, adding to the genetic heterogeneity of the disorder.

Material and Methods

Patient material

The pedigrees of four families are shown in Fig. 1.

All affected family members show a typical EA–2 phenotype, consisting of childhood-onset attacks of ataxia lasting hours to days, triggered by stress and/or physical exercise. Interictal neurological examination in all patients revealed nystagmus and/or unsteadiness. Acetazolamide reduced the frequency or the severity in a proportion of patients. In addition to episodic attacks of ataxia, the proband of family 3 has moderate mental retardation.

Family 1

The proband (II–2), a 40-year-old male, had a long history starting in childhood, of episodic ataxia accompanied by visual aura-like disturbances and a number of migrainous features. Later in life he was free of attacks for a prolonged period. Recently, attacks have re-occurred. Acetazolamide treatment has some beneficial effects on the attacks. Interictal examination revealed mild unsteadiness only. His brother (II–1) and mother (I–2) are also affected. His eldest daughter (III–1) has similar symptoms.

Family 2

The proband (II–1) was a 23-year-old man who, at the age of five, began to have multiple, weekly, episodes of severe ataxia lasting several hours. The ataxia was associated with vomiting but not with headaches. Interictal examination revealed nystagmus only. Cerebral MRI was normal. He responded well to acetazolamide treatment (500–750 mg daily), commenced at the age of 17, and attacks are now only precipitated by sleep deprivation and fasting for more than 12 hours. An attempt to lower the dose because of renal colic (a known complication of long-term treatment with acetazolamide), was stopped as the attack frequency increased instantly. Neither parent (both aged over 45) or all other family members has experienced similar symptoms.

Family 3

The proband (III–2), a 29-year-old woman, had, since the age of two years, weekly episodes of gait ataxia, vertigo and nystagmus, associated with headache and vomiting. She also experienced migrainous headache. From approximately 20 years of age, the episodes became less striking. Interictal examination revealed moderate mental retardation, a broad-based gait, saccadic smooth ocular pursuit, positional nystagmus, and upbeat nystagmus on fixation. Acetazolamide (400 mg daily) had no effect on attack frequency or severity.

Her brother (III–3) had experienced similar episodes, which started in the first year of his life. Acetazolamide reduced the attack frequency to 50 %. Their father (II–3) also had EA–2 attacks and had cortical and cerebellar atrophy on cerebral MRI. A paternal uncle of the proband (II–1) had been diagnosed with small cell lung carcinoma complicated by a Lambert Eaton myasthenic syndrome, an autoimmune disorder caused by circulating autoantibodies against voltage-gated calcium channels, including P/Q-type calcium channels. He also had a history of episodes of a "drunken feeling" and unsteadiness after prolonged exercise.

Family 4

The proband (III–4) was a 47-year-old man, with attacks of gait ataxia and dysarthria that started at the age of ten. The attacks occurred once or twice weekly, typically during exertion and after alcohol intake, and lasted approximately 30 minutes. From the age of 40, he developed a slowly progressive, mild gait imbalance. Interictal examination revealed saccadic pursuit eye movements, lateral gaze nystagmus, and mild truncal ataxia. The daily use of 250 mg acetazolamide markedly reduced the attack frequency. Of his family, his mother (II–2), a maternal uncle (II–3), three out of six sibs (III–1, III–3, III–6) a nephew (IV–1) and his son (IV–2) suffered from identical attacks.

Genetic analysis

From the persons marked "+" in the pedigrees in Fig. 1, genomic DNA was isolated from peripheral blood cells according to standard methods [13]. All 47 exons and flanking intron sequences of the CACNA1A gene were analysed for mutations using single strand conformation polymorphism (SSCP) analysis, essentially according to Ophoff et al. [14]. In brief, intronic primers were used to amplify the coding regions of the CACNA1A gene, using genomic DNA as template. Primary PCR products were labelled by incorporation of [α]-^{32}P] dCTP in a second round of PCR. Samples were diluted and denatured in formamide buffer and electrophoresed on 8 % polyacrylamide (19:1) gels containing 10 % glycerol. Electrophoresis was carried out at room temperature at a constant power of 28 W. Finally, SSCP gels were dried and exposed for autoradiography.

When aberrant banding patterns were observed, the respective PCR products were directly sequenced using the BigDyeTM Terminator Cycle Sequencing kit (PE Applied Biosystems, Foster City, CA), using the amplification primers. Sequencing reaction fragments were then run on an ABI 377 DNA automated sequencer (PE Applied Biosystems), to reveal the underlying nucleotide sequence and mutations. The samples harbouring deletion mutations were cloned in vector pCR®II-TOPO (Invitrogen, Carlsbad, CA, USA), and inserts of recombinants were sequenced as described above, but now using standard M13 vector primers. For each mutation, we analysed a panel of 96 control DNAs from the Dutch population.

To determine the number of CAG repeats at the 3' end of the CACNA1A gene, a PCR reaction was performed using the forward primer 5'-GCCCCCTCAACATCTGGTA–3' and reverse primer 5'-GACCCGCCTCTCCATCCT–3' [14]. PCR products were electrophoresed on the same sequencer as described above and the number of repeats determined by direct comparison with an internal standard for size determination.

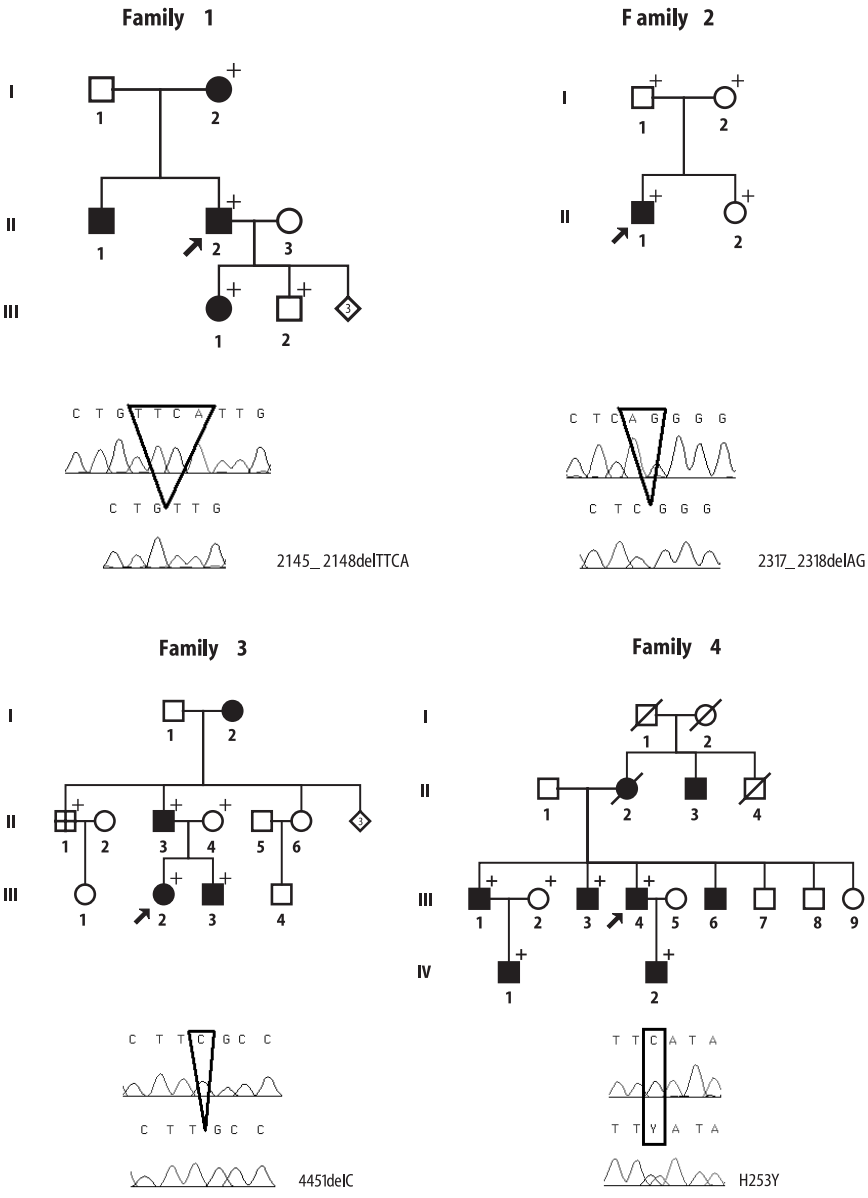

Fig. 1a+b Pedigrees of family 1 to 4. Affected members with an EA–2 phenotype are indicated by filled boxes (male) or circles (female). The box with cross symbol identifies an individual with Lambert-Eaton myasthenic syndrome and 'unsteadiness', but no diagnosis of EA–2. An arrow indicates the index case of the family. A plus indicates that DNA is available. A dash indicates a deceased person. Below the pedigrees an electropherogram of the relevant part of the CACNA1A sequence harbouring the mutation is shown, with the normal sequence above and the mutant sequence below.

Results

Mutations in the calcium channel pore-forming subunit CACNA1A were found in the four index cases (Fig.s 1 and 2).

A four base pair-deletion in exon 14, c.2145_2148delTTCA (nomenclature according to [4]; accession number X99 897 was used as the reference sequence), was identified in the index case from *family 1* (II–2). As a consequence of the deletion the predicted putative protein would be truncated in exon 15 at position c. 2248. The same mutation was also detected in samples from his mother and daughter (I–2 and III–1, respectively).

In the index case from *family 2* (II–1), a frameshift mutation (c.2145_2148delAG) was detected in exon 16. This mutation resulted in a putative protein that terminates prematurely in exon 19 at position c. 2615. Because neither of the parents carried the mutation, and non-paternity was ruled out by testing additional markers (data not shown), this has to be a *de novo* mutation.

In the index case of *family 3*, a single nucleotide deletion c. 4451delC was identified in exon 26. The frameshift resulted in a putative truncated protein because of a premature termination occurring in exon 27 (position c. 4564). The mutation was also found in the brother (III–3) and the father (II–3) of the proband. Although the paternal uncle (II–1) did experience several clinical symptoms that are reminiscent of EA–2 (f. i.

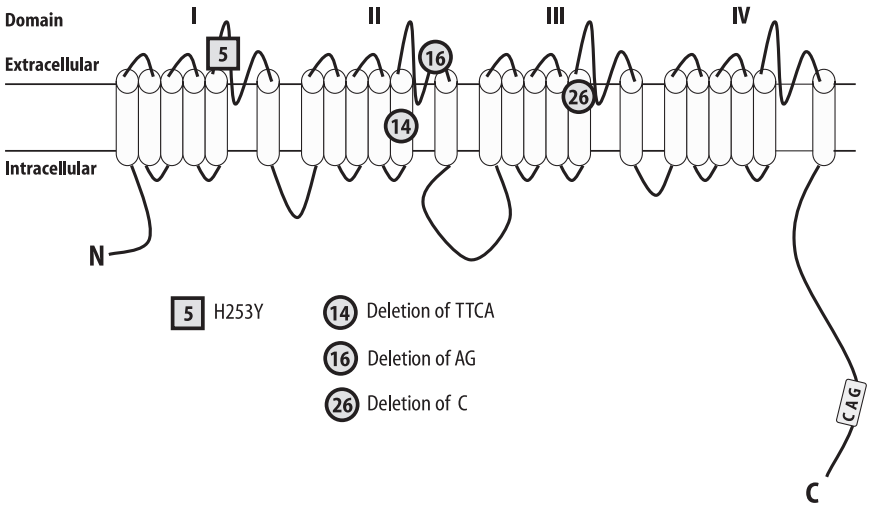

Fig. 2 Schematic representation of the CACNA1A calcium channel subunit protein.
This protein is the pore-forming, main unit of the P/Q type calcium channel. It forms the calcium channel, together with auxiliary subunits (β and α2δ).
The position of the three deletions are indicated by grey circles (with affected exon). The missense mutation is indicated by a grey box (with affected exon).

'unsteady gait') and LEMS, he did not have the mutation.

In the index case from *family 4*, a nucleotide substitution was detected in exon 5 (c. 1032C > T) substituting a histidine for a tyrosine (H253Y). The H253Y mutation is located in the highly conserved IS5-IS6 P-loop domain of the protein (Fig. 2). Conservation of histidine within this functional domain is preserved in both paralogues and ortologues of human CACNA1A (Fig. 3). DNA was available of four additional affected members of this pedigree (III–1, III–3, IV–1, IV–2) and an unaffected spouse (III–2). The affected members were found to be carriers of this missense mutation, and the spouse was not.

None of the mutations mentioned above were found in a set of 192 chromosomes of the general population that were tested as controls. In addition, we investigated whether any of the patients carried expansions of the CAG sequence that is present in the carboxy terminal region of the CACNA1A protein and that potentially could contribute to the observed variable expression of the EA–2 phenotype. All patients had CAG repeats that were within the normal range (4–18 CAG units; [21]) (data not shown).

	IS5	P-LOOP
Human CACNA1A	GLEFYM	GKFHTTCFEEGTD
Mouse CACNA1A	GLEFYM	GKFHTTCFEEGTD
Human CACNA1B	GLEFYM	GKFHKACFPNSTD
Human CACNA1E	GLEFYS	GKLHRACFVNNSG

Fig. 3 Sequence alignment of a part of IS5 segment and the relevant part of the P-loop between 1S5 and 1S6 of the CACNA1A protein harbouring the affected histidine residue. The position of the mutated histidine residue is indicated by an arrow. The conserved amino acids in this section of the P-loop are depicted by short bars. The following cDNA sequences (accession numbers) were used for the alignment: human CACNA1A (X99897), mouse CACNA1A (U76716), human CACNA1B (M94172) and human CACNA1E (L29384).

Discussion

We have characterised one novel missense and three truncating mutations within the CACNA1A gene. Including these newly characterised mutations, a total of 22 mutations has now been identified in patients with EA–2. Seventeen (77 %) of these are truncating mutations. All but two reported families have a typical EA–2-phenotype, with acetazolamide-responsive episodes of ataxia triggered by physical exercise, starting in childhood. In one atypical family with a c. 4914C > T mutation, resulting in an early stop R1547X, affected members also experienced hemiplegia during the EA–2 attacks [9]. In a second atypical family attacks of absence epilepsy were present in addition to the EA–2 phenotype and caused by a R1820X mutation [11]. It is now apparent that a remarkable variation in the clinical expression of the EA–2 phenotype exists [5, 9–11] suggesting that other modifying genetic and/or environmental factors play a role.

We confirm this inter-familial variation of clinical symptoms in the present study. For instance, family 3 displays a severe phenotype with a very early onset of the disease in the proband (III–2) at the age of two, and the brother (III–3) even in his first year, both with weekly episodes, which last up to hours. Currently, at the age of 16, the brother shows an impaired VOR suppression and saccadic dysmetria at interictal neurological examination. In contrast, the phenotype in the proband of family 1 (II–2) is less severe: the first attacks occurred in childhood, but there were prolonged attack-free periods. The patient only shows some mild unsteadiness at interictal examination at the age of 46. The five missense

mutations now known to be associated with an EA–2 phenotype are H253Y, F1491S, R1662H, F1406C and E1757K. The H253Y mutation removes a conserved amino acid in the P-loop between transmembrane segment S5 and S6 of repeat I, a region that contributes to ion-selectivity of the pore [3]. Unlike familial hemiplegic migraine, where several recurrent CACNA1A mutations have been reported (e. g. T666M and R583Q), no recurrent mutations have yet been observed for EA–2. This has important implications for genetic analysis of samples from patients with presumed EA–2 patients and their families. In cases of EA–2 with a suspected CACNA1A mutation, it is apparent that the entire gene must be screened for mutations.

Acknowledgments This work was supported by the Netherlands Organisation for Scientific Research (NWO) (nr. 903–52–291), EC Research Network HPRN-CT–2000–00082, and the Migraine Trust. We thank Prof PS Harper and Dr C M Wiles (University Hospital of Wales, UK) for their help in collecting one of the families.

References

1. Baloh RW, Jen JC (2000) Episodic ataxia type 2 and spinocerebellar ataxia type 6. Klockgether, T and Dekker, M Handbook of ataxia disorders. New York. pp 0.447–68
2. Brandt T, Strupp M (1997) Episodic ataxia type 1 and 2 (familial periodic ataxia/vertigo). Audiol Neurootol 2: 373–383
3. Catterall WA (1998) Structure and function of neuronal Ca2+ channels and their role in neurotransmitter release. Cell Calcium 24: 307–323
4. Den Dunnen JT, Antonarakis SE (2001) Nomenclature for the description of human sequence variations. Hum Genet 109: 121–124
5. Denier C, Ducros A, Vahedi K, Joutel A, Thierry P, Ritz A, Castelnovo G, Deonna T, Gerard P, Devoize JL, Gayou A, Perrouty B, Soisson T, Autret A, Warter JM, Vighetto A, Van Bogaert P, Alamowitch S, Roullet E, Tournier-Lasserve E (1999) High prevalence of CACNA1A truncations and broader clinical spectrum in episodic ataxia type 2. Neurology 52: 1816–1821
6. Denier C, Ducros A, Durr A, Eymard B, Chassande B, and Tournier-Lasserve E (2001) Missense CACNA1A mutation causing episodic ataxia type 2. Arch Neurol 58: 292–295
7. Friend KL, Crimmins D, Phan TG, Sue CM, Colley A, Fung VS, Morris JG, Sutherland GR, and Richards RI (1999) Detection of a novel missense mutation and second recurrent mutation in the CACNA1A gene in individuals with EA–2 and FHM Hum Genet 105: 261–265
8. Guida S, Trettel F, Pagnutti S, Mantuano E, Tottene A, Veneziano L, Fellin T, Spadaro M, Stauderman K, Williams M, Volsen S, Ophoff R, Frants R, Jodice C, Frontali M, and Pietrobon D (2001) Complete loss of P/Q calcium channel activity caused by a CACNA1A missense mutation carried by patients with episodic ataxia type 2. Am J Hum Genet 68: 759–764
9. Jen J, Wan J, Graves M, Yu H, Mock AF, Coulin CJ, Kim G, Yue Q, Papazian DM, and Baloh RW (2001) Loss-of-function EA2 mutations are associated with impaired neuromuscular transmission. Neurology 57: 1843–1848
10. Jen J, Yue Q, Nelson SF, Yu H, Litt M, Nutt J, and Baloh RW (1999) A novel nonsense mutation in CACNA1A causes episodic ataxia and hemiplegia. Neurology 53: 34–37
11. Jouvenceau A, Eunson LH, Spauschus A, Ramesh V, Zuberi SM, Kullmann DM, and Hanna MG (2001) Human epilepsy associated with dysfunction of the brain P/Q-type calcium channel. Lancet 358: 801–807
12. Kramer PL, Yue Q, Gancher ST, Nutt JG, Baloh RW, Smith E, Browne D, Bussey K, Lovrien E, Nelson SF, and Litt M (1995) A locus for the nystagmus-associated form of episodic ataxia maps to an 11-CM region on chromosome 19p. Am J Med Genet 57: 182–185 (letter)
13. Miller SA, Dykes DD, and Polesky HF (1988) A simple salting out procedure for extracting DNA from human nucleated cells. Nucleic Acids Res 16: 1215
14. Ophoff RA, Terwindt GM, Vergouwe MN, van Eijk R, Oefner PJ, Hoffman SM, Lamerdin JE, Mohrenweiser HW, Bulman DE, Ferrari M, Haan J, Lindhout D, van Ommen GJ, Hofker MH, Ferrari MD, Frants RR (1996) Familial hemiplegic migraine and episodic ataxia type–2 are caused by mutations in the Ca2+ channel gene CACNL1A4. Cell 87: 543–52
15. Scoggan KA, Chandra T, Nelson R, Hahn AF, and Bulman DE (2001) Identification of two novel mutations in the CACNA1A gene responsible for episodic ataxia type 2. J Med Genet 38: 249–253
16. Teh BT, Silburn P, Lindblad K, Betz R, Boyle R, Schalling M, and Larsson C (1995) Familial periodic cerebellar ataxia without myokymia maps to a 19-CM region on 19p13. Am J Hum Genet 56: 1443–1449
17. Vahedi K, Joutel A, Bogaert van P, Ducros A, Maciazeck J, Bach JF, Bousser MG, and Tournier-Lasserve E (1995) A gene for hereditary paroxysmal cerebellar ataxia maps to chromosome 19p. Ann Neurol 37: 289–293
18. Von Brederlow B, Hahn AF, Koopman WJ, Ebers GC, and Bulman DE (1995) Mapping the gene for acetazolamide responsive hereditary paroxysmal cerebellar ataxia to chromosome 19p. Hum Mol Genet 4: 279–284
19. Wappl E, Koschak A, Poteser M, Sinnegger MJ, Walter D, Eberhart A, Groschner K, Glossmann H, Kraus RL, Grabner M, Striessnig J (2002) Functional consequences of P/Q-type Ca2+ channel Cav2.1 missense mutations associated with episodic ataxia type 2 and progressive ataxia. J Biol Chem 277: 6960–6966
20. White JC (1969) Familial periodic nystagmus, vertigo, and ataxia. Arch Neurol 20: 276 280
21. Zhuchenko O, Bailey J, Bonnen P, Ashizawa T, Stockton DW, Amos C, Dobyns WD, Subramony SH, Zoghby HY, and Lee CC (1997) Autosomal dominant cerebellar ataxia (SCA6) associated with small polyglutamine expansions in the 1A-voltage-dependent calcium channel. Nat Genet 15: 62–69