Mutation Spectrum in the CACNA1A Gene in 49 Patients with Episodic Ataxia

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Episodic ataxia is an autosomal dominant ion channel disorder characterized by episodes of imbalance and incoordination. The disease is genetically heterogeneous and is classified as episodic ataxia type 2 (EA2) when it is caused by a mutation in the CACNA1A gene, encoding the α1A subunit of the P/Q-type voltage-gated calcium channel CaV2.1. The vast majority of EA2 disease-causing variants are loss-of-function (LoF) point changes leading to decreased channel currents. CACNA1A exonic deletions have also been reported in EA2 using quantitative approaches. We performed a mutational screening of the CACNA1A gene, including the promoter and 3′UTR regions, in 49 unrelated patients diagnosed with episodic ataxia. When pathogenic variants were not found by sequencing, we performed a copy number variant (CNV) analysis to screen for duplications or deletions. Overall, sequencing screening allowed identification of six different point variants (three nonsense and three missense changes) and two coding indels, one of them found in two unrelated patients. Additionally, CNV analysis identified a deletion in a patient spanning exon 35 as a result of a recombination event between flanking intronic Alu sequences. This study allowed identification of potentially pathogenic alterations in our sample, five of them novel, which cover 20% of the patients (10/49). Our data suggest that most of these variants are disease-causing, although functional studies are required.

Episodic ataxia type 2 (EA2, MIM #108500) is a rare autosomal dominant ion channel disorder caused by mutations in the CACNA1A gene and characterized by episodes of midline cerebellar disturbance manifesting as ataxia, imbalance, vomiting, oscillopsia1 and interictal nystagmus; progressive ataxia can eventually develop2. EA2 has a wide phenotypic spectrum which includes paroxysmal neurological features other than ataxia. Around 50% of patients also experience migraine, and 80% suffer from rotational vertigo during the attacks3. Ataxia episodes last from hours to 2–3 days and are usually triggered by emotional stress, physical exercise, alcohol or caffeine. Onset usually occurs during the second decade of life, although later onsets have been reported3, 4. Acetazolamide administration can stop or diminish the frequency and severity of the attacks3.

CACNA1A encodes the pore-forming α1 subunit of the neuronal voltage-gated P/Q-type calcium channel (CaV2.1), which is widely expressed in the central nervous system (CNS), especially in Purkinje cells and...
| Patient | Age of onset (years) | Frequency of the episodes (per month) | Duration | Trigger(s) | MRI | Other features | cDNAa | Exon | Proteinb | Domain | Restriction enzyme | PhyloPc/PhastConsd scores | SIFT scoree | PolyPhen-2 scoref | Reference |
|---------|---------------------|--------------------------------------|----------|------------|-----|----------------|-------|------|----------|--------|-------------------|-----------------------------|------------|------------------|-----------|
| EP-004  | infancy             |                                       |          | NA         | NA   | NA             | c.749delG | 5    | p.G250Efs*60 | DL, SS-S6 loop | + Tsugl (mismatch) | 5.84/1                        |            |                  |           |
| A03_44  | NA                  | 15’–3 h                              |          | stress, fatigue | normal | ACZ responsive | c.G959A   | 6    | p.W320†       | DL, SS-S6 loop | + Acrl          | 5.78/1                        |            |                  |           |
| A98_279 | 58                  | ~30                                  | 30’–1 h  | NA         | normal | episodic hand dystonia | c.G1913A | 14   | p.G638D     | DL, SS-S6 loop | − Acrl      | 5.63/1                        |            |                  |           |
| 7A806   | 5                   | 4                                    | NA       | NA         | NA   | MO             | c.2042-43delAG | 16   | p.Q681Rfs*110 | DL, SS-S6 loop | − Ddel         | −                  | −            | −                |           |
| 474     | 8                   | 1–4                                  | 3–6 h    | NA         | deep WM hyperintensities | worsen on CBZ and PHT, ACZ responsive | c.5253-2259, 5403 + 1133del | 35   | p.S1753Cfs*2 | DIV, SS-S6 loop | −            | −                  | −            | −                |           |
| 335a    | <1                  | 48 h                                 | NA       | NA         | normal | migraine, visual aura | c.T5347A | 37   | p.Y1849*     | Cytoplasmic tail | − Pshl         | 4.32/1                        |            |                  |           |
| 340     | 4                   | NA                                   | exercise, fatigue, emotional stress | temporal parahippocampal cortex | congenital squint, nystagmus, ACZ responsive | c.C5569T | 37   | p.R1857*    | Cytoplasmic tail | + Bp-Hl (mismatch) | 3.33/1                        |            |                  |           |
| 389A    | 2                   | 8–12                                 | 6–8 h    | upon sleep | stress, exercise, coffee, tea | mild cerebellar atrophy | c.C5569T | 37   | p.R1857*    | Cytoplasmic tail | + Bp-Hl (mismatch) | 3.33/1                        |            |                  |           |
| 349A    | 1.5                 | 1–2                                  | 30’      | stress     | normal | nystagmus | c.C6665T | 46   | p.P2222L     | Cytoplasmic tail | − Fuel        | 2.83/1                        | Tolerated (1.00) | Benign (0.06) |           |

Table 1. Clinical information and results from the CACNA1A mutational screening in episodic ataxia patients with identified variants. MRI: Magnetic Resonance Imaging; NA: not available; ACZ: acetazolamide; MO: migraine without aura; FHM: Familial Hemiplegic Migraine; WM: white matter; CBZ: carbamazepine.; PHT: phenytoin; D: domain; S: segment. aReference sequence for cDNA nomenclature: NM_001127221 (nucleotide c.279A corresponding to the initiation codon, ATG). bReference sequence for protein nomenclature: NP_001120693. cPhyloP score: Positive scores indicate sites that are predicted to be conserved, whereas negative scores indicate sites predicted to be fast-evolving. dPhastCons score: It ranges from 0 (non conserved amino acid positions) to 1 (highly conserved amino acid positions). eSIFT score: It ranges from 0 to 1. The amino acid substitution is predicted as damaging if the score is < 0.05, and tolerated if the score is > 0.05. fPolyPhen-2 score: It ranges from 0 to 1. The aminoacid substitution is predicted as probably damaging if the score is > 0.85, possibly damaging (score is between 0.15 and 0.85) or benign (score < 0.15). gVariants p.T501M and p.P2222L have the rs codes rs121908240 and rs77855191, respectively.

Granule cells of the cerebellum. The Ca2.1 channel is responsible for the coupling of calcium influx to vesicular exocytosis, mediating neurotransmission*. Since the first CACNA1A disease-causing variants were described in EA27, over 80 EA2 alterations have been reported in the gene*. Several other neurological disorders are caused by pathogenic variants in CACNA1A, including familial hemiplegic migraine (FHM1, MIM #301011) and spinocerebellar ataxia type 6 (SCA6, MIM #183086), and the gene has also been related to other hemiplegic migraine (HM)-associated phenotypes like alternating hemiplegia of childhood8, acute striatal necrosis9, and spinocerebellar ataxia type 6 (SCA6, MIM #183086), and the gene has also been related to other hemi-plegic migraine (HM)-associated phenotypes like alternating hemiplegia of childhood, acute striatal necrosis, hemiplegia-hemiconvulsion-epilepsy10 or recurrent ischemic stroke11.

The vast majority of EA2-causing variants in the CACNA1A gene are predicted to cause loss of function of the channel, since nonsense, splicing and indel variants have been extensively reported. Furthermore, missense variants leading to decreased channel currents have been described and over the past years deletions in CACNA1A have been reported in EA2 patients using quantitative approaches, such as Multiplex Ligation dependent Probe Amplification (MLPA)14,15 or Quantitative Multiplex PCR of Short fluorescent Fragments (QMPSF)16. Functional studies have been carried out to investigate the pathogenic mechanism of EA2 mutations by expressing Ca2.1 channels carrying either missense or truncating CACNA1A changes in mammalian cells17,18 and Xenopus oocytes19. Two main hypotheses, negative dominance and haploinsufficiency, have been tested.

In this study we aimed to perform an extensive mutation analysis of the CACNA1A gene in 49 unrelated patients with episodic ataxia by means of sequencing and CNV analyses to identify both disease-causing point variants and structural variants.

**Results**

Patients. Ten out of 49 patients were found to harbour potentially pathogenic heterozygous CACNA1A variants (Table 1). Their clinical signs were prototypical for episodic ataxia. However, since most patients were adults when interviewed, complete information regarding their clinical presentation and the periods with higher attack frequencies was difficult to retrieve. Acetazolamide was generally effective in preventing the spells. Brain MRI documented vermian cerebellar atrophy in just one of the ten cases with molecular diagnosis.
Sequencing analysis of CACNA1A. Sanger-sequencing analysis of the CACNA1A gene in a cohort of 49 Spanish individuals diagnosed with episodic ataxia allowed the identification of eight heterozygous variants in nine patients (Figs 1 and 2). These included three missense changes, three nonsense variants leading to premature stop codons (one of them found in two unrelated patients) and two indels causing frameshift (Table 1). All variants were confirmed by restriction analysis (Table 1). Four of them are novel and four have been reported previously. None of the variants were present in a screening of 200 Spanish control individuals and all but two were absent from two different exome databases, one specific for the Spanish population (http://csvs.babelomics.org), including 576 subjects, and the ExAC resource (http://exac.broadinstitute.org) that includes more than 60,000 individuals. The p.T501M variant was found once in ExAC and p.P2222L was detected in four subjects in the same database, whereas the rest of the changes were not present in either dataset. In the nine families where a rare CACNA1A variant was identified, seven relatives were also screened for the particular change identified in the index case (Fig. 2). These relatives, five with episodic ataxia, one with migraine with aura and one healthy subject, belong to four of the families. The six relatives with a paroxystic phenotype bear the same mutation as the index case, whereas the healthy individual is not a carrier. In all four pedigrees the variant was transmitted, so no de novo changes were revealed by our analyses.

Two of the nonsense variants, p.W320* and p.Y1849*, are described here for the first time. The first one is located in the extracellular loop between S5 and S6 from domain I, maintaining only the N-terminus and part of the first domain of the protein (Fig. 1). Variant p.Y1849*, in contrast, leads to the truncation of the cytoplasmic tail, leaving out of the subunit the calcium binding domain (Fig. 1). Two other patients in our series carried the p.R1857* change, reported previously by other authors.20 We identified two coding indels leading to frameshifts and predicting truncated proteins. The novel genetic variant c.749delG (p.G250fs*60) is located in the S5-S6 extracellular loop from domain I, the same domain where the p.W320* change is located. The second one, c.2042-43delAG (p.Q681Rfs*100) produces a frameshift in domain II, also in the extracellular loop S5-S6, with a premature stop codon 100 codons downstream. This variant had been described in previous works.21-24.

Finally, three missense variants were identified. Two variants had already been reported by our group, but they were added to the present study to provide a complete view of our episodic ataxia series: Patient A98_279 carries variation p.G638D and patient A03_44, diagnosed with both episodic ataxia and FHM carries the p.T501M change.25 Both reports were accompanied by functional assays. The third variation, p.P2222L, described here for the first time, is located in a poly-proline region in the cytoplasmic tail of the protein.

CNV analysis of CACNA1A. The study of potential structural variants in the CACNA1A gene by MLPA allowed the identification of a deletion of the entire exon 35 (Fig. 3) in patient 474, which was confirmed by QMPSF.

In order to map the deletion breakpoint, a genomic region covering exons 34 to 36 was PCR-amplified and sequenced. A recombination occurred between two identical fragments located within Alu sequences in introns 34 (AluY) and 35 (AluSz), proximal and distal from exon 35 (Fig. 3), resulting in a 3.5-kb deletion. This novel deletion disrupts the coding frame from amino acid position 1,752 and is predicted to lead to a premature truncation only one residue downstream (p.S1752Cfs*2) in the extracellular S5-S6 loop of domain IV.
Nonsense mediated mRNA decay (NMD) assay. An NMD assay was performed from skin fibroblasts of patient 432B, bearing a heterozygous p.W320* stop variant. Sanger sequencing and RFLP analysis were performed and the mutated allele was shown to be present both in cells treated with cycloheximide (an inhibitor of mRNA decay) and those untreated. Gel quantification showed that the transcript produced from the mutated allele accounts for 55% of the total amount of product, indicating that this process does not trigger degradation of the mRNA encoding the truncated protein (Supplementary Fig. S1).

Discussion
Here we report an exhaustive screening of the \( CACNA1A \) gene in a large sample of patients with episodic ataxia, which was addressed through Sanger sequencing and CNV analyses. We identified nine different potentially pathogenic genetic variants in 10 of the 49 patients studied, five of which are novel (Table 1). This represents resolution of 20.4% of our patients sample, a percentage that is similar to others reported elsewhere. \(^1\) \(^2\) \(^6\) All these variants map to exons that are present in the main \( CACNA1A \) isoform (NM_001127221), which is expressed in brain, with highest expression in the cerebellar cortex, according to the public transcriptomic datasets Allen Brain Atlas (http://human.brain-map.org) and Human Brain Transcriptome (http://hbatlas.org/pages/hbtd). Although most of the previously reported changes in episodic ataxia type 2 (EA2) are point variations, deletions encompassing one or more \( CACNA1A \) exons have also been described. \(^14\) \(^16\) \(^27\) For this reason, in addition to sequencing exons, splice sites, branch points and the promoter region of the gene, we used two complementary quantitative approaches, MLPA and QMPSF, to extend the mutational screening of deletions and duplications within the gene. MLPA and QMPSF allowed inspection of 40 out of 48 \( CACNA1A \) exons (Supplementary Fig. S2). Thus, an exhaustive genetic diagnostic protocol consisting of two sequential approaches offered a wider and more comprehensive view of the genetic background of EA2.

We identified a total of three nonsense and three missense variants, two \emph{indels} (one of them found in two patients) and a deletion encompassing exon 35 of the gene. From these, six were predicted to truncate the protein, either by introducing a stop codon (p.W320*, p.Y1849* and p.R1857*) or by causing a frame shift (p.G250Efs*60, p.Q681Rfs*100 and p.S1752Cfs*2). Disrupting variants that result in a loss of function of the \( Ca_2.1 \) calcium channel are the most commonly reported changes in EA2 patients. Functional analyses of the truncated \( CACNA1A \) subunit have showed diminished or null activity of the channel for EA2 mutations. \(^18\) Thus, although truncating mutations have usually shown to cause a loss of function of the mutated subunit, the underlying...
Pathophysiological mechanism that causes the EA2 phenotype remains still unclear. Two hypotheses have been proposed: haploinsufficiency, supported by the finding of nonsense mediated RNA decay (NMD)\(^{15,28}\), and a dominant negative effect of the mutated subunit. The latter is more generally accepted as the major mechanism on the basis of functional studies that support an altered interaction between the WT and the mutated allele that would retain the complex in the endoplasmic reticulum, affecting protein trafficking and activating the proteasome response\(^{17,29,30}\). The dominant negative effect of the interaction may require the presence of the N-terminus of the protein in the mutant form, leading to a suppression of the Cav2.1 channel expression due to the interaction between truncated and full-length subunits\(^{18}\). All six truncating changes reported here are located beyond the N-terminus of the channel and so are candidates to undergo dominant negative effects.

In our study, since skin fibroblasts from patient 432B (p.W320*) were available, the hypothesis of haploinsufficiency caused by a possible degradation of the mutated mRNA by NMD could be tested. No mRNA degradation was observed for this particular genetic variant in these cells (see Supplementary Fig. S1), although this result may not reflect what actually occurs in brain. NMD could not be tested in the rest of the patients from our collection, as biological samples were not accessible for analysis.

Figure 3. CNV studies in the CACNA1A gene of patient 474. (a) Results from the MLPA analysis performed with Coffalyser. The deleted exon 35 is indicated with an arrow. C: control probe. (b) Deletion breakpoint mapping. Sequence chromatogram corresponding to the homologous fragment from AluY and AluSz sequences. The identical shared sequence by the 5′ and 3′ Alu elements located in introns 34 and 35 that mediated the recombination is framed. Location of fragments taken from UCSC Genome Browser on Human Feb. 2009 Assembly (GRCh37/hg19).
Three heterozygous missense variants (p.T501M, p.G638D and p.P2222L) were identified in three patients. The effect of missense variants are not easy to predict functionally. Two of the variants that we describe in this study, p.G638D and p.T501M, had been reported in previous works from our group, where functional analyses were performed. Variant p.G638D showed a loss of function of the channel, in agreement with the most commonly described effect for EA2-causing mutations. On another hand, the patient bearing variant p.T501M, a change reported previously in EA2, presents with a phenotype that combines EA2 and HM. The functional analysis of this missense change revealed a gain of function of the channel, a pathogenic mechanism typical of HM rather than EA2. There are other reported cases where related phenotypes overlap, such as HM with progressive ataxia or EA2 with migraine. The third missense variant, p.P2222L, was not functionally characterized. Although this variation was also detected in the index case, also with episodic ataxia (Fig. 2), neither SIFT nor PolyPhen-2 predicted a damaging effect, whereas PhyloP scored it as a moderately conserved residue, and PhastCons as a highly conserved one (Table 1). The variant, rare, was not found in a set of around 600 exomes of the CIBERER Spanish Variant Server nor among 200 healthy Spanish individuals screened by us, and it was found at heterozygosity in only 4 out of 6,000 subjects from the ExAC database (although this last figure should be taken with caution as the relevant position is covered only in 10% of the individuals, possibly indicating low-quality sequences). This indicators (a rare conserved variant that cosegregates with the disorder but has no favourable damaging effect predictors) are contradictory and thus a functional test is needed to shed light on the impact of the p.P2222L change.

Taking together this and other studies, no specific prevalent variants are found in EA2, which results in large allelic heterogeneity: However, there are some regions in the CACNA1A gene that are found to be more frequently mutated in EA2 patients and also in other patients presenting ataxic features (cerebellar ataxia). In our study, five out of nine variants are located in the S5-S6 extracellular loop of different domains (I, II and IV) (Fig. 1). Many disease-causing changes, both nonsense and missense, in EA2 seem to be preferentially located in these areas. Therefore, this S5-S6 linker may represent a key region that influences the proper functionality of the subunit. This effect has also been seen in mouse models bearing changes in this region, which present a mild ataxia phenotype. Only one variant in our study, p.T501M, present in a patient with EA2 and HM, is located in a transmembrane domain (DII-S1) that belongs to the voltage sensor part, affecting both activation and inactivation of the channel.

Three other variants identified here are located in the C-terminal tail. The two nonsense variants p.Y1849* and p.R1857* are in exon 37, within the EF-hand responsible for calcium binding, so the IQ-like CaM interaction domain (IQ) and the Calmodulin Binding Domain (CBD), located downstream, are also lost. Finally, although the missense variant p.P2222L is located downstream from these interaction regions (EF-hand and calmodulin binding domains), it may affect the conformation of the tail and disturb the interaction between the CACNA1A subunit and other elements that bind the C-terminal region, such as auxiliary β-subunits, leading to an impaired function of the channel.

Genotype-phenotype correlations were not apparent in our cohort: all pathogenic variants described in this study produced comparable EA2 phenotypes regardless of their molecular nature or they location in transmembrane or cytoplasmic tail protein domains. Of note, two patients (cases 340 and 389A) displayed the same variant p.R1857*, in that particular instance the clinical presentation was similar in the early age of onset and presence of interictal cerebellar signs.

In summary, we have identified nine potentially disease-causing variants in ten patients with episodic ataxia. However, there is still a significant proportion of subjects with this phenotype that bear no mutations in the CACNA1A gene. Although there might be some degree of missing allelic heterogeneity in CACNA1A, possibly other genes are involved in the disorder in our sample, which may be uncovered by means of next generation sequencing approaches. Indeed, genes other than CACNA1A have previously been involved in episodic ataxia, including KCNA1 (episodic ataxia type 1, EA1), CACNB4 (EA4), SLC1A3 (EA6) and FGF14. More recently, other potential EA genes have been reported, including SCN2A, FGF14 and SLC2A1. With the exception of CACNA1A and KCNA1, the rest have been found mutated in only one or a few patients, indicating that the number of genes involved in this neurologic phenotype may be high.

Methods

Patients. All 49 patients were diagnosed with episodic ataxia on clinical grounds by expert neurologists. Central to the diagnosis was eliciting a history of recurrent paroxysmal attacks of ataxia, vertigo, and nausea or vomiting typically lasting minutes to days in duration. Additional supporting criteria were (i) the presence of interictal ataxia and nystagmus; (ii) a history of the attacks being triggered by exercise, emotional stress, alcohol, caffeine, fever, or heat; (iii) reduction of attack frequency/severity by acetazolamide; (iv) absence of myokymia and (v) a family history consistent with autosomal dominant inheritance.

Sampling and mutation screening. Peripheral blood samples were collected from all probands and genomic DNA was isolated using a standard salting-out method. All 48 exons, splice sites and branch points from the CACNA1A gene were sequenced. The promoter (894 bp upstream from the translation initiation codon) and the 3′UTR region, containing exon 48, were also screened as previously described. All variants were assessed by bidirectional sequencing and confirmed by restriction fragment length polymorphism (RFLP) analysis. Two hundred control individuals were screened by Sanger sequencing, and the presence of the identified mutations was investigated at the Exome Aggregation Consortium dataset (ExAC, http://exac.broadinstitute.org) and the CIBERER Spanish Variants Server (http://cvs.babelomics.org). Seven relatives from four of the families with identified CACNA1A mutations were also screened by Sanger sequencing and RFLP analysis. Variant nomenclature follows Human Genome Variation Society (HGVS) guidelines (http://www.hgvs.org/).
CNV analysis. Multiplex Ligation-dependent Probe Amplification (MLPA) was the first approach used for the CNV analysis. We used the MLPA CACNA1A kit SALSA-P279-A2 (MRC Holland, Amsterdam) that contains 25 probes covering 24 exons of the CACNA1A gene. This test was performed according to the manufacturer’s instructions. Quantitative Multiplex PCR of Short fluorescent Fragments (QMPSF) was used as a complementary approach to cover most of the exons that were not covered by the MLPA assay. Four sets of probes targeting 16 additional exons were designed. For five additional exons, since they were located approach to cover most of the exons that were not covered by the MLPA assay. Four sets of probes targeting 16 additional exons were designed. For five additional exons, since they were located

Nonsense-mediated mRNA decay (NMD) assay. Only in one case biological material was available to perform NMD assays. Fibroblast primary cultures from a control individual and from patient 432B (bearing the nonsense variant p.W320*) were obtained from skin biopsies, cultured in a monolayer at 37 °C under 5% CO₂ in T25 flasks (Greiner Bio-One, North America, Inc.) with DMEM medium (Sigma-Aldrich, Steinheim, Germany) containing 12% fetal bovine serum (FBS) (Gibco, Invitrogen Life Technologies, Heidelberg, Germany). After three weeks of maintenance, fibroblasts were trypsinized and cultured in 35mm dishes with DMEM medium containing

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Author Contributions
C.S., O.C., R.C., N.F.-C., M.V.-P., E.C.-L. and I.B. performed the sequencing analysis of the CACNA1A gene; C.S., O.C. and C.T. did the C.N.V. study; C.R., V.V. and A.M. participated in the recruitment of patients and clinical assessment and coordinated the clinical research; C.S. prepared the first draft of the manuscript and all figures and tables; C.S., O.C., B.C. and A.M. designed the study; B.C. and A.M. coordinated the study and supervised the manuscript preparation. All authors contributed to and approved the final manuscript.

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