A Novel Null Homozygous Mutation Confirms CACNA2D2 as a Gene Mutated in Epileptic Encephalopathy

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Abstract

Contribution to epileptic encephalopathy (EE) of mutations in CACNA2D2, encoding α2δ-2 subunit of Voltage Dependent Calcium Channels, is unclear. To date only one CACNA2D2 mutation altering channel functionality has been identified in a single family. In the same family, a rare CELSR3 polymorphism also segregated with disease. Involvement of CACNA2D2 in EE is therefore not confirmed, while that of CELSR3 is questionable. In a patient with epilepsy, dyskinesia, cerebellar atrophy, psychomotor delay and dysmorphic features, offspring to consanguineous parents, we performed whole exome sequencing (WES) for homozygosity mapping and mutation detection. WES identified extended autozygosity on chromosome 3, containing two novel homozygous candidate mutations: c.1295delA (p.Asn432fs) in CACNA2D2 and c.G6407A (p.Gly2136Asp) in CELSR3. Gene prioritization pointed to CACNA2D2 as the most prominent candidate gene. The WES finding in CACNA2D2 resulted to be statistically significant (p = 0.032), unlike that in CELSR3. CACNA2D2 homozygous c.1295delA essentially abolished α2δ-2 expression. In summary, we identified a novel null CACNA2D2 mutation associated to a clinical phenotype strikingly similar to the Cacna2d2 null mouse model. Molecular and statistical analyses together argued in favor of a causal contribution of CACNA2D2 mutations to EE, while suggested that finding in CELSR3, although potentially damaging, is likely incidental.

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Introduction

Epileptic Encephalopathies (EEs) are severe brain disorders in which the seizures and the epileptic activity itself may cause severe psychomotor impairment. EEs may arise from the neonatal to the early infancy period as recurrent, prolonged or drug resistant seizures, resulting in devastating permanent global developmental delay with brain atrophy. Occasionally, EEs can be associated to brain lesions or malformations of cortical development [1]. EEs are genetically heterogeneous. Numerous genes, all involved in diverse primary developmental processes of the brain, have been already identified and their number and that of the associated clinical spectrum is expanding continuously [1]. Among these the so-called “channelopathies”, originating from defects in genes coding for neuronal ion channels, play a prominent role in monogenic epilepsies, among which EEs [2].

Mutations in CACNA1A (MIM 601011), [3] encoding the transmembrane pore-forming subunit Ca2.1 of Voltage Dependent Calcium Channels (VDCCs), [4] have been associated to the peculiar phenotypic combination of absence epilepsy and cerebellar ataxia. Auxiliary regulatory subunits α2δ, β and γ associate with the pore forming α1 subunit and modulate its function [5,6]. Several mouse models, all characterized by homozygous mutations in one of the genes encoding VDCC subunits, share similar phenotypes including cerebellar ataxia, paroxysmal dyskinesia and seizures similar to those of absence epilepsy as well as other forms of generalized epilepsy. Among these the ducky mutant mice, which carry null alleles in Cacna2d2, represent a model for absence epilepsy characterized by behavioral arrest synchronous with spike-wave discharges and cerebellar ataxia [7,8]. α2δ is encoded by a single gene and is post-translationally modified to form two proteins, δ and α2: the δ piece, a single-pass trans-membrane portion, anchors the α2 protein to the membrane [9]. It acts mainly by enhancing the trafficking or reducing the turnover of the channel complex in the plasma membrane [8,10]. The α2δ-2 subunit is involved in the composition of a variety of different VDCCs, but it contributes mainly to Cav2.1/B4 (P-type current) in central synapses [9].

Recently, a homozygous CACNA2D2 (Calcium Channel, Voltage Dependent, α2δ subunit 2; MIM 607082) mutation was identified in a family with 3 siblings, offspring to consanguineous parents, who presented with early-onset epileptic encephalopathy...
and global developmental delay. This mutation, a probably pathogenic missense p.Leu1040Pro substitution, affected a highly conserved residue and was shown to cause dysfunction of α2δ-2, resulting in reduced current density and slow inactivation in neuronal calcium channels (Table 1) [11]. Another possibly detrimental rare p.Met2630Ile polymorphism (rs149614835) in CELSR3 (Cadherin EGF LAG 7-pass G-type receptor 3; MIM 604264) (Table 1) segregated with the disease phenotype in the same family. These findings supported the hypothesis that defective α2δ-2 may underlie the epileptic phenotype. However, this observation was not confirmed in independent patients and a role in disease pathogenesis of the concomitant CELSR3 variant could not be ruled out [11]. Here we report on abolished expression of α2δ-2 in a patient, offspring to consanguineous parents characterized by the association of epilepsy with also absence seizures, dyskinetic movements and cerebellar atrophy, a clinical picture closely resembling the phenotype displayed by the ducky mouse.

### Materials and Methods

#### Case Report

The proband is an Italian 9-years-old boy, offspring to first cousins, with an older and healthy sister. We followed this patient since the age of 2 years. He was born at 40 weeks after an uneventful pregnancy and delivery. During the first months of life, the child presented hypertonus and eye rolling movements. Afterwards, a severe delay of psychomotor development became apparent, characterized by legs hypertonia, axial hypotonia, dyskinetic movements and myoclonic jerks of the arms and the head, no eye contact, and uncoordinated eye movements. The first epileptic seizure occurred at 5 months without fever, characterized by salivation, loss of contact and clonic jerks on the left side of the

### Table 1. Features of the CACNA2D2 and CELSR3 mutations.

| Gene  | Present work | Edvardson et al., 2013 (11) |
|-------|--------------|----------------------------|
| Gene  | CACNA2D2     | CELSR3                     | CACNA2D2     | CELSR3         |
| Frequency | Novel       | Novel                      | Novel       | <0.001 (rs149614835) |
| Genomic position | chr3:50416390 | chr3:48687978 | chr3:50402595 | chr3:48682550 |
| cDNA change | c.1295delA  | c.341G>A                  | c.3119A>G    | c.7890G>A      |
| Protein change | p.N432fs*  | p.G114D                    | p.L1040P    | p.M2630I       |
| GERP score | –           | 5.07                       | 4.76        | 5.24           |
| Pathogenicity prediction | –           | Tolerated (SIFT) Damaging (MT) | Probably Damaging (Polyphen2) | Damaging (SIFT, MT) |

1GERP (Genomic Evolutionary Rate Profiling) is a measure of the nucleotide evolutionary conservation. It ranges from −12.3 to 6.17, with 6.17 being the most conserved [30].

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**Figure 1. Instrumental findings in the proband.** Left panel: EEG recording during wakefulness showing generalized spike-wave complex at 4 Hz lasting thirteen seconds with absence, eye up deviation and eyelid myoclonia. Right panel: Sagittal T1 MRI showing prominent cerebellar atrophy. [doi:10.1371/journal.pone.0082154.g001](https://doi.org/10.1371/journal.pone.0082154.g001)
body. The first seizure lasted 20 minutes and the interictal EEG showed slow waves on the left occipital regions. Over time, several seizure types occurred, characterized by: a) left eye and head deviation with clonic jerks on the left side of the body; b) generalized hypertonia, loss of contact, uncoordinated movements of eye and limbs, cyanosis, and facial and arms twitching; or c) loss of contact, generalized hypotonia and clonic jerks, and tachycardia or bradycardia. Occasionally, at the end of the ictal event, dyskinetic (particularly choreiform) movements of the limbs appeared, without recovery of consciousness. The seizures, with or without fever, could be prolonged (20–30 minutes) and were frequently followed by sleep. Between seizures, the child always showed head tonic extension, erratic limb movements and tremor. The interictal EEG showed multifocal spikes over the right centro-temporal and the left parieto-occipital regions.

At three years of age, absences with eye up deviation and eyelid myoclonus appeared (Figure 1). The other seizure types persisted, although reduced in frequency. Seizures were resistant to many drugs (phenobarbital, benzodiazepine, valproic acid, levetiracetam, lamotrigine); absences frequency was improved by ethosuximide. The EEG recordings showed slowing background activity, multifocal, diffuse paroxysmal abnormalities, and a transitory photosensitivity.

Clinical examination showed dysmorphisms (bilateral epicanthus, arched palate, pronounced Cupid’s bow, narrow naris, clinodactyly of the IV and V fingers) and head circumferences between 3 and 10 percentiles. Neurologic examination showed oculo-motor apraxia, strabismus, nystagmus, axial and leg hypertonia, head tonic extension, erratic limb movements and tremor. At the most recent follow-up, it was substantially unchanged, and the child had two types of seizures: very brief absences during wakefulness and tonic-clonic seizures during sleep, which were prevalent on the left side. Laboratory investigations showed hyperglycaemia, and glycosuria. Genetic (high resolution karyotype, analysis for Angelman and Dravet syndromes) and metabolic investigations (urine organic acids, amino acids, isoelectric focusing of transferrins), including those for mitochondrial pathology, were all negative. The ECG and cardiac examination were normal. Brain MRI was remarkable for cerebellar atrophy (Figure 1).

Whole Exome Sequencing for Variant Detection and Autozygosity Mapping

Whole exome DNA from patient’s whole blood was captured using the TruSeq exome enrichment kit (Illumina Inc., San Diego, CA, USA) and sequenced as 100 bp paired-end reads on Illumina HiSeq2000 platform (Illumina Inc., San Diego, CA, USA).

Generated reads have been deposited in the European Nucleotide Archive with accession number PRJEB4676 (http://www.ebi.ac.uk/ena/data/view/PRJEB4676). Reads were checked with FastQC [http://www.bioinformatics.babraham.ac.uk/publications.html] and aligned with BWA [12] to the reference genome hg19. Aligned reads were treated for realignment and base quality score recalibration with GATK, [13] and for

![Figure 2. Genetic mapping and segregation of CACNA2D2 c.C1295delA mutation. Upper panel: barcode plot showing EX-HOM on chromosome 3. If the SNP is heterozygous, y = 0, if it is homozygous y = 1. Black bars represent regions of mixed heterozygous/homozygous SNPs, whilst white bars regions of contiguous homozygous SNPs. Different colors reflect the type of mutation: CELSR3 has a missense variant (black), CACNA2D2 has a loss-of-function variant (red). Lower panel: the CACNA2D2 c.C1295delA mutation segregates with the disease in the nuclear pedigree (parents are 1st cousins). doi:10.1371/journal.pone.0082154.g002]
duplicate removal with PicardTools (http://picartools.sourceforge.net). Alignment statistics were collected by SAMtools [14] and GATK. Coverage statistics over the targeted regions were calculated with GATK. Variant calling and filtering by quality were performed by GATK. Variants passing quality filters were annotated separately against NCBI RefGene (http://www.ncbi.nlm.nih.gov) and UCSC KnownGene (http://genome.ucsc.edu).

WES genotypes of polymorphic sites present in dbSNP135 (http://www.ncbi.nlm.nih.gov/snp/) were retrieved to create a genetic map consisting of 1673111 sites in the targeted exome, in order to perform autozygosity mapping in the proband as described elsewhere [15].

Gene Prioritization

To enhance prioritization of candidate genes harboring novel, probably detrimental variants detected by WES, we used Exomiser (http://www.sanger.ac.uk/resources/databases/exomiser/) [16], an on-line tool that functionally annotates and prioritizes mutated genes using criteria as variant frequency, predicted pathogenicity, inheritance pattern and model organism phenotype data. Scores are based on Mutation Taster [17], SIFT [18] and Polyphen2 [19] for predicted pathogenicity of mutations and on Mouse PhenoDigm [20] for phenotypical overlap with the animal model. We adopted the following criteria: 1) autosomal recessive model; 2) phenotypic classification according to the HPO (Human Phenotype Ontology) terms (http://www.human-phenotype-ontology.org): a) epileptiform EEG discharges, b) cerebellar atrophy and c) dyskinesia; 3) removal of variants with allele frequency >1% and predicted as non-pathogenic. To calculate the probability that a mutation falling in a candidate gene is truly associated with disease, we used the on-line tool Exome Power Calculator [21] (exomepower.ssg.ucsf.edu/) to obtain p-values as measure of statistical significance of the WES findings. Exome Power Calculator provides a simple statistical framework to guide quantitative data analysis by setting the following parameters: 1) Ps, sequence sensitivity: the probability that a variant in the targeted regions is correctly called; 2) m, per individual number of candidate variants; 3) w, relative gene length (ratio of the protein length to the genomic average); 4) n, sample size (number of unrelated patients sequenced); 5) M, total number of genes in the mutational target; 6) a, significance level of the test after Bonferroni correction; 7) o, observed value for the statistic; 8) T, underlying genetic model (dominant/recessive/additive). We assumed Ps = 1 (100% sensitivity), a = 0.05, M = 20653 [22], the underlying genetic model Tr. From the Uniprot database (http://www.uniprot.org/), we retrieved the protein product length of CACNA2D2 (1150 amino acids) and CELSR3 (3312 amino acids). Based on the average genomic protein length of 447 amino acids [23], we calculated w = 2 for CACNA2D2 (1150/447) and w = 7 for CELSR3 (3312/447). We calculated m as the mean individual number of “candidate variants” from our internal exome database (n = 50 exomes). Since we found a loss-of-function mutation in CACNA2D2, we calculated m either including all the coding nonsynonymous, canonical splice-site and coding small indels mutations with population frequency <1% (m_all), or applying a more stringent filter by selecting only loss-of-function (nonsense

| m (n’ variants) | CACNA2D2 (w = 2) | CELSR3 (w = 7) |
|-----------------|------------------|----------------|
| mall (584)      | 0.999            | 1,000          |
| mlof (19)       | 0.032            | 0.109          |

Table 2. Calculation of the level of statistical significance of WES findings in candidate genes.
mutations and coding small frameshift indels with population frequency <1%) mutations (m_null). m_all resulted to be 504, while m_null 19.

**Muscle Biopsy**

Muscle biopsy of the proband was performed by open surgery after informed consent. Four normal muscle biopsies were used as controls. Muscle specimens were frozen in cooled isopentane and stored in liquid nitrogen. Standard staining (H&E, Gomori modified trichrome, Oil Red O, PAS) and histoenzymatic activities (SDH, COX, NADH, ATPase pH 9.4 and 4.3) were performed following standard protocol [24].

**Expression Analysis**

Total RNA was extracted from 150 muscle slices (20 µm thick) by TriPure isolation reagent (Roche) and 1 µg of total RNA was reverse transcribed using the Transcriptor First Strand cDNA Synthesis Kit (Roche). CACNA2D2 expression was evaluated by real time-PCR, using the Universal Probe Library (Roche) system. The analysis has been conducted in triplicate and the CACNA2D2 concentration has been calculated through a standard curve by absolute quantification and normalized on TUBB expression.

Total proteins were extracted from 30 muscle slices (20 µm thick) adding 100 µl of RIPA buffer (30 mM Tris-Cl pH 7.6, 150 mM NaCl, 1% NP-40, 1% NaDOC, 0.1% SDS, 5 mM EDTA) and 100 µl/ml of protease inhibitor cocktail (Roche). The lysates were sonicated and centrifuged at 10000 g and the protein content of the supernatant was determined according to Bradford method.

**Discussion**

Our study strengthens the evidence that CACNA2D2 loss-of-function mutation causes EE. As highlighted by gene prioritization using the Exomiser tool, p.Asn432fs in CACNA2D2 emerged as the most prominent candidate from the WES performed in the proband. Expression data clearly showed that CACNA2D2 is mostly abundant in the cerebellum and particularly in Purkinje cells, consistent with the patient’s cerebellar atrophy.

The muscle biopsy from the proband, histologically characterized by some variability of fiber size with occasional hypotrophic type II fibers and signs of mitochondrial subsarcolemmal proliferation (data not shown), demonstrated that CACNA2D2 expression was dramatically reduced compared to controls (Figure 3). mRNA quantification showed a ~70% of reduction of CACNA2D2 gene expression in the proband compared to control individuals (p<0.001), whereas the father and the mother showed a reduction of ~70% and ~50% (p<0.001) respectively (Figure 3, left panel). Accordingly, the protein in the proband resulted almost absent (3% of expression, p<0.05), and both the parents presented ~50% of CACNA2D2 protein expression (Figure 3, right panel).

These findings enforced the hypothesis that dysfunction of CACNA2D2 is a cause of EE. However, as in the previous report of a CACNA2D2 mutation in early onset EE, [11] we noticed the concomitant occurrence of a CELSR3 variant that segregated with disease in the family (data not shown). This was a nonsynonymous mutation c.6407G>T (p.Gly2136Asp) change, highly conserved and predicted to be deleterious by three algorithms (SIFT, PolyPhen and AlignGO). The analysis in the proband increased the probability of finding a CACNA2D2 variant in an individual WES experiment using Exome Power Calculator. Only the occurrence of a recessive loss-of-function mutation in CACNA2D2 reached statistical significance (p = 0.032) (Table 2). Based on molecular and statistical evaluation we concluded that the loss-of-function variant in CACNA2D2, leading to abolishment of expression of the encoded protein, was causative of EE in the proband while the nonsynonymous change in CELSR3, although potentially damaging, was likely an incidental finding.
and mutant transcripts would encode proteins that are unlikely functional.

In the 3 previously reported affected siblings mutated in CACNA2D2, the p.Leu1040Pro nonsynonymous change was associated with a clinical picture slightly different from the case described here: the three affected siblings had earlier onset of epileptic seizures (20–60 days of age vs. 5 months of the present case); they displayed atomic, clonic and tonic attacks without focality rather than partial or absence seizures; they did not show dysmorphic features; their EEG picture was consistent with a Lennox-Gastaut syndrome rather than being characterized by multifocal paroxysmal abnormalities and typical absences as observed in the present case. Epilepsy with different type of seizures, including partial, hemiclonic, and typical absence seizures was observed in the present case. EEG with different type of abnormalities in cerebral cortex connectivity [29]. The two families with EE and CELSR3 mutations will provide confirmation of this hypothesis. The presence of variants in CELSR3 in two unrelated families with EE and CACNA2D2 mutations could in principle be explained by a joint contribution of these two genes to the EE phenotype. Although CELSR3+/− mice display gross structural brain alterations that do not resemble any features of the affected children in the two families, [28] a role for the CELSR3 mutations cannot be ruled out. Inactivation of CELSR3 in mice causes abnormalities in cerebral cortex connectivity [29]. The two CELSR3 mutations, p.Met2630Ile in the previously reported family and p.Gly2136Asp in the present one, are rare or novel nonsynonymous changes affecting highly conserved residues, and therefore are both variants with a putative detrimental role. However the present CELSR3 mutation was not predicted as pathogenic by all the predictors used, unlike the one previously reported (Table 1) [11]. Therefore, in light of our results clearly showing the disruptive effect of the p.Asn432fs mutation, we explored the possibility that the CELSR3 variants were only incidentally detected in the two families. Focusing on the analysis of the present single proband alone, we obtained that the detection of the p.Gly2136Asp in CELSR3 was not statistically significant. This is due to the relatively excessive length of its encoded protein (about 7-fold the genomic average), that inflates the number of variants that can be randomly drawn from this gene in a WES setting. Conversely, detection of p.Asn432fs in CACNA2D2, which encodes a shorter protein (about 2-fold the genomic average) resulted to be statistically relevant applying a stringent loss-of-function filtering. CACNA2D2 and CELSR3 are only 1.7 megabases apart on chromosome 3. It follows that a haplotype harboring a CACNA2D2 disease-causing mutation can be found to coincidentally carry a CELSR3 rare or novel polymorphism, and that due to recent parental relatedness both the two variants appear in the homozygous state. In order to prove with certainty that the CELSR3 does not contribute to cause the EE pathogenesis, further patients should be recruited that do show mutations in CACNA2D2 but not in CELSR3. However, it is often difficult to recruit additional patients or families with an ultra-rare disorder such as the one affecting the present proband. Here, we demonstrated that functional and statistical validation together can be valuable resource to collect evidence of causality or non-causality of reliable WES variant findings.

In conclusion, our results strengthened the association of CACNA2D2 mutations to EE. The data we collected suggested that p.Asn-2 genotype-phenotype correlation may depend on different levels of residual p.Asn-2 activity. Our analyses indicated that the CELSR3 variants were potentially deleterious but likely incidental findings, demonstrating that molecular and statistical reasoning can assist in discriminating the true disease-causing from the reliable but non causative candidate variants that emerge from the individual WES data.

Supporting Information

Table S1 Extended autozygous regions identified in the proband.

Table S2 Results of the variant prioritization by Exomiser.

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Author Contributions

Conceived and designed the experiments: TP MS VC. Performed the experiments: TP FP AM MLV RL. Analyzed the data: TP FP AM. Contributed reagents/materials/analysis tools: AA LC FC. Wrote the paper: TP AP VC.

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