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Loss of SYNJ1 dual phosphatase activity leads to early onset refractory seizures and progressive neurological decline

Katia Hardies,[1,2] Yiying Cai,[3] Claude Jardel,[4,5] Anna C. Jansen,[6] Mian Cao,[3] Patrick May,[7,8] Tania Djémié,[1,2] Caroline Hachon Le Camus,[9] Kathelijn Keymolen,[10] Tine Deconinck,[1,2] Vikas Bhambhani,[11] Catherine Long,[11] Samin A. Sajan,[12] Katherine L. Helbig,[12] AR working group of the EuroEPINOMICS RES Consortium,* Arvid Suls,[1,2,13] Rudi Balling,[7] Ingo Helbig,[14,15] Peter De Jonghe,[1,2,16] Christel Depienne,[17,18] Pietro De Camilli[3] and Sarah Weckhuysen[1,2,16]

*See Appendix 1.

SYNJ1 encodes a polyphosphoinositide phosphatase, synaptojanin 1, which contains two consecutive phosphatase domains and plays a prominent role in synaptic vesicle dynamics. Autosomal recessive inherited variants in *SYNJ1* have previously been associated with two different neurological diseases: a recurrent homozygous missense variant (p.Arg258Gln) that abolishes Sac1 phosphatase activity was identified in three independent families with early onset parkinsonism, whereas a homozygous nonsense variant (p.Arg136*) causing a severe decrease of mRNA transcript was found in a single patient with intractable epilepsy and tau pathology. We performed whole exome or genome sequencing in three independent sib pairs with early onset refractory seizures and progressive neurological decline, and identified novel segregating recessive *SYNJ1* defects. A homozygous missense variant resulting in an amino acid substitution (p.Tyr888Cys) was found to impair, but not abolish, the dual phosphatase activity of SYNJ1, whereas three premature stop variants (homozygote p.Trp843* and compound heterozygote p.Gln647Argfs*6/p.Ser1122Thrfs*3) almost completely abolished mRNA transcript production. A genetic follow-up screening in a large cohort of 543 patients with a wide phenotypical range of epilepsies and intellectual disability revealed no additional pathogenic variants, showing that *SYNJ1* deficiency is rare and probably linked to a specific phenotype. While variants leading to early onset parkinsonism selectively abolish Sac1 function, our results provide evidence that a critical reduction of the dual phosphatase activity of *SYNJ1* underlies a severe disorder with neonatal refractory epilepsy and a neurodegenerative disease course. These findings further expand the clinical spectrum of synaptic dysregulation in patients with severe epilepsy, and emphasize the importance of this biological pathway in seizure pathophysiology.

1 Neurogenetics Group, Department of Molecular Genetics, VIB, Antwerp, Belgium
2 Laboratory of Neurogenetics, Institute Born-Bunge, University of Antwerp, Antwerp, Belgium
3 Departments of Neuroscience and Cell Biology, Howard Hughes Medical Institute, Kavli Institute for Neuroscience and Program in Cellular Neuroscience, Neurodegeneration and Repair, Yale University School of Medicine, New Haven, CT 06510, USA
4 Inserm U 1016, CNRS UMR 8104, Institut Cochin, F-75013, Paris, France
5 AP-HP, Hôpital de la Pitié-Salpêtrière, Service de Biochimie Métabolique et Centre de Génétique moléculaire et chromosomique, F-75013, Paris, France
6 Paediatric Neurology Unit, Department of Pediatrics Neurology, UZ Brussel, Brussels, Belgium
7 Luxembourg Centre for Systems Biomedicine, University of Luxembourg, Luxembourg
8 Institute for Systems Biology, Seattle, USA

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9 Unité de neurologie pédiatrique, hôpital des enfants de Toulouse, 330 avenue de Grande-Bretagne, 31059 Toulouse cedex 09, France
10 Centre for Medical Genetics, Reproduction and Genetics; Genetics and Regenerative Medicine, UZ Brussel, Vrije Universiteit Brussel (VUB), Brussels, Belgium
11 Department of Medical Genetics, Children's Hospitals and Clinics of Minnesota, Minneapolis, USA
12 Division of Clinical Genomics, Ambry Genetics, Aliso Viejo CA, USA
13 GENOMED, Centre for Medical Genetics, University of Antwerp, University Hospital, Antwerp, Belgium
14 Department of Neuropaediatrics, University Medical Centre Schleswig-Holstein, Christian Albrechts University, Kiel, Germany
15 Division of Neurology, The Children's Hospital of Philadelphia, Philadelphia, USA
16 Department of Neurology, Antwerp University Hospital, Antwerp, Belgium
17 Inserm U 1127, CNRS UMR 7225, Sorbonne Universités, UPMC Univ Paris 06 UMR S 1127, Institut du Cerveau et de la Moelle épinière (ICM), F-75013, Paris, France
18 AP-HP, Hôpital de la Pitié-Salpêtrière, Département de Génétique, F-75013, Paris, France

Correspondence to: Prof. Dr. Peter De Jonghe,
Neurogenetics group,
VIB-Department of Molecular Genetics,
University of Antwerp – CDE (Building V),
Antwerp, Belgium
E-mail: peter.dejonghe@molgen.vib-ua.be

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Abbreviations: 5'PP = 5'-phosphatase domain; $PI(4,5)P_2$ = phosphatidylinositol 4,5-bisphosphate

Introduction

Synaptic transmission is a highly complex process that mediates rapid propagation of information in the nervous system. Synaptic vesicles play a key role in this process, as they are stored in the presynaptic compartment and release their content by exocytosis in the synaptic cleft. Endocytosis and recycling of synaptic vesicle membranes allow their reuse in multiple cycles of secretion, hence maintain the synapse function during high frequency stimulation (Sudhof, 2004). Synaptic vesicle endocytosis is dynamically regulated and requires transient recruitment to the membrane of a variety of cytosolic factors, which interact with both membrane proteins and phospholipids to control budding and fission (Brodin *et al.*, 2000; Di Paolo and De Camilli, 2006; Saheki and De Camilli, 2012; Posor *et al.*, 2013).

A major pathway of synaptic vesicle recycling involves clathrin-mediated endocytosis. Synaptojanin 1 (SYNJ1), a protein encoded by the *SYNJ1* gene on human chromosome 21, is a highly conserved polyphosphoinositide phosphatase that is concentrated at synapses (Takei *et al.*, 1995; Cremona *et al.*, 1999; Mani *et al.*, 2007). In particular, its 145 kDa isoform is enriched on clathrin-coated intermediates (Haffner *et al.*, 1997) where one of its major interactors is endophilin, an adaptor protein that binds the highly curved membrane of endocytotic buds (de Heuvel *et al.*, 1997; Ringstad *et al.*, 1997; Farsad *et al.*, 2001; Gallop *et al.*, 2006; Milosevic *et al.*, 2011). A main function of SYNJ1 is to couple the endocytic reaction to the dephosphorylation of phosphatidylinositol 4,5-bisphosphate [$PI(4,5)P_2$], a phosphoinositide concentrated at the plasma membrane. Dephosphorylation is critical for shedding of the clathrin coat and other endocytic factors whose recruitment to endocytic sites is mediated by $PI(4,5)P_2$ in the bilayer (Di Paolo and De Camilli, 2006). This function is facilitated by two consecutive phosphatase domains in the SYNJ1 protein: an N-terminal Sac1-like inositol domain (Sac1) and a central 5'-phosphatase domain (5'PP), which enable the removal of a phosphate group from the 4 and 5 position of $PI(4,5)P_2$, respectively. This functional tandem is followed by a C-terminal proline-rich domain (PRD) that primarily functions as a protein-protein interactor and binds SH3 domain-containing proteins such as endophilin and amphiphysin (McPherson *et al.*, 1996; Ramjaun and McPherson, 1996; de Heuvel *et al.*, 1997; Cestra *et al.*, 1999; Cremona *et al.*, 1999; Guo *et al.*, 1999; Gad *et al.*, 2000; Haffner *et al.*, 2000; Harris *et al.*, 2000; Kim *et al.*, 2002; Verstreken *et al.*, 2003; Perera *et al.*, 2006; Mani *et al.*, 2007; McCrea and De Camilli, 2009; Soda *et al.*, 2012; Posor *et al.*, 2013; Dong *et al.*, 2015).

Genetic defects affecting components of the endocytic machinery are well known to cause neurological disorders of variable severity in both humans and model organisms. Animal models with loss-of-function genetic deficits in *SYNJ1* homologues display neurological features and early death (Cremona *et al.*, 1999; Harris *et al.*, 2000; Luthi *et al.*, 2001; Kim *et al.*, 2002; Verstreken *et al.*, 2003; Van Epps *et al.*, 2004; Sato *et al.*, 2009). In humans, autosomal recessive inherited variants in *SYNJ1* have recently been associated with two different neurological disorders. A recurrent homozygous missense variant located in the Sac1 domain (p.Arg258Gln), which selectively abolishes the phosphatase function of this specific domain, was identified in three independent families with early onset parkinsonism (Krebs *et al.*, 2013; Quadri *et al.*,

2013; Olgiati *et al.*, 2014); whereas a homozygous nonsense variant (p.Arg136*) expected to result in loss of protein formation, was found in a single patient with intractable epilepsy and tau pathology (Dyment *et al.*, 2015*a*, *b*). Here, we describe two consanguineous families of Moroccan ethnicity and a non-consanguineous Caucasian family with two affected siblings each presenting early onset refractory seizures and a neurodegenerative disease course. Using next generation sequencing technologies biallelic *SYNJ1* variants were identified in all three families. We further examined the frequency of *SYNJ1* defects in a large epilepsy cohort and studied the effect of the identified variants on *SYNJ1* mRNA or phosphatase activity.

Patients and methods

Family collection and clinical description

This study was a combined effort of the EuroEPINOMICS RES consortium working group on recessive epilepsies, a project on presumed mitochondrial diseases from La Pitié-Salpêtrière Hôpital in Paris, and the medical genetics unit of the Children's Hospitals and Clinics of Minnesota. The study was approved by the Ethical Committees of the local institutes. Parents of each patient signed an informed consent form for participation.

Case report Family A

The two siblings of Family A are the only offspring of a healthy consanguineous couple of Moroccan origin (Fig. 1A). Both were born after a normal pregnancy. The girl, currently 7 years of age, was admitted to the neonatal unit 3 days after birth for poor feeding and hypotonia. Flexion spasms were diagnosed at the age of 10 months, but in retrospect they probably first occurred at the age of 2.5 months. Her younger brother, currently 6 years of age, also presented with hypotonia at birth and was moved to the neonatal unit 2 days after birth for an apparent life-threatening event. Epileptic spasms occurred at the age of 6 months. Neither sibling showed noticeable development after that. Seizure frequency progressively increased despite polytherapy and both patients developed multiple seizures daily, consisting of myoclonic seizures, tonic seizures and eye blinking. EEG showed a modified hypsarrhythmia or multifocal epileptic activity on a slow background, whereas brain MRI was normal. Overall, both children showed a neurodegenerative disease course with a progressive spastic quadriplegia, severe intellectual disability, central visual impairment, and progressive feeding problems for which tube feeding was started around the age of 5 years. The girl recently became oxygen-dependent. Extended clinical information can be found in Supplementary Table 1. This family was included in a project of the EuroEPINOMICS RES consortium that studied five consanguineous and 17 non-consanguineous families with at least two siblings presenting with unexplained early onset seizures and intellectual disability.

Case report Family B

The two sisters of Family B are the only offspring of a healthy Moroccan couple, who are second-degree relatives (Fig. 1A). Both were born after a normal pregnancy, had onset of clonic seizures during the first day of life and subsequently developed myoclonic and tonic-clonic seizures. The oldest sister, currently 5 years of age, had several episodes of convulsive status epilepticus. Although seizure frequency stabilized in the oldest girl, they both developed monthly seizures despite polytherapy. Both have profound intellectual disability, and developed a progressive spastic quadriplegia and feeding problems for which they received a gastrostomy. EEG showed modified hypsarrhythmia or focal spikes on a slow background, whereas brain MRI was normal. Increased serum creatine kinase was suggestive of myopathy. Due to high lactate and a phenotype associating liver disease and myoclonic epilepsy, a mitochondrial origin was suspected. Spectrophotometry of respiratory chain complexes showed a combined deficiency in complex III and IV activity in liver and fibroblasts. Metabolic investigations remained inconclusive. Extended clinical information can be found in Supplementary Table 1. This family was included in a French research study on the genetic aetiology of presumed mitochondrial disorders. Nineteen families including 25 patients were recruited according to strong arguments of mitochondrial diseases (MRI, muscle or liver biopsy, hyperlactataemia), and negative whole mitochondrial DNA screening. The cohort included three consanguineous sib-pairs, 13 sporadic consanguineous cases, and three non-consanguineous sib-pairs.

Case report Family C

Family C consists of a non-consanguineous Caucasian couple with two affected sons and a healthy daughter (Fig. 1A). The eldest brother presented seizures from Day 12, consisting of eye blinking and shoulder movements. Seizures evolved to tonic-clonic and myoclonic seizures and were difficult to treat despite combination therapy with anti-epileptic drug. EEGs were not available for review. MRI at the age of 6 months showed no abnormalities. Whereas he was hypotonic in the neonatal period, he became increasingly hypertonic with tendency to opisthotonus and co-occurring dystonia. He died at the age of 2.5 years after a progressive neurological decline from birth. At that time, he had profound intellectual disability and was tube fed. No autopsy was performed, but he was thought to have died from cardiopulmonary arrest after aspiration in the context of a gastro-intestinal infection. His younger brother had onset of seizures with eye deviation on the first day of life, and subsequently developed refractory generalized tonic-clonic and myoclonic seizures. He developed a progressive spastic quadriparesis and feeding problems for which he received a gastrostomy. He was profoundly impaired, non-verbal and did not present eye contact. He died recently at the age of 8 years, probably from cardiopulmonary impairment in the context of an unspecified infection too. No autopsy was done. EEGs in both sibs showed multifocal epileptic activity on a slow background. MRI was normal in the eldest sib, and showed a thin corpus callosum and limited gliosis and atrophy of the periventricular white matter in the youngest brother. Extended clinical information can be found in Supplementary Table 1. The parents and youngest brother

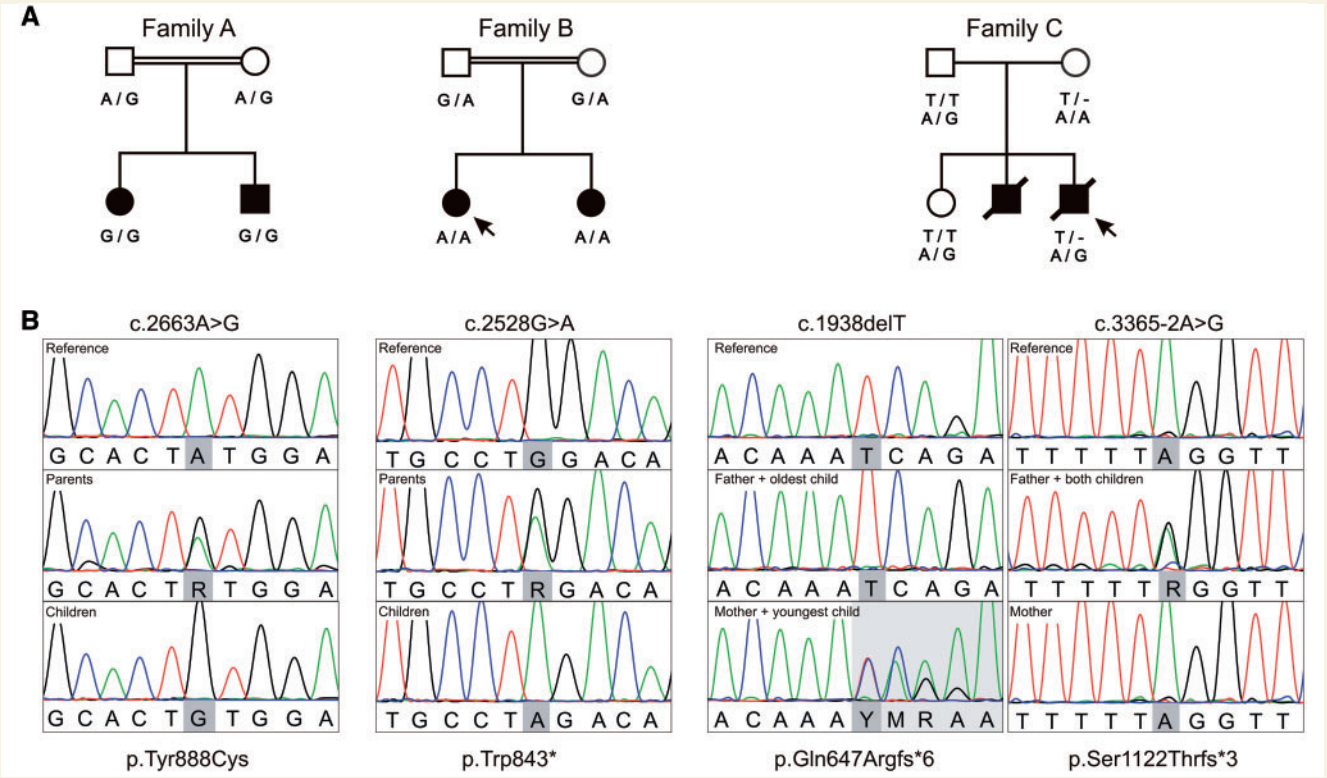

Figure 1 Families with recessive inherited *SYNJ1* variants reported here. (**A**) Pedigrees of Families A–C with an indication of the segregating *SYNJ1* variants (NM_203446.2; NP_982271.2). All parents are healthy, affected children are indicated by filled symbols. Double horizontal lines indicate consanguinity between the parents and arrows point towards the patients whose fibroblasts were used in the mRNA expression studies. (**B**) Visualization of sequencing reads produced by direct Sanger sequencing during variant validation.

underwent whole exome sequencing as part of the diagnostic workup.

Next generation sequencing and data analysis

Families A and B were included in two different next generation sequencing research studies, and Family C in a clinical diagnostic workup, with the common goal to identify the underlying genetic defect in the respective siblings. Genomic DNA was isolated from whole blood from patients and all available family members. Differences in the initial study designs led to variability in the number of individuals sequenced (four for Family A, two for Family B, and three for Family C), the choice of sequencing technique (exome or genome sequencing), and analysis platforms. Nevertheless, similar filtering cascades for variant prioritization were used. A schematic overview of the genetic workflow can be found in Supplementary Fig. 1. The most promising candidate variants were confirmed by direct Sanger sequencing.

Whole genome sequencing of Family A

Whole genome sequencing was performed by Complete Genomics on DNA from the two affected children and both healthy parents of Family A ($n = 4$). Data generation, annotation and variant prioritization was done as previously described (Schubert *et al.*, 2014; Hardies *et al.*, 2015). The pairwise population concordance (Supplementary material) and runs of homozygosity were determined with PLINK v1.07 (http://pngu.mgh.harvard.edu/purcell/plink/) (Purcell *et al.*, 2007).

Whole exome sequencing of Family B

Whole exome sequencing was performed on DNA from the two affected children from Family B ($n = 2$) by the IGBMC Microarray and Sequencing platform (Strasbourg, France). Exon-capture was performed using the SureSelect XT2 Human all exon V5 enrichment System (Agilent) followed by HiSeq 2500 sequencing (Illumina Inc.) according to the manufacturer's protocol. Image analysis and base calling were performed using the Real-Time Analysis software from Illumina. DNA sequences were aligned to the reference genome hg19 using Burrows-Wheeler Aligner (BWA) v0.6.1 (Li and Durbin, 2009). Aligned data were then refined with the use of Picard v1.68 (http://picard.sourceforge.net/) to flag duplicate reads, GATK v2.5.2 (DePristo *et al.*, 2011) to perform local realignments and recalibrate base qualities, and Samtools v0.1.18 (Li and Durbin, 2009) to filter out multi-mapped reads. Variant calling was done by combining results of four variant callers i.e. GATK UnifiedGenotyper, GATK HaplotypeCaller, Samtools v0.1.18 mpileup and Samtools 0.1.7 pileup. Variant quality scores were recalibrated using

GATK. Variants were annotated using SnpEff v2.0.5, GATK v2.5.2 and SnpSift v3.3c (Cingolani *et al.*, 2012*a*, *b*). Finally, VaRank (Geoffroy *et al.*, 2015) was used to rank discovered variants, which were filtered on recessive state in the two affected children.

Whole exome sequencing of Family C

Whole exome sequencing was performed on DNA of the youngest patient of Family C and his parents ($n = 3$) at Ambry Genetics (Aliso Viejo, CA). Library preparation, sequencing, bioinformatics, and data analysis were performed as previously described (Farwell *et al.*, 2015). Briefly, samples were prepared and sequenced using paired-end, 100 cycle chemistry on the Illumnia HiSeq 2500. Enrichment was performed using the IDT xGen Exome Research Panel V1.0. Data were annotated with the Ambry Variant Analyzer tool.

Genetic follow-up

To investigate the frequency of recessive *SYNJ1* variants in a larger epilepsy cohort, a 3-fold genetic follow-up was performed (Supplementary Fig. 2): (i) we re-examined the *SYNJ1* region within whole exome sequencing data of 97 patients with epileptic encephalopathies and their healthy parents (previously described in Suls *et al.*, 2013); (ii) we developed a targeted Multiplex Amplification of Specific Targets for Resequencing (MASTR) assay (Multiplicom) and screened all coding regions as well as flanking intronic segments of *SYNJ1* in 154 index patients with epilepsy and developmental delay (previously described in Hardies *et al.*, 2015); and (iii) we screened 292 additional patients with a larger gene panel covering both known and candidate genes for neurodevelopmental disorders, including *SYNJ1*. Ninety of these patients (31%) were suspected to have a recessively inherited genetic defect based on the presence of consanguinity or multiple affected siblings. Target genes were captured with a custom made kit (Agilent) and sequenced on a SOLiD™ 4 System (Applied Biosystems). Reads were aligned with the BWA (Li and Durbin, 2009). Variant calling and analysis was done with a custom pipeline.

Evaluating the effect of missense variants on the enzymatic activity of SYNJ1

To study the effect of identified missense variants in *SYNJ1* on protein function, the dephosphorylation by recombinant purified proteins of domain-specific substrates, diC8 PI(4,5)P_2 and diC8 PI(3,4,5)P_3 for 5'PP, and diC8 phosphatidylinositol 4-phosphate [PI(4)P] for Sac1 was quantified. To obtain wild-type and mutant proteins, the coding sequence of human 145 kD SYNJ1 was cloned into the p3XFLAG-CMV10 vector. Substitution p.Tyr888Cys and p.Met1020Ile/p.Tyr1057Ser were further generated by site-directed mutagenesis from the wild-type constructs (QuikChange® system, Stratagene). Plasmids harbouring wild-type or mutant *SYNJ1* were transfected into Expi293F cells for over-expression (Thermo Fisher Scientific). Proteins were first purified by pull down using anti-FLAG M2 beads (Sigma), eluted with the FLAG peptide and further purified by gel filtration on a Superdex™ 200 column (GE Healthcare). The gel filtration buffer contained 20 mM HEPES at pH 8.0, 150 mM NaCl, and 0.5 mM TCEP [tris(2-carboxyethyl)phosphine].

The enzymatic activity of wild-type and mutant proteins was measured by a malachite-green assay as previously described (Maehama *et al.*, 2000). In short, 5 nM SYNJ1 (wild-type or mutant) was incubated with 1 µg of substrate PI(4)P, PI(4,5)P_2 or phosphatidylinositol (3,4,5)-trisphosphate [PI(3,4,5)P_3] at room temperature for 5 min in a total volume of 25 µl reaction mixture, which also contained 20 mM HEPES at pH 7.4, 100 mM KCl, 2 mM $MgCl_2$, and 1 mM EGTA. After addition of 75 µl of malachite green reagent, phosphate release was measured by absorbance at OD_{620} (Echelon Biosciences Inc.).

Evaluating the effect of premature stop variants on *SYNJ1* mRNA formation

Fibroblasts were generated from skin biopsies of the oldest affected sister from Family B and the youngest affected brother from Family C (Fig. 1A). Cells were cultivated at 37°C in either Dulbecco's modified Eagle medium (DMEM) high glucose (Gibco®) enriched with 10% heat-inactivated foetal calf serum, 1% L-glutamine and 1% penicillin-streptomycin or minimum essential medium (MEM) enriched with 10% heat-inactivated foetal calf serum, 1% penicillin-streptomycin and 100× MEM non-essential amino acid solution (all from Thermo Scientific). To confirm the presence of nonsense-mediated mRNA decay, fibroblasts were split into three subcultures. One subculture remained untreated, whereas the other two were exposed for 4 h at 37°C to 150 µg/ml cycloheximide or an equal volume of solvent (1% dimethyl sulphoxide). After incubation, cells were extensively washed with Dulbecco's phosphate-buffered saline (DPBS, Thermo Scientific) and harvested by centrifugation.

RNA was extracted with the RNeasy® Mini Kit (Qiagen) from 2×10^6 pelleted fibroblasts, according to the manufacturer's instructions. The extracted RNA was treated with the TURBO DNA-free™ Kit (Ambion) and 1 µg was converted to cDNA using the SuperScript® III First-Strand Synthesis System (Life Technologies) with both oligo-dT and random hexamer primers. RNA concentrations were measured with the NanoDrop1000 (Thermo Scientific). To visualize *SYNJ1* mutant transcripts and confirm cell lineage, direct Sanger sequencing was performed on 50 ng cDNA from cycloheximide treated cells (data not shown). Quantitative polymerase chain reaction (qPCR) for relative quantification of SYNJ1 transcript levels was performed on 10 ng cDNA using a Fast SYBR® Green master mix (Life Technologies) with primer pairs specific for *SYNJ1* and five housekeeping genes (primers available on request). Resultant data were analysed with the ViiATM7 PCR system (Life Technologies). Measurements were normalized against the two most stable housekeeping genes determined by geNorm (Vandesompele *et al.*, 2002). Levels of mutant transcript from patient-derived fibroblasts were relatively quantified to the level of wild-type transcript from a control cell line. Unpaired *t*-testing compared the normalized and relative mean ΔCt values. Statistical analyses were done with GraphPad Prism version 6.05 for Windows (La Jolla California USA, www.graphpad.com).

Results

Next generation sequencing data analysis

Different next generation study strategies were applied in Families A–C and identified potentially causal recessive variants in *SYNJ1* (NM_203446.2; NP_982271.2) in all three. Additional variants are listed in Supplementary Table 2.

Whole genome sequencing of Family A

On average, 91.3% of the genome and 93.5% of the exome was covered at 20× or higher for the two siblings and their parents. The common ancestry of Family A was confirmed with a calculated pairwise population concordance value of 1 (Supplementary material) and the identification of 30 homozygous regions covering 160.477 Mb genome wide. Whole genome sequencing of both healthy parents and the two affected children resulted in an average of 4 318 298 variant callings per sample; taken together a total of 6 644 778 different variants were called in at least one family member. Of the latter, only 154 variants were retained after initial prioritization on quality, frequency in control databases, and predicted impact. The only homozygous candidate variant present in both patients was located in *SYNJ1* c.2663A>G, which was validated by direct Sanger sequencing (Fig. 1B). The resulting amino acid substitution, p.Tyr888Cys, is conserved through evolution (Supplementary Fig. 3) and was unanimously predicted to be damaging by PolyPhen-2, MutationTaster, and likelihood ratio test. The two siblings did not share any compound heterozygous variants, nor did they share novel heterozygous variants that were absent in both parents (possible germline mosaicism).

Whole exome sequencing of Family B

On average, 93% of the exome was covered at 20× or higher for the two siblings. Whole exome sequencing data analysis of the two affected children of Family B resulted in an average of 66 671 variant callings per sample. Of these, 175 variants were retained after initial prioritization on quality, frequency in control databases, and predicted impact. Seven were homozygous in both sisters and an additional three compound heterozygotes were identified (Supplementary Table 2). Extensive data mining on the remaining genes' function, disease association, expression and *in silico* variant predictions, implied a premature stop codon in *SYNJ1* (c.2528G>A; p.Trp843*) as best candidate. Using Sanger sequencing (Fig. 1B) this variant was confirmed in the patients and found in a heterozygous state in the parents.

Whole exome sequencing of Family C

On average, 97% of the exome was covered at 20× or higher. Whole exome sequencing of this trio resulted in an average of 127 577 variant callings per sample. Of these 2165 were retained after filtering on quality, frequency in controls and impact on the protein. One homozygous and three compound heterozygous genetic defects were identified in the index patient (Supplementary Table 2). Again, extensive data mining pointed towards two variants located in *SYNJ1* (c.1938delT and c.3365-2A>G; p.Gln647Argfs*6 and p.Ser1122Thrfs*6) as best candidates. Recessive segregation was confirmed in the family using Sanger sequencing (Fig. 1B); the proband's unaffected older sister was found to be a heterozygous carrier of the c.3365-2A>G splice site alteration.

Given the comparable phenotype of the affected siblings in all three families, and the similarities with a previously reported patient (Dyment *et al.*, 2015*a*, *b*), the bi-allelic genetic disruption of *SYNJ1* was considered likely pathogenic in all three families (Supplementary Fig. 1 and Supplementary Table 2).

Genetic follow-up analyses

Using three different methods, a total of 543 index patients with various forms of epilepsy and/or developmental delay were screened for recessive variants in *SYNJ1*. Data analysis of the MASTR assay revealed a potentially compound heterozygous *SYNJ1* defect in an isolated patient with a severe, early onset fever-sensitive epileptic encephalopathy resembling Dravet syndrome (Supplementary Fig. 2). Sanger sequencing of the patients' parents confirmed paternal inheritance of variant c.3060G>T (p.Met1020Ile) and maternal inheritance of variant c.3170A>C (p.Tyr1057Ser). Whereas the latter variant was novel, the first has been reported (rs115683257) in a heterozygous state by both the Exome Variant Server (0.48%) and the ExAC Browser (0.07%). *In silico* prediction programs were inconsistent for both substitutions: predicted tolerated by SIFT, disease causing by MutationTaster and benign to possibly damaging by PolyPhen-2. We did not identify any other potential deleterious variants in *SYNJ1*.

Enzymatic activity is reduced for the p.Tyr888Cys variant (Family A)

We tested the effect of the identified substitutions (p.Tyr888Cys and p.Met1020Ile/p.Tyr1057Ser) on the enzymatic activity of SYNJ1 *in vitro*, using purified proteins from transfected Expi293F cells (Fig. 2). A clear reduction ($P < 0.0001$–0.0003) towards all tested substrates was found for the p.Tyr888Cys substitution located in the 5'PP domain of the protein (Fig. 3). We did not find any difference in enzymatic activity for aberrant proteins carrying the p.Met1020Ile/p.Tyr1057Ser substitution identified during our genetic follow-up study. These findings are consistent with (i) the localization of these two amino acids in a predicted unfolded region of the protein outside the phosphatase domains (Fig. 3); (ii) the non-conservation of this residue between SYNJ1 and SYNJ2, two enzymes with similar catalytic activities (Supplementary Fig. 3); (iii) the

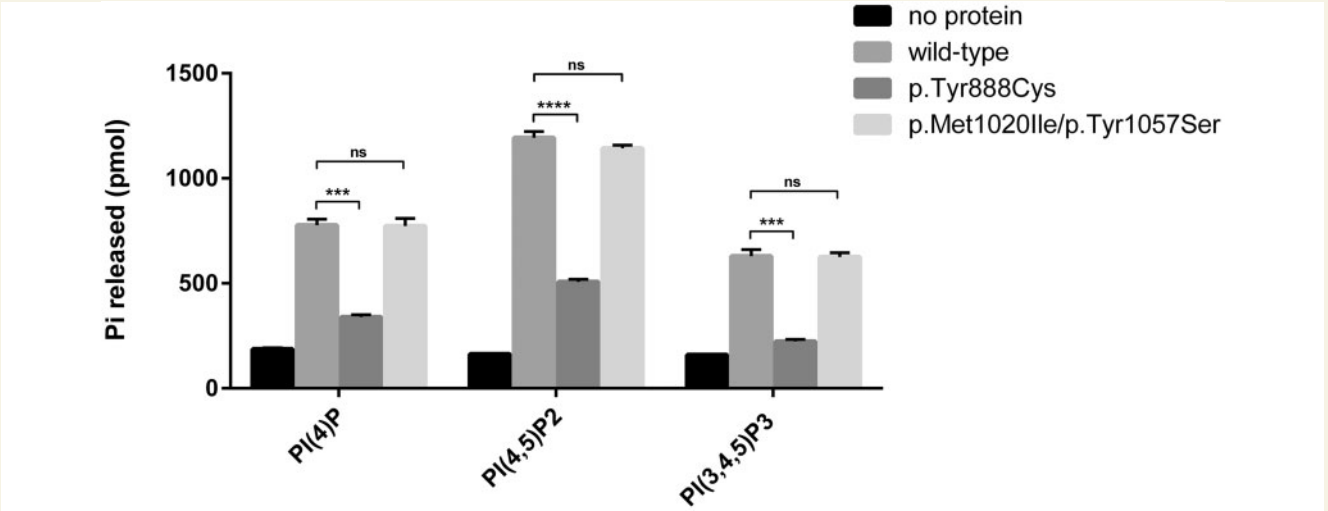

Figure 2 Phosphatase activity assay of the identified *SYNJ1* missense variants. The effect on enzymatic activity of SYNJ1 was tested for mutants resulting from missense variants. Wild-type or mutant FLAG-tagged SYNJ1 (NM_203446.2) was expressed in Expi293F cells, purified and tested for enzymatic activity against short chain phosphoinositides using a malachite-based assay (Maehama *et al*., 2000). The dephosphorylation of domain specific substrates was investigated: PI(4)P for the Sac1 domain, and $PI(4,5)P_2$ and $PI(3,4,5)P_3$ for the 5'PP domain. Because dephosphorylation of $PI(4,5)P_2$ by the 5'PP domain results in PI4P, which can then be dephosphorylated by the Sac1 domain, the phosphate released when using $PI(4,5)P_2$ as substrate reflects the action of both phosphatase domains. When $PI(3,4,5)P_3$ was used as a substrate, selective action of the 5'PP domain could be assessed. Graphics depict mean values of released inorganic free phosphate from three independent experiments (bars reflect standard error of mean). A clear reduction, but not a complete loss, of phosphate activity towards all tested substrates was found for the p.Tyr888Cys substitution identified in Family A. Compared to wild-type expressing cells, no differences in enzymatic activity was found for cells expressing the p.Met1020Ile/p.Tyr1057Ser substitutions. Unpaired *t*-test was used to analyse the difference between wild-type and mutants (***P = 0.0002–0.0003, ****$P < 0.0001$). ns = non-significant.

Figure 3 Protein structure of human SYNJ1. Domains of the protein are shown in different colours. The positions of newly identified (*above*) and previously reported (*below*) pathogenic variants are indicated (NP_982271.2). Variants identified in patients with early onset refractory epilepsy and progressive neurological decline are marked in red, the recurrent substitution identified in three families with early onset parkinsonism in blue, and the compound heterozygous substitutions that are likely to be polymorphisms (p.Met1020Ile/p.Tyr1057Ser), in green. Sac = Sac1 domain; 5PPtase = 5-phosphatase domain; RRM = RNA recognition motif; PRD = proline rich domain.

difference in phenotype compared to other patients; and (iv) the relative high frequency of one of the variants in population databases (rs115683257; 0.07–0.48%).

Messenger RNA level is diminished by the premature stop variants (Families B and C)

The p.Trp843* variant identified in Family B is located in the 5'PP domain and is predicted to result in a truncated protein, and thus a misfolded/inactive 5'PP domain. Premature stop variants can, however, induce mRNA degradation via nonsense-mediated mRNA decay. Fibroblasts carrying the p.Trp843* variant indeed contained only 15% (mutant) *SYNJ1* mRNA transcript ($P < 0.0001$) compared to wild-type expressing cells (Fig. 4). Transcript levels could be increased after treatment with the translation inhibitor cycloheximide that blocks nonsense-mediated mRNA decay (Lejeune *et al*., 2003) (data not shown). Likewise, the p.Gln647Argfs*6/p.Ser1122Thrfs*3 genetic defect identified in Family C resulted in only 5% of mRNA transcript ($P < 0.0001$; Fig. 4).

Discussion

We describe the clinical and molecular findings of three families with four different autosomal recessive inherited

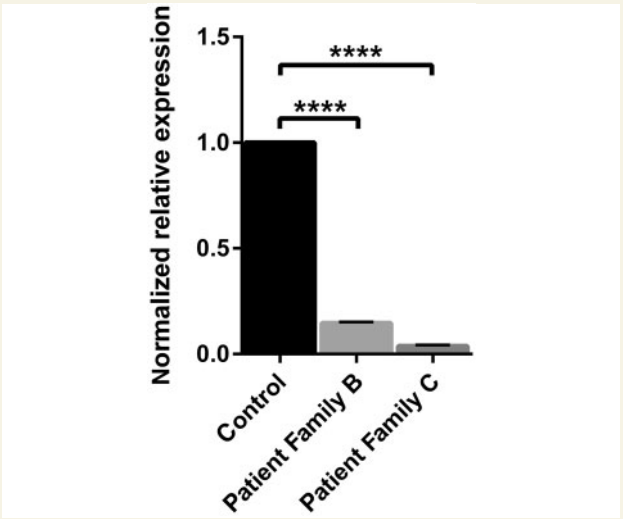

Figure 4 Levels of *SYNJ1* mRNA in patient and control fibroblasts. The effect of the premature stop variants identified in Families B and C was evaluated by the amount of mutant mRNA transcript in patient fibroblasts. Messenger RNA was extracted from human fibroblasts expressing either homozygous wild-type (control) or mutant SYNJ1: patient Family B homozygous for p.Trp843* and patient Family C compound heterozygous for p.Gln647Argfs*6/p.Ser1122Thrfs*3. Quantitative PCR was performed on the converted cDNA. Graphics depict mean values of normalized ΔCt, relatively compared to wild-type mRNA levels, from at least two independent experiments (bars reflect standard error of mean). *P*-values were determined by comparing the normalized ΔCt (unpaired *t*-test, ****$P < 0.0001$). A significant reduction in *SYNJ1* transcript expression was found in both patients' fibroblasts compared to control cells.

SYNJ1 variants and a devastating phenotype with early onset treatment-resistant seizures and progressive neurological decline. The homozygous p.Tyr888Cys substitution identified in Family A and localized in the 5'PP domain, surprisingly showed an impairment of the dephosphorylation activity of both the 5'PP and the Sac1 domain. The unexpected impact on Sac1 domain function suggests the occurrence of an intramolecular regulation. We further showed a significant loss of mutant *SYNJ1* transcript for the premature stop variants in *SYNJ1,* namely a homozygous p.Trp843* variant in Family B and the compound heterozygous variant p.Gln647Argfs*6/p.Ser1122Thrfs*3 in Family C. A similar reduction in *SYNJ1* transcript was shown for the previously reported premature stop variant p.Arg136*, identified in a patient with a comparable phenotype (Dyment *et al.*, 2015*a*, *b*). A large genetic follow-up study ($n = 543$) identified one additional patient who was compound heterozygous for two *SYNJ1* substitutions (p.Met1020Ile/p.Tyr1057Ser). Phosphatase activity of purified proteins carrying these latter variants, located in the C-terminal of the protein and downstream of the catalytic domains, did not differ from wild-type proteins. Although it remains possible that these variants disrupt unknown binding sites and subsequently disable protein-protein interactions or mistarget SYNJ1 (Haffner *et al.*, 1997), we concluded that there is currently no evidence that these variants are pathogenic. Our follow-up screening indicates that bi-allelic defects in *SYNJ1* are not a frequent cause of severe epilepsy, even though the disease variant yield might be higher in a more selected cohort of patients with early onset epilepsy and progressive neurological decline.

Bi-allelic genetic disruption of *SYNJ1* in animal models results in impaired synaptic vesicle recycling dynamics and accumulation of clathrin-coated vesicles (Cremona *et al.*, 1999; Harris *et al.*, 2000; Kim *et al.*, 2002; Verstreken *et al.*, 2003; Van Epps *et al.*, 2004). Knock-out mice that survive longer than 24 h after birth (maximum up to 15 days) progressively develop a variety of neurological manifestations, including ataxia and generalized seizures (Cremona *et al.*, 1999). Zebrafish larvae with synaptojanin mutations present with peripheral blindness and abnormal swimming behaviour (Van Epps *et al.*, 2004; Jia *et al.*, 2014). In humans, recessive variants in *SYNJ1* have been associated with two different neurological disorders, which seem to be related to the variant type and their effect on protein function. A first disease entity consists of refractory epileptic seizures with neonatal or early infancy onset, little or no development after birth, and a progressive neurodegenerative disease course leading to spastic quadriplegia and loss of feeding capacities, as seen in the affected children reported here and in a previously reported patient (Supplementary Table 1; Dyment *et al.*, 2015*a*, *b*). Seizure types vary and include clonic, myoclonic, tonic and/or tonic-clonic seizures. The progressive neurological decline in the presence of myoclonic seizures even led to the suspicion of a progressive myoclonic epilepsy of potential mitochondrial origin in one family (Family B). Three children died between the ages of 2.5 and 8 years, probably due to cardiorespiratory arrest, whereas the oldest living child reported here is currently 7 years old and recently became oxygen-dependent. The *SYNJ1* variants associated with this severe phenotype include two homozygous nonsense variants [the novel p.Trp843*, Family B; and the previously reported p.Arg136* (Dyment *et al.*, 2015*a*, *b*)] and two premature stop variants in a compound heterozygous state (p.Gln647Argfs*6/p.Ser1122Thrfs*3, Family C) leading to loss of functional *SYNJ1* expression, plus a homozygous missense variant (p.Tyr888Cys, Family A) located in the 5'PP domain leading to a decreased dephosphorylation capacity towards all substrates. A second disease entity was recently described in three independent families, and consists of parkinsonism with onset in the third decade of life. Half of the patients with parkinsonism (3/6) also had infrequent (or even a single) generalized tonic-clonic seizures with onset during infancy or adolescence (Krebs *et al.*, 2013; Quadri *et al.*, 2013; Olgiati *et al.*, 2014). All three families carried the same homozygous missense variant (p.Arg258Gln) located in the Sac1 domain. This missense variant specifically abolishes the capacity of the protein to

dephosphorylate Sac1-specific substrates, whereas the 5'PP domain activity remains unchanged (Krebs *et al.*, 2013). Hence, the phenotypic presentation associated with autosomal recessive *SYNJ1* variants in humans seems to correlate with the residual *SYNJ1* phosphatase activity: variants resulting in a (nearly) complete loss of SYNJ1 dual phosphatase activity, either by loss of protein formation or protein function, lead to a devastating phenotype with early onset refractory seizures, progressive neurological decline and early mortality; whereas loss of dephosphorylation activity limited to the Sac1 domain leads to parkinsonism with onset in early adulthood and an apparent mild increase in seizure susceptibility. The identification of additional patients will help addressing full phenotype–genotype correlations.

Our results add new evidence to the concept that dysregulation of endocytic recycling of synaptic vesicles in neurons is an important biological pathway in the aetiology of epilepsy with disturbance of neurodevelopment. Consistent with these findings, mice knock-out studies have shown that loss of function variants in endophilin and amphiphysin, two SYNJ1 binding proteins, also lead to severe epilepsy (Di Paolo *et al.*, 2002; Milosevic *et al.*, 2011), and *de novo* mutations in *DNM1*, which impairs synaptic vesicle endocytosis, were shown to cause a severe epileptic encephalopathy in humans (EuroEPINOMICS RES Consortium *et al.*, 2014). Increased understanding of the different pathways leading to neurodevelopmental disorders will guide future development of novel treatments for these severely affected patients.

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Supplementary material

Supplementary material is available at *Brain* online.

Conflict of interest

Since mid-October 2015, K. Hardies is under employment of UCB Pharma (Braine-l'Alleud, Belgium). The company had no part in this study, all the data was generated and analysed before enrolment. K. L. Helbig and S.A. Sajan are employed by Ambry Genetics, a company that provides exome sequencing among its commercially available tests (here used for Family C). None of the other authors have disclosures.

Appendix 1

The AR working group of the EuroEPINOMICS RES Consortium consists of the following consortium members:

Zaid Afawi; Stéphanie Baulac; Nina Barisic; Hande Caglayan; Dana Craiu; Carolien G. De Kovel; Rosa Guerrero Lopez; Renzo Guerrini; Helle Hjalgrim; Holger Lerche; Johanna Jahn; Karl Martin Klein; Bobby C. Koeleman; Eric Leguern; Johannes Lemke; Carla Marini; Hiltrud Muhle; Felix Rosenow; José M. Serratosa; Katalin Štěrbová; Rikke S. Møller; Aarno Palotie; Pasquale Striano; Yvonne Weber; Federico Zara

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