Mutations in *TNK2* in Severe Autosomal Recessive Infantile Onset Epilepsy

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We identified a small family with autosomal recessive, infantile onset epilepsy and intellectual disability. Exome sequencing identified a homozygous missense variant in the gene *TNK2*, encoding a brain-expressed tyrosine kinase. Sequencing of the coding region of *TNK2* in 110 patients with a similar phenotype failed to detect further homozygote or compound heterozygote mutations. Pathogenicity of the variant is supported by the results of our functional studies, which demonstrated that the variant abolishes NEDD4 binding to TNK2, preventing its degradation after epidermal growth factor stimulation. Definitive proof of pathogenicity will require confirmation in unrelated patients.

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In recent years, a growing list of genes have been identified in monogenic forms of epilepsy.[1] Nevertheless, the causative gene remains unidentified in a considerable proportion of familial epilepsies. Thanks to the advent of next generation sequencing technology, it is now possible to identify the underlying mutations in small pedigrees, even in cases where conventional linkage analysis is underpowered.[2]

Here we applied exome sequencing to identify the causative gene in a small pedigree with a novel phenotype of severe infantile onset focal epilepsy and cognitive regression.

Subjects and Methods

Patients

We identified a nonconsanguineous, Belgian–Italian family in which all 3 siblings presented with infantile onset epilepsy and cognitive regression (Fig 1).

Written informed consent was obtained from all family members or their legal representatives. The study was approved by the Ethics Commission of Erasme Hospital, Brussels, Belgium. Clinical data were collected from medical records.

Exome Sequencing

Exome sequencing was performed on DNA extracted from lymphocytes of the proband and the oldest brother using an Agilent (Santa Clara, CA) All-Exon 50Mb capture kit on an Illumina (San Diego, CA) HiSeq2000 with a coverage of $>50\times$. Paired-end reads were aligned to the human reference genome (National Center for Biotechnology Information Build 36) with Burrows–Wheeler Aligner software.[3] Variant calling was performed with SAMtools software,[4] and SequenceVariantAnalyzer[5] was used for annotating and listing identified variants (Ensembl 50_36l). Controls consisted of 403 individuals nonenriched for epilepsy or other neuropsychiatric phenotypes who underwent exome or whole genome sequencing as part of other studies at the Duke Center for Human Genome Variation. We identified all shared homozygous and compound heterozygous variants predicted to disrupt the protein (nonsense, missense, or splice site mutations) with a minor allele frequency of $\leq 1\%$ in the controls.

Follow-up Sequencing and Genotyping

The identified candidate mutation was confirmed by Sanger sequencing in the 3 affected children and both parents. Further genotyping of the variant was performed using a custom-

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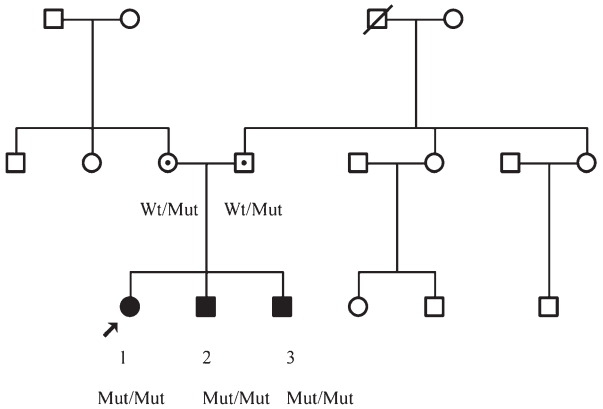

FIGURE 1: Family pedigree and genotypes. The proband is indicated by an arrow. Wt = wild type (chr3:197079609_C, hg18); Mut = mutated (chr3:197079609_T).

designed TaqMan assay (Applied Biosystems, Foster City, CA) in 3,140 patients with epilepsy from the EpiGen cohort (www.epilepsygenetics.eu), which comprises mainly adult Caucasians with predominantly focal epilepsy, and 1,693 controls (1,309 from Duke University Genetics of Memory Cohort and 384 with documented Belgian ancestry from the Occupational Health Department of Erasme Hospital, Brussels, Belgium). Primer sequences are available in Supplementary Table 1. In an attempt to identify other pathogenic variants in the *TNK2* gene, we performed Sanger sequencing of all 15 exons and splice sites in 110 patients with infantile onset epilepsy and intellectual disability (94 from Australia and 16 from Belgium). Clinical details are provided in Supplementary Table 2.

Functional Studies

PLASMIDS. cDNAs encoding human *TNK2,* neural precursor cell expressed developmentally down-regulated 4-1 (*NEDD4-1*), and *NEDD4-2* were amplified from first-strand cDNA derived from the human neuroblastoma A172. The *TNK2* 716Met allele was made by polymerase chain reaction-directed mutagenesis on the pCR-Blunt II-TOPO vector (Invitrogen-Life Technologies, Carlsbad, CA). cDNAs were subcloned into pcDNA3.1^{+} vector (Invitrogen-Life Technologies). Primer sequences are shown in Supplementary Table 1.

WESTERN BLOTTING. Plasmids were transfected into human kidney cell line HEK-293 using Lipofectamine 2000 (Invitrogen-Life Technologies). After 48 hours of transfection, the cells were lysed, and the lysates were subjected to sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis gel and transferred to a polyvinylidene difluoride membrane (Millipore, Billerica, MA). The membranes were incubated with anti–human-TNK2 polyclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA) or anti–glyceraldehyde-3-phosphate dehydrogenase (Cell Signaling Technology, Danvers, MA). Proteins were visualized with the enhanced chemiluminescence plus Western blotting detection system (GE Healthcare, Piscataway, NJ).

IMMUNOPRECIPITATION. Plasmids were transfected into monkey kidney cell line COS-7. After 48 hours of transfection, the cells were lysed, and the lysates were incubated with anti–human-TNK2 monoclonal antibody (Santa Cruz Biotechnology) and protein G sepharose (Sigma-Aldrich, St Louis, MO). Protein G Sepharose was washed after 16 hours and mixed with SDS sample buffer (Invitrogen-Life Technologies), and subjected to Western blotting with anti-NEDD4 WW domain antibody (Millipore) or anti–human-TNK2 polyclonal antibody.

TNK2 DEGRADATION BY EPIDERMAL GROWTH FACTOR. Plasmids were transfected into COS-7. After 30 hours of transfection, cells were incubated with serum-free Dulbecco modified Eagle medium (0% fetal bovine serum). After 48 hours of transfection, cells were incubated with recombinant human epidermal growth factor (EGF; Sigma-Aldrich) for 1, 2, or 4 hours. Cells were subjected to Western blotting.

Results

Clinical Features

The proband was a girl who presented to the Neuropediatrics Department of Erasme Hospital at the age of 26 months (Supplementary Table 3). She had focal seizures since age 19 months, characterized by unresponsiveness, hypertonia, and automatisms, with occasional secondary generalization. Seizures occurred several times per day and were refractory to multiple antiepileptic drugs (AEDs). Birth and neonatal history were unremarkable. Early development was considered normal by the parents. Cognitive regression occurred soon after seizure onset. She also developed autistic features. Neurological examination was normal. At the time of presentation, there was no family history of epilepsy.

Neuropsychological testing at 31 months showed an overall developmental age of 16 months and language development of 13 months. Brain magnetic resonance imaging (MRI) was normal. Preoperative investigations including surface and intracranial video-electroencephalographic (EEG) monitoring and 18F-fluorodeoxyglucose positron emission tomography were consistent with right anteromesial temporal lobe seizure onset. The patient underwent a right temporal lobectomy at the age of 4.5 years. Pathological examination of the resected tissue was unremarkable. Seizures recurred after a honeymoon period of 3 months. At last follow-up, the patient was 9 years old and had 15 to 20 seizures per month.

The patient's younger brother developed epilepsy at the age of 21 months, with focal seizures characterized by unresponsiveness, pallor, hypertonia, and automatisms. Early development was considered normal by the parents, but speech and cognitive regression occurred soon after epilepsy onset. His developmental quotient was 78 at 3 years and 33 at 5 years. He also exhibited behavioral disturbance and hyperactivity. Neurological examination was normal. Brain MRI and interictal

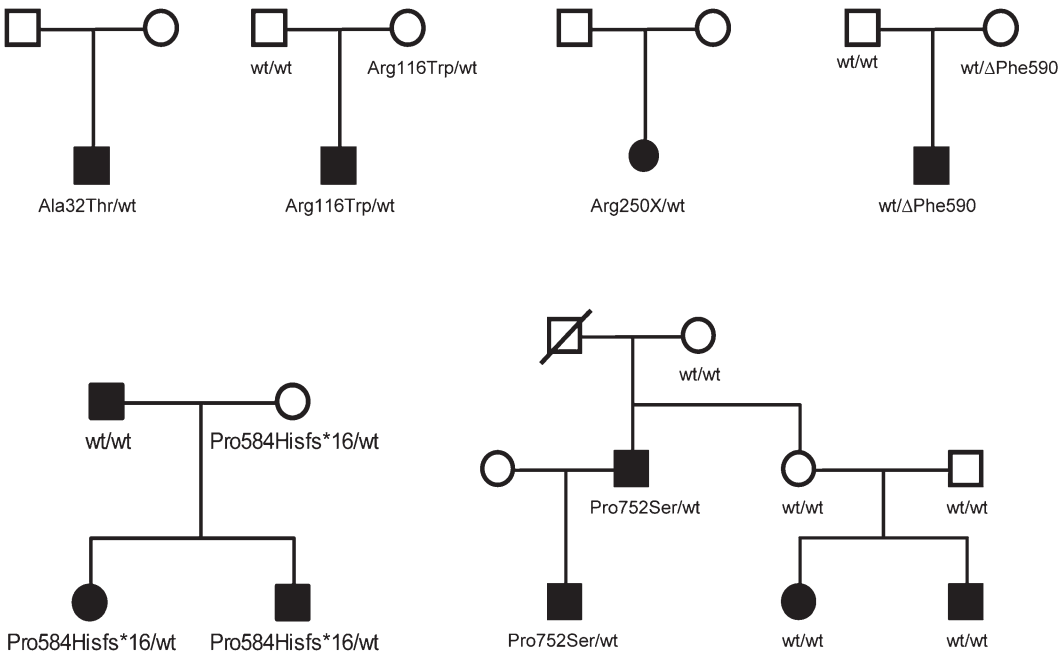

FIGURE 2: Family pedigrees and genotypes for detected heterozygous variants in TNK2. wt= wild type.

EEG were normal. At last follow-up, he was 8 years old and had about 2 seizures per month despite AED bitherapy.

The youngest child developed epilepsy at age 35 months. Seizures were characterized by unresponsiveness, pallor, and automatisms. Early development was considered normal by the parents, but speech delay was noted from the age of 18 months. Cognitive regression occurred soon after epilepsy onset. His developmental quotient was 30 at 5 years. He also had behavioral problems and autistic features. Neurological examination was normal. Brain MRI and interictal EEG were normal. At last follow-up, he was 7 years old and had about 4 seizures per year on AED monotherapy.

Exome Sequencing

Two high-quality single-nucleotide variants were shared, homozygous, and not observed in a homozygous state in

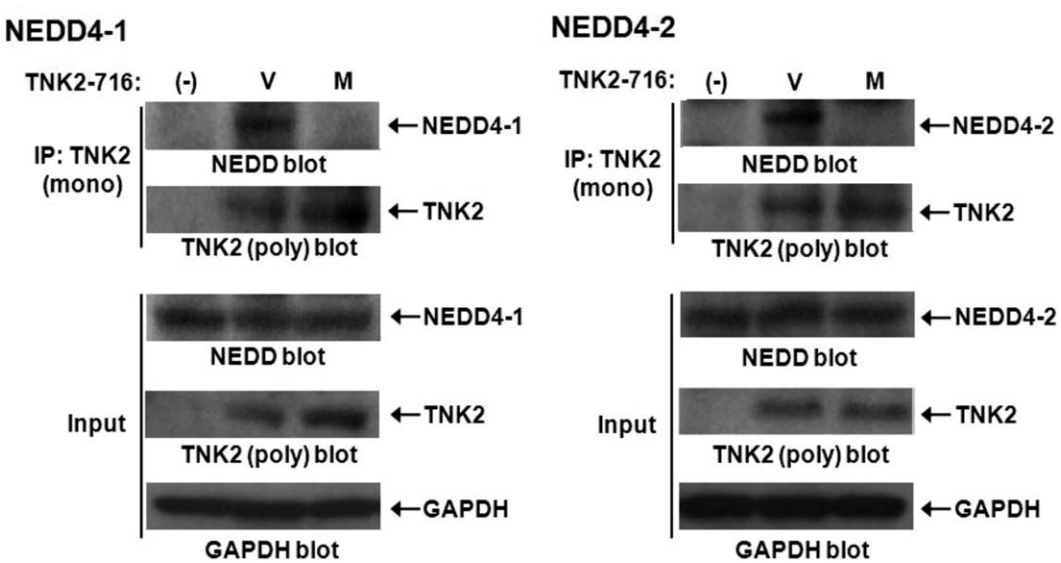

FIGURE 3: Loss of binding between TNK2-716Met and neural precursor cell expressed developmentally down-regulated 4-1 (NEDD4-1) or NEDD4-2. cDNA of each TNK2 allele and NEDD4-1 or NEDD4-2 was cotransfected into COS-7 cells, and binding was detected by immunoprecipitation (IP) using an anti-TNK2 monoclonal antibody after 48 hours of transfection. The top row of both parts shows binding of wild-type TNK2 (716V) to NEDD4-1 and NEDD4-2, whereas binding of variant TNK2 (716M) is abolished. GAPDH = glyceraldehyde-3-phosphate dehydrogenase.

controls: variant *15_73439034_A in the gene *MAN2C1* has a minor allele frequency of 0.03 in controls and is therefore unlikely to be causal. Variant * 3_197079609_T in the gene *TNK2* was absent in the 403 controls. The variant is also absent from the Exome Variant Server (http://evs.gs.washington.edu/EVS/). *TNK2* is located on chromosome 3q29 and encodes a cytosolic, non-receptor tyrosine kinase. The gene has at least 14 alternatively spliced transcript variants, but the full-length nature of only 2 isoforms has been determined (http://www.ensembl.org). Variant *3_197079609_T is located in a highly conserved region in exon 13 (Supplementary Fig 1) and is predicted to result in a Val → Met substitution (c.2146 G>T/Val716Met), which is predicted to be damaging.[6] We identified no good candidate compound heterozygote variants.

Follow-up Sequencing and Genotyping

Sanger sequencing of the *3_197079609_T variant in the family showed that the 3 affected children were homozygotes and both parents were heterozygotes. Genotyping of *3_197079609_T in 3,140 patients with epilepsy and 1,693 controls showed no further homozygotes and very low frequencies of heterozygotes (0.002) in both groups. The frequency of the variant in the 384 Belgian controls was not significantly different from the overall frequency (0.003).

Sanger sequencing of the coding region of *TNK2* in 110 patients with infantile onset epilepsy and intellectual disability identified no further homozygous mutations and 6 novel heterozygous variants (Fig 2). Further genotyping of these variants in the 4 families in which additional subjects were available showed no cosegregation of the variant with the epilepsy phenotype in 2 cases and transmission of the variant from an unaffected parent in 3 instances.

Functional studies

Western blotting showed no difference in protein expression between TNK2 wild-type (716Val) and variant (716Met; Supplementary Fig 2). Because the Val716Met variant is located in the binding region of the E3 ubiquitin protein ligases NEDD4-1 and NEDD4-2, we checked the molecular association between TNK2-716Val and 716Met and ubiquitin ligases by immunoprecipitation.[7,8] Figure 3 demonstrates binding of wild-type but not variant TNK2 with NEDD4-1 and NEDD4-2. TNK2 is degraded by activated NEDD4-1 and NEDD4-2, which are activated by EGF receptor after binding with its ligand EGF.[7,8] EGF stimulation of TNK2 transfectants showed normal degradation of wild-type TNK2 but absence of degradation of the TNK2 variant protein (Fig 4). These results indicate that

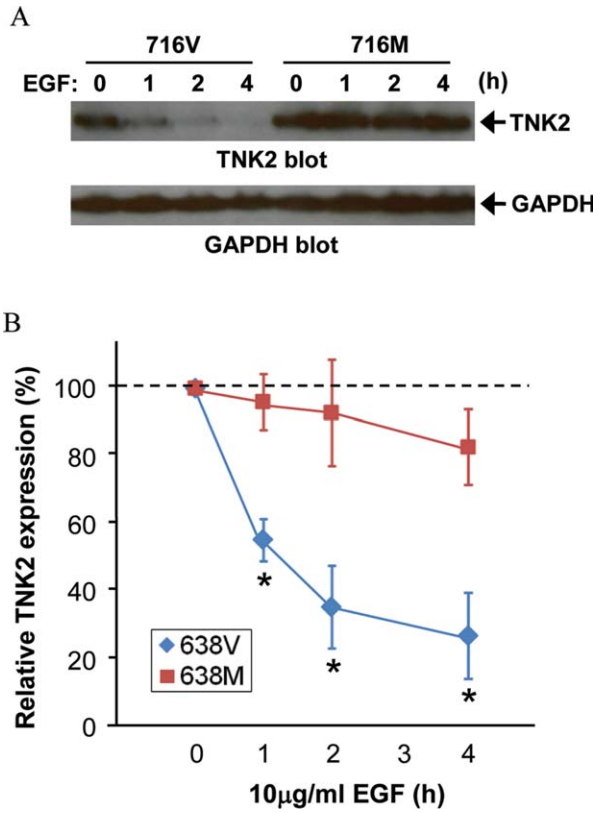

FIGURE 4: Loss of TNK2-716Met degradation after epidermal growth factor (EGF) treatment. cDNA of each TNK2 allele was transfected into COS-7 cells and 10μg/ml of recombinant human EGF was added after 48 hours of transfection. (A) After 1, 2, and 4 hours of incubation, expression of TNK2 was detected by Western blotting. Degradation of wild-type TNK2 (716V) but not of variant TNK2 (716M) is observed. (B) Plotted data represent averages and standard error of triplicated assays. *$p < 0.01$ (Student t test). GAPDH = glyceraldehyde-3-phosphate dehydrogenase. [Color figure can be viewed in the online issue, which is available at www.annalsofneurology.org.]

TNK2 Val716Met disrupts the molecular associations with these ubiquitin ligases, thus leading to its loss of degradation by EGF-mediated activation.

Discussion

We report the clinical, genetic, and functional findings in a family with a distinct phenotype of autosomal recessive, infantile onset epilepsy and intellectual disability. Seizures were reminiscent of mesial temporal lobe seizures. Seizure onset was accompanied by cognitive regression evolving to severe intellectual disability. Behavioral problems and autistic features were additionally noted. The cognitive regression with absence of myoclonus, normal brain MRI, and unremarkable interictal EEG distinguish the phenotype from known infantile onset epileptic syndromes. Although the phenotype in the 2 oldest siblings could be compatible with an epileptic encephalopathy,

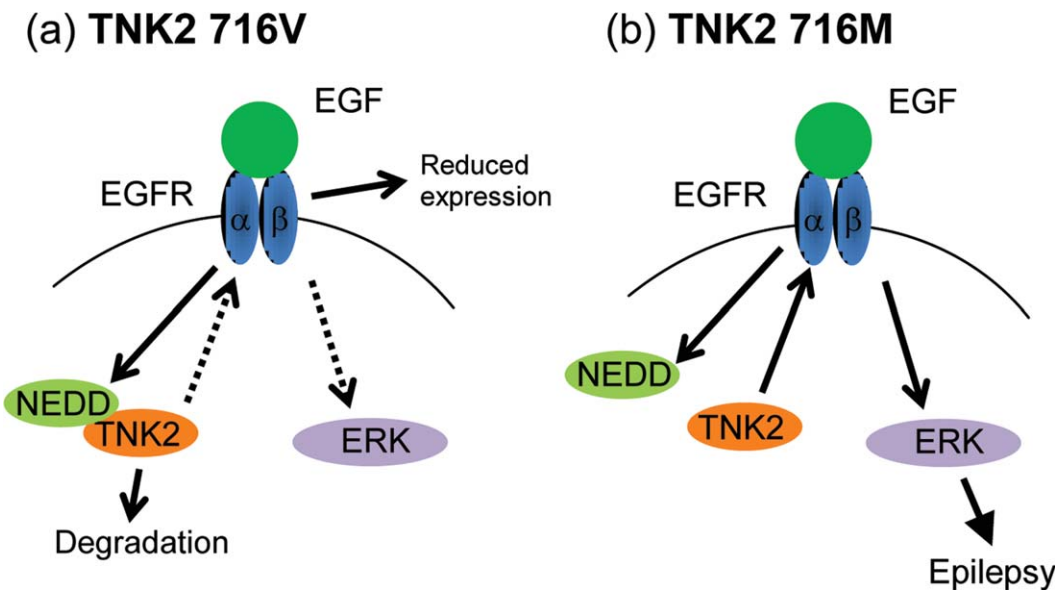

FIGURE 5: Postulated functional effects of TNK2 wild-type and Val716Met variant. (A) Binding of wild-type TNK2 (716V) to neural precursor cell expressed developmentally down-regulated 4-1 (NEDD4-1) and NEDD4-2 results in TNK2 degradation in response to epidermal growth factor (EGF) stimulation. (B) The 716M variant abolishes TNK2 binding with NEDD4-1 and NEDD4-2 and inhibits TNK2 degradation in response to EGF stimulation. Increased expression of TNK2 and EGF receptor induces epilepsy through enhanced extracellular signal-regulated kinase (ERK) activity. EGFR = epidermal growth factor receptor. [Color figure can be viewed in the online issue, which is available at www.annalsofneurology.org.]

we believe that the presence of severe intellectual disability despite the relatively benign epilepsy in the youngest child argues against a diagnosis of epileptic encephalopathy in this family.

Exome sequencing followed by Sanger sequencing identified a homozygous missense mutation that segregated with the phenotype in the gene *TNK2*. Genotyping of this variant in large cohorts of patients with epilepsy and controls confirmed its rarity and identified no further homozygotes.

The TNK2 protein is brain expressed and developmentally regulated in mice, where it is thought to be involved both in adult synaptic function and plasticity and in brain development.[9,10] In humans, TNK2 has been related to tumor invasiveness and metastasis.[11] TNK2 binding to NEDD4-1 and NEDD4-2 results in TNK2 degradation in response to EGF stimulation.[7,8] We demonstrate that the Val716Met variant abolishes TNK2 binding with NEDD4-1 and NEDD4-2 and inhibits TNK2 degradation in response to EGF stimulation. TNK2 has been reported to be necessary for the stability of the EGF receptor at the cell surface.[12] Activation of extracellular signal-regulated kinase (ERK), the downstream molecule of EGF signaling, results in epileptic seizures in mice through activation of the NR2B N-methyl-D-aspartate receptor.[13] We therefore postulate that increased expression of TNK2 and EGF receptor induces epilepsy through enhanced ERK activity (Fig 5). Interestingly, our results thus suggest a gain of function mechanism, as opposed to other recessive disorders, which are usually caused by a loss of function. Furthermore, TNK2 is known to interact with SEZ6 (seizure related 6 homolog),[14] which has been implicated in febrile seizures and epilepsy in humans.[15,16]

The heterozygous *TNK2* variants identified in cases of infantile onset epilepsy are unlikely to be pathogenic, because they do not segregate with the phenotype and are also detected in unaffected parents. However, our sequencing results do not exclude the presence of undetected recessive alleles, for example, variants located in introns or regulatory regions. Likewise, we cannot definitively exclude the presence of large deletions or insertions.

In conclusion, the combined results of our genetic and functional analyses suggest that the phenotype observed in our family is a consequence of a homozygous mutation in the *TNK2* gene, resulting in a gain of function. Definitive proof of pathogenicity will require identification of further homozygote or compound heterozygote mutations in individuals with a similar phenotype.

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Potential Conflicts of Interest

H.-H.D.: employment, NHMRC. S.F.B.: honoraria, UCB; patents, patent for PCDH19 testing planned, patent for SCN1A testing held by Bionomics and licensed to various diagnostic companies (no financial return); paid educational presentations, UCB Pharma, Novartis Pharmaceuticals, Sanofi-Aventis, Janssen-Cilag. I.E.S.: editorial boards, *Annals of Neurology*, *Epileptic Disorders*; speaking fees, UCB, Janssen-Cilag, Athena Diagnostics; patents filed, WO61/010176 (therapeutic compound); paid educational presentations, UCB, Athena Diagnostics; travel expenses, Athena Diagnostics, UCB, Biocodex, GlaxoSmithKline. B.L.: board membership, GSK, UCB; consultancy, Pfizer; grants/grants pending, speaking fees, UCB; travel expenses, GSK, UCB, Janssen-Cilag. M.R.: grants/grants pending, EC 7th Framework Programme. P.D.J.: grants/grants pending, Fund for Scientific Research Flanders research project on rare epilepsies (2010–2013). M.P.: grants/grants pending, Repligen; speaking fees, Santhera; royalties, Athena Diagnostics. D.B.G.: board membership, Biogen Idec, Knome; consultancy, Vertex Pharmaceuticals, Hoffman LaRoche, Severe Adverse Events Consortium; grants/grants pending, Gilead Sciences, SAIC, Eisai, Biogen Idec, CB Pharma; patents, Merck USA; stock/stock options, Knome. C.D.: grants/grants pending, paid educational presentations, travel expenses, UCB.

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