De Novo Mutations in *YWHAG* Cause Early-Onset Epilepsy

Ilaria Guella,[1,9] Marna B. McKenzie,[1,9] Daniel M. Evans,[1] Sarah E. Buerki,[2] Eric B. Toyota,[3] Margot I. Van Allen,[4] Epilepsy Genomics Study, Mohnish Suri,[5] Frances Elmslie,[6] Deciphering Developmental Disorders Study, Marleen E.H. Simon,[7] Koen L.I. van Gassen,[7] Delphine Héron,[8] Boris Keren,[8] Caroline Nava,[8] Mary B. Connolly,[3] Michelle Demos,[3,*] and Matthew J. Farrer[1,*]

Massively parallel sequencing has revealed many de novo mutations in the etiology of developmental and epileptic encephalopathies (EEs), highlighting their genetic heterogeneity. Additional candidate genes have been prioritized in silico by their co-expression in the brain. Here, we evaluate rare coding variability in 20 candidates nominated with the use of a reference gene set of 51 established EE-associated genes. Variants within the 20 candidate genes were extracted from exome-sequencing data of 42 subjects with EE and no previous genetic diagnosis. We identified 7 rare non-synonymous variants in 7 of 20 genes and performed Sanger sequence validation in affected probands and parental samples. De novo variants were found only in *SLC1A2* (aka *EAAT2* or *GLT1*) (c.244G>A [p.Gly82Arg]) and *YWHAG* (aka 14-3-3γ) (c.394C>T [p.Arg132Cys]), highlighting the potential cause of EE in 5% (2/42) of subjects. Seven additional subjects with de novo variants in *SLC1A2* (n = 1) and *YWHAG* (n = 6) were subsequently identified through online tools. We identified a highly significant enrichment of de novo variants in *YWHAG*, establishing their role in early-onset epilepsy, and we provide additional support for the prior assignment of *SLC1A2*. Hence, in silico modeling of brain co-expression is an efficient method for nominating EE-associated genes to further elucidate the disorder's etiology and genotype-phenotype correlations.

Developmental and epileptic encephalopathies (EEs) are a genetically heterogeneous group of severe epilepsy syndromes characterized by early-onset, treatment-resistant seizures and developmental slowing or regression.[1] There are currently 51 established EE-associated genes, in which many causal mutations arise de novo.[2–13] However, with the advent of high-throughput sequencing, an increasing number of candidate genes are emerging with only a single reported de novo variant.[4,5] The interpretation of these findings remains challenging given that on average ~0.5 de novo exonic mutations are expected per individual.[14–16] Co-segregation analyses in large pedigrees, which usually provide strong genetic evidence for pathogenicity, are not applicable in the case of de novo mutations. Rather, identification of recurrent mutations or different mutations in the same gene helps provide evidence for causality. Such candidate genes can also be prioritized in silico if they belong to the same biological networks, including ion channels and synaptic and regulatory pathways.

Of 179 candidate genes with a single de novo variant reported,[4] Oliver et al.[17] prioritized 19 on the basis of brain-specific co-expression with a reference gene set of 29 established EE-associated genes. Five of the 19 candidate genes have since been confirmed as disease causing, demonstrating the validity of this approach. Using an updated reference gene set of 51 established EE-associated genes, Oliver et al.[13] recently highlighted six more genes that are co-expressed. Here, we evaluate rare coding variability in all co-expressed candidate genes (n = 20) nominated by Oliver et al.[13,17] in 42 EE-affected individuals (14 males and 28 females) selected from 149 epileptic individuals hospitalized and/or attending BC Children's Hospital neurology, medical genetic, and biochemical clinics and enrolled in the Epilepsy Genomics (EPGEN) Study. Eligible subjects had a clinical diagnosis of epilepsy[1] with onset by the age of 5 years and an unknown cause after appropriate investigations, including electroencephalography (EEG), head MRI, neurometabolic testing, and chromosome microarray. Seizure types and electroclinical syndromes were classified according to the International League Against Epilepsy (ILAE).[1] Individuals with self-limiting benign electroclinical syndromes, such as childhood absence epilepsy (onset > 4 years), were excluded on the basis of its more likely multifactorial inheritance. Individuals were identified to have an epileptic encephalopathy if the epileptic activity itself was determined to or was at risk of (e.g., new onset of infantile spasms) contributing to severe cognitive and behavioral impairments above and beyond what might be expected from the underlying pathology alone.[1] The average age of seizure onset for the 42 selected EE subjects was 20.6 ± 18.9 months (1 week to 5 years). Focal seizures with impaired awareness were the most common seizure type (20 subjects [47.6%]), followed by generalized

[1]Centre for Applied Neurogenetics, University of British Columbia, Vancouver, BC V5R 6H8, Canada; [2]Department of Neuropediatrics, Development, and Rehabilitation, University Children's Hospital, Inselspital, 3010 Berne, Switzerland; [3]Division of Neurology, Department of Pediatrics, University of British Columbia and BC Children's Hospital, Vancouver, BC V6H 3V4, Canada; [4]Department of Medical Genetics, University of British Columbia, Vancouver, BC V6H 3N1, Canada; [5]Regional Clinical Genetics Service, Nottingham University Hospitals NHS Trust, Nottingham NG5 1PB, UK; [6]South West Thames Regional Genetics Service, St. George's University Hospitals NHS Foundation Trust, London SW17 0QT, UK; [7]Department of Medical Genetics, University Medical Centre Utrecht, 3508 AB Utrecht, the Netherlands; [8]Département de Génétique et Centre de Référence Déficiences Intellectuelles de Causes Rares, Hôpital de la Pitié-Salpêtrière, Assistance Publique – Hôpitaux de Paris, Paris 75651, France
[9]These authors contributed equally to this work
*Correspondence: mdemos@cw.bc.ca (M.D.), mfarrer@can.ubc.ca (M.J.F.)
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myoclonic seizures (16 subjects [40%]). Informed consent was obtained for all subjects in accordance with site-specific institutional review boards.

Our cohort represents a highly selected group of individuals without mutations in genes known to be associated with epilepsy (n = 545), including the 51 established EE-associated genes. Variants within co-expressed candidate genes (n = 20; Table S1) were extracted from exome sequencing data. On average, 96% (83%–100%) of the coding region was sequenced >10-fold (Table S1). Variant annotation was performed with ANNOVAR,[18] which integrated data from a variety of public databases. Highly penetrant de novo mutations are not expected in healthy individuals, and we assessed only extremely rare variants (≤1 carrier in the Exome Aggregation Consortium [ExAC] Browser), including small indels and missense, nonsense, and splicing changes (±5 bp). Sanger sequencing in probands and both parents (when available) validated a total of seven non-synonymous variants in seven different genes (Table 1). Variants in four genes, *GABBR2* (MIM: 607340), *ANK3* (MIM: 600465), *NBEA* (MIM: 604889), and *ACOT4* (MIM: 614314), were inherited from unaffected parents, so pathogenicity is doubtful. A variant of unknown significance was identified in *PLXNA1* (MIM: 601055); whereas the mother is not a carrier, paternal DNA was not available. The remaining two mutations were de novo: (1) c.244G>A (p.Gly82Arg) in *SLC1A2* (solute carrier family 1 member 2 [MIM: 600300; GenBank: NM_004171.3]) was identified in a 6-year-old boy (subject A) with seizure onset at 6 weeks and (2) c.394C>T (p.Arg132Cys) in *YWHAG* (tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein gamma, also known as 14-3-3γ [MIM: 605356; GenBank: NM_012479.3]) was identified in an 18-year-old woman (subject B) with seizure onset at 12 months (Table 2).

In an attempt to identify additional carriers of de novo variants in *SLC1A2* and *YWHAG*, we queried biomedical literature on PubMed and three online resources: (1) GeneMatcher,[21] a freely accessible tool that connects clinicians and researchers with interests in the same gene; (2) DECIPHER, which contains exome sequencing data from nearly 4,300 families affected by developmental disorders;[22,23] and (3) AnnEX, a collaborative platform that interrogates exomes specializing in neurologic and neurodegenerative disorders. We identified seven additional probands: subject C (*SLC1A2* c.866C>G [p.Pro289Arg]), subject D (*YWHAG* c.44A>C [p.Glu15Ala]), AU027A with autism (*YWHAG* c.148A>C [p.Lys50Gln]),[20] ND27637 with Lennox-Gastaut syndrome (*YWHAG* c.387C>G [p.Asp129Glu]),[4] subjects E and F (*YWHAG* c.394C>T [p.Arg132Cys]), and DDD4K.00159 with a neurodevelopmental disorder (*YWHAG* c.398A>C [p.Tyr133Ser])[19] (Table 1). All mutations were de novo and resulted in substitutions that involve highly conserved amino acids and are predicted to be damaging by multiple in silico algorithms (Combined Annotation Dependent Depletion [CADD] and Rare Exome Variant Ensemble Learner [REVEL]). None have been reported in public databases (gnomAD Browser), and one mutation (*YWHAG* c.394C>T) is recurrent in three subjects (B, E, and F). We used denovolyzeR[24] to evaluate the excess of de novo mutations in *YWHAG*. Assuming that 6,869 individuals were included, we observed a highly significant enrichment of heterozygotes (0.2 expected versus 7 observed; $p = 5.1 \times 10^{-9}$; 30.8×; Table 1). Although the number of subjects in GeneMatcher was not defined, these results remained significant ($p = 0.009$) even when 100,000 individuals were included.

Table 2 summarizes the main clinical features of the six affected individuals who have not previously been described in the literature. All probands underwent WES and had no other pathogenic or likely pathogenic mutations in genes known to be associated with seizure disorders. Detailed case reports are provided in the Supplemental Note.

SLC1A2 encodes the excitatory amino acid transporter EAAT2 (also known as GLT-1), a glutamate transporter that is primarily expressed in astrocytes and is responsible for ~95% of glutamate uptake activity in the mammalian brain. Lethal spontaneous seizures and progressive neuronal death due to excitotoxicity were reported in a *Slc1a2* knockout mouse model.[25] Elevated extracellular glutamate levels have also been reported in animal models of epilepsy and epilepsy patients alike, particularly in individuals with temporal lobe epilepsy.[26–29]

EAAT2 p.Gly82Arg (subject A) substitutes a small hydrophobic and neutrally charged glycine with hydrophilic, positively charged arginine within the cytoplasmic loop joining the first pair of transmembrane domains (Figure 1). The same substitution is reported in the Epi4K-EPGP dataset in a subject with infantile spasms[4] and two additional individuals with a severe EE, and de novo variants (EAAT2 p.Gly82Arg and p.Leu85Pro) were reported[33] during the preparation of this manuscript.

EAAT2 p.Pro289Arg (subject C) affects a highly conserved residue in all human EAATs (EAAT1–EAAT5) and their ancestral homolog. Pro289 leads to a hinge in the middle of the fifth transmembrane domain (Figure 1). Its substitution to positively charged arginine is predicted to cause a major structural change of the protein. The homologous mutation (c.1047C>G [p.Pro290Arg]) in *SLC1A3* (MIM: 600111), encoding EAAT1, has been described in a subject with episodic ataxia, seizures, migraine, and alternating hemiplegia (MIM: 612656).[34] In addition to mediating secondary-active glutamate uptake, EAATs also function as anion channels.[35–38] The EAAT1 p.Pro290Arg variant decelerates a conformational change associated with Na^+ binding, causing a decrease in glutamate transport rates and a significant increase in EAAT1-associated anion current amplitudes.[39] EAAT1 and EAAT2 have similar expression profiles (mainly expressed in astrocytes and localized to plasmalemma), share basic mechanisms of glutamate transport, and exhibit similar anion conduction properties. Therefore, it is likely that

Table 1. Rare Coding Variants

ID	Gene[a]	hg19 Position	rsID	MAF	Nucleotide Change	Amino Acid Change	CADD	REVEL	Inheritance	Study
109	*ACOT4*	chr14: 74,059,006	rs762048021	4.09×10^{-6}	c.343G>A	p.Glu115Lys	13.3	0.100	paternal	this study (n = 42)
044	*ANK3*	chr10: 61,828,433	NA	NA	c.12206A>G	p.Glu4069Gly	13.3	0.111	paternal	this study (n = 42)
015	*GABBR2*	chr9: 101,073,426	NA	NA	c.1955 A>G	p.His652Arg	23.2	0.830	maternal	this study (n = 42)
062	*NBEA*	chr13: 35,770,172	NA	NA	c.5099T>C	p.Met1700Thr	13.2	0.079	paternal	this study (n = 42)
133	*PLXNA1*	chr3: 126,752,801	rs149124469	8.51×10^{-6}	c.5632C>T	p.Arg1878Trp	33.0	0.149	unknown (not maternal)	this study (n = 42)
Subject A	*SLC1A2*	chr11: 35,336,636	NA	NA	c.244 G>A	p.Gly82Arg	32.0	0.864	de novo	this study (n = 42)
Subjects B, E, and F	*YWHAG*	chr7: 75,959,244	NA	NA	c.394C>T	p.Arg132Cys	33.0	0.791	de novo	this study (n = 42), DDD[19] (n = 4,293), GeneMatcher (NA)
Subject C	*SLC1A2*	chr11: 35,314,059	NA	NA	c.866C>G	p.Pro289Arg	26.9	0.911	de novo	DDD[19] (n = 4,293)
Subject D	*YWHAG*	chr7: 75,988,082	NA	NA	c.44A>C	p.Glu15Ala	27.2	0.417	de novo	GeneMatcher (NA)
AU027A	*YWHAG*	chr7: 75,959,490	NA	NA	c.148A>C	p.Lys50Gln	25.4	0.597	de novo	De Rubeis et al.[20] (n = 2,270)
ND27637	*YWHAG*	chr7: 75,959,251	NA	NA	c.387C>G	p.Asp129Glu	26.9	0.524	de novo	Epi4K[4] (n = 264)
DDD4K.00159	*YWHAG*	chr7: 75,959,240	NA	NA	c.398A>C	p.Tyr133Ser	27.1	0.911	de novo	DDD[19] (n = 4,293)

Abbreviations are as follows: MAF, minor allele frequency from gnomAD; CADD, Combined Annotation Dependent Depletion (v.1.3); REVEL, Rare Exome Variant Ensemble Learner; NA, not available.
[a]GenBank accession numbers: *ACOT4*, NM_152331.3; *ANK3*, NM_001204403.1; *GABBR2*, NM_005458; *NBEA*, NM_015678.4; *PLXNA1*, NM_032242.3; *SLC1A2*, NM_004171.3; and *YWHAG*, NM_012479.3.

Table 2. Clinical Features of Subjects with *SLC1A2* and *YWHAG* Mutations

	SLC1A2		*YWHAG*			
	Subject A	**Subject C**	**Subject B**	**Subject D**	**Subject E**	**Subject F**
Genetic findings	c.244G>A (p.Gly82Arg)	c.866C>G (p.Pro289Arg)	c.394C>T (p.Arg132Cys)	c.44A>C (p.Glu15Ala)	c.394C>T (p.Arg132Cys)	c.394C>T (p.Arg132Cys)
Sex	male	male	female	female	female	female
Seizure onset	6 weeks	5 days	12 months	6 months	<6 months	<6 years (unknown)
Age	6 years	10 years	18 years	10 years	22 years	15 years
Diagnosis	developmental and EE (treatment resistant)	developmental and EE (treatment resistant)	developmental and EE	unclassified epilepsy	unclassified or genetic generalized epilepsy	unclassified or genetic generalized epilepsy
Seizure type at onset	focal motor (left leg, right arm) initially without impaired awareness	focal motor (face, eyes, and right foot)	generalized myoclonic	prolonged focal motor seizure with fever	myoclonic	absence, eyelid myoclonia, myoclonic
Course of seizures	focal motor ± impaired awareness evolving to bilateral tonic-clonic	multiple seizure types	generalized myoclonic, atypical absence, GTC, no febrile seizure	second prolonged seizure with fever and then two episodes of status epilepticus associated with regression and hemiparesis	prolonged GTC with fever, generalized myoclonic, absence, GTC	persistence of absence seizures
Ictal EEG	not available, but capture of right arm jerks and chin twitching showed no EEG correlate	spike-and-wave discharges (age 7 weeks)	myoclonic jerks, generalized spike-and-wave discharge	not available	spike-and-slow wave, polyspike, and slow-wave discharges	not available
Interictal EEG	2 months: paroxysmal delta and frequent bilateral mid-central parietal spikes and sharp waves, occasional right temporal spikes; 4 years: slow dysrhythmic background, multifocal epileptiform discharges	very high amplitude (>500 μV), 0.7–3.3 Hz delta and very slow waves with intermixed generalized sharp waves (age 8 months)	2 years: dysrhythmic background, generalized atypical spike wave, frequent bifrontal spikes; 14 years: dysrhythmic background, rare sharp waves in bianterior quadrants	not available	21 months: generalized 3 Hz spike wave with absence seizures; 10 years: generalized polyspike wave with myoclonic seizures; 14 years: occasional spike wave	8 years: bilateral frontotemporal spikes, generalized spike waves
Timing of seizure occurrence	diurnal or nocturnal	not provided	diurnal	not provided	frequently early morning	not provided
Head circumference	45.1 cm at 8 months (50%), 52 cm at 5 years (50^{th} percentile)	44 cm at 4 months (91^{st} percentile)	normal	∼+1 SD (mother has macrocephaly)	53.7 cm at 15 years (20^{th} percentile)	normal (50^{th} percentile)
Neurologic findings	visually inattentive with sluggish pupils, axial hypotonia, mild limb spasticity, dyskinetic movements of arms	hypotonia with up-going plantars and severe thoraco-lumbar kyphoscoliosis	action tremor in upper extremities and mild coordination difficulties	hypotonia, joint hyperlaxity	ataxia, thoracolumbar scoliosis	thoracolumbar kyphoscoliosis

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Table 2. ***Continued***

	SLC1A2		***YWHAG***			
	Subject A	**Subject C**	**Subject B**	**Subject D**	**Subject E**	**Subject F**
Response to treatment	partial response to phenobarbital, levetiracetam, and clobazam; longest seizure-free period was 16 months	partial response to ketogenic diet and phenytoin, no response to other antiseizure medications	responded to clonazepam, which was stopped at 8 years; lamotrigine at 10 years was ineffective for atypical absence and GTC seizure; seizure free since 11 years on divalproex sodium and ethosuximide; ethosuximide was weaned off by 17 years	epilepsy under control with divalproex sodium	partial response to divalproex sodium and stiripentol	epilepsy under control with divalproex sodium and lamotrigine
ID	yes (profound)	yes (severe)	mild to moderate ID	yes (IQ ca. 55)	yes (moderate to severe)	WPPSI III (6 years), VIQ 73, PIQ 58
Development	delayed (severe), nonverbal, unable to sit unsupported	yes (severe)	early language delay, fine motor difficulties with tremor, ADHD	delayed	delayed	mildly delayed
Brain MRI	6 weeks: normal; 2 years: incomplete myelination, generalized atrophy, thin corpus callosum, abnormal signal in thalami, putamen, and caudate, and abnormal peritrigonal white-matter signal; 4 years: progressive generalized atrophy, persistent abnormal signal in basal ganglia and thalami, and periventricular white-matter signal abnormalities	2 months: normal; 2.5 years: severely delayed myelination, cerebral atrophy, and bilateral T2 prolongation in caudate heads and putamina	3 years: asymmetric brainstem not thought to be significant	generalized atrophy with diffuse loss of white matter	10 years: normal	normal

Abbreviations are as follows: ADHG, attention-deficit hyperactivity disorder; EE, epileptic encephalopathy; EEG, electroencephalography; GTC, generalized tonic-clonic; ID, intellectual disability; PIQ, performance IQ; VIQ, verbal IQ; WPPSI, Wechsler Preschool and Primary Scale of Intelligence.

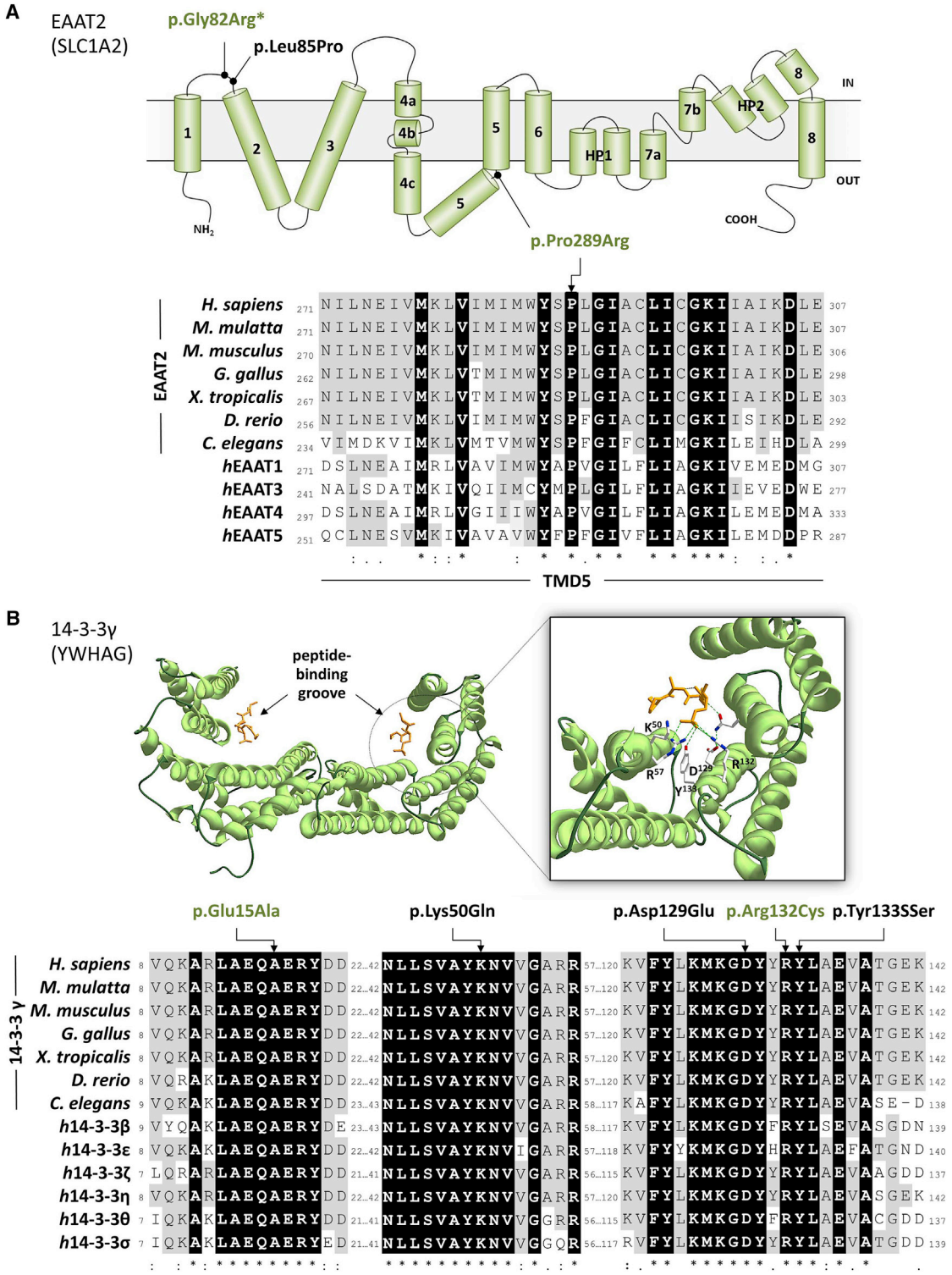

Figure 1. De Novo *SLC1A2* and *YWHAG* Mutations in Individuals with Early-Onset Epilepsy

(A) Top: transmembrane topology of EAAT2 (adapted from Yernool et al.[30]) and localization of the variants identified in this study (green) or previously reported (black) (the asterisk indicates a recurrent variant). Bottom: partial sequence alignment of EAAT2 orthologs and different human EAAT proteins surrounding p.Pro289Arg (indicated by an arrow). Identical residues across all proteins are shown in black, and residues identical to the human EAAT2 are in gray. GenBank accession numbers are as follows: *Homo sapiens*, NP_004162.2; *Macaca mulatta*, NP_001248598.1; *Mus musculus*, NP_001070982.1; *Gallus gallus*, NP_001012917.1; *Xenopus tropicalis*, XP_002937340.1; *Dario rerio*, NP_956273.1; *Caenorhabditis elegans*, NP_001024393.1; human EAAT1, NP_004163.3; human EAAT3, NP_004161.4; human EAAT4, NP_005062.1; and human EAAT5, NP_006662.3.

(B) Top: crystal structure of 14-3-3γ (PDB: 5D3E). Left: dimeric 14-3-3γ is shown as green ribbons, and the phosphopeptide ligand is shown as an orange stick. Right: close-up view of the binding groove, side chains of the residues crucial for the phosphopeptide binding,

(legend continued on next page)

the EAAT2 p.Pro289Arg variant would have an effect similar to that of the homologous mutation.[33] Similar to three previously reported individuals with *SLC1A2* mutations, our subjects have a severe phenotype consisting of early-onset epilepsy and severe development delay. Four of five individuals have multiple seizure types, but subject A (who has the same mutation as the subject reported to have infantile spasms)[4] primarily has focal motor seizures. This subject also experienced regression with an episode of lethargy and irritability, whereas no definite regression occurred in the other subjects. Neuroimaging revealed brain atrophy and abnormalities in white matter and basal ganglia in subjects A and C (Figure 2). The atrophy is possibly due in part to glutamate-induced excitotoxicity.[25]

Moreover, we identified four subjects (B and D–F) with de novo variants in *YWHAG*. The 14-3-3 family has seven conserved isoforms that are highly expressed in the mammalian brain. They are involved in a wide variety of biological processes, including intracellular signaling, cell-cycle control, apoptosis, and protein trafficking.[40] Knockdown of 14-3-3γ in zebrafish resulted in delayed brain development, reduced brain size, and increased diameter of the heart tube.[41] Curiously, in mice, a decrease or increase in 14-3-3γ leads to delayed neuronal migration of pyramidal neurons in the cerebral cortex.[42,43] Hence, normal neuronal migration in the developing brain is exquisitely sensitive to 14-3-3γ levels. Atypical neuronal migration has previously been implicated in epilepsy. Haploinsufficiency of *YWHAG* has been proposed as a potential cause of infantile spasms in individuals with Williams-Beuren syndrome (WBS [MIM: 194050]),[41] a multisystem developmental disorder caused by a recurrent de novo hemizygous deletion of ~1.5 Mb in chromosomal region 7q11.23.[44,45] Atypical distal 7q11.23 deletions ranging in size from 180 kb to 19.6 Mb have been described in several individuals presenting with a more severe phenotype that includes developmental delay, autistic features, and epilepsy. Three genes—*MAGI2* (MIM: 606382), *HIP1* (MIM: 601767), and *YWHAG*—have been suggested as possible candidates for these atypical features.[46–53] Ramocki et al.[51] suggested that haploinsufficiency of *HIP1* is sufficient to alter neuronal homeostasis and cause focal and generalized epilepsies and cognitive dysfunction. However, their findings do not exclude the possibility that *YWHAG* loss of function is sufficient to cause neurological phenotypes alone, as proven by our results. Epilepsy and infantile spasms were also described in WBS individuals harboring typical 7q11.23 deletions,[54] albeit in relatively few subjects.[55] Moreover, a recent paper[56] described a subject harboring a WBS-typical 1.41 Mb deletion and presenting with additional infantile-onset refractory EE and severe developmental delay caused by a second, independent de novo mutation in *GABRA1* (MIM: 137160). Hence, atypical aspects seen in individuals with a typical 7q11.23 deletion might also be caused by a second co-occurring but unrelated genetic defect.

Subject D harbors a de novo 14-3-3γ p.Glu15Ala variant. Native 14-3-3 exists in monomeric and dimeric states as homo- and heterodimers, respectively, although 14-3-3γ is almost entirely dimeric.[57] Glu15 is part of a triad of residues (Leu13, Ala14, and Glu15) necessary for 14-3-3γ dimerization,[58,59] and its ability to complex with other proteins is potentially inhibited by substitution at this site.

The same recurrent de novo 14-3-3γ p.Arg132Cys variant was identified in subjects B, E, and F. The arginine residue at position 132 is part of a highly conserved triad of two arginines and a tyrosine (Arg132-Arg57-Tyr133) (Figure 1) that normally form a positively charged pocket within a binding groove for interacting phosphopeptides.[57,60] An individual (ND27637) with Lennox-Gastaut syndrome and a de novo p.Asp129Glu variant is present in the Epi4K-EPGP dataset.[4] The Asp129 residue is also located within the 14-3-3γ binding groove (Figure 1) and plays an important role in determining the orientation of the phosphorylated peptides.[57] A de novo mutation affecting position p.Tyr133Ser has been reported in a Deciphering Developmental Disorders cohort individual (DDD4K.00159) severely affected by a neurodevelopmental disorder,[19] but we were unable to contact the clinician who followed this subject. Finally, a p.Lys50Gln de novo variant was identified in a subject with autism (AU027A).[20] The Lys50 residue is located on the charged face of the binding groove (together with Arg57, Arg61, Arg132, and Tyr133) (Figure 1) and is essential for ligand binding.[61–63]

All four subjects (B and D–F) from this study are females with seizure onset in the first year of life, although a precise age of onset for subject F is unknown. Subjects B, E, and F have the same mutation and similar epilepsy phenotype, which includes generalized epilepsy with myoclonic, (atypical) absence, and generalized tonic-clonic (GTC) seizures. Subject E also has prolonged GTC seizures and

and hydrogen bonds (green dashed lines). Images were generated with Swiss-Pdb Viewer v.4.10.[31] Bottom: partial sequence alignment of 14-3-3γ orthologs and different human 14-3-3 proteins surrounding the mutated residues identified in this study (green) or previously reported (black). Identical residues across all proteins are shown in black, and residues identical to the human 14-3-3γ are in gray. GenBank accession numbers are as follows: *Homo sapiens*, NP_036611.2; *Macaca mulatta*, NP_001181365.1; *Mus musculus*, NP_061359.2; *Gallus gallus*, NP_001026648.1; *Xenopus tropicalis*, NP_001072309.1; *Dario rerio*, NP_998187.1; *Caenorhabditis elegans*, AAA61872.1; human 14-3-3β, NP_003395.1; human 14-3-3ε, NP_006752.1; human 14-3-3 ζ, NP_001129174.1; human 14-3-3 η, NP_003396.1; human 14-3-3 θ, NP_006817.1; and human 14-3-3 NP_006133.1.
Sequences were aligned with CLUSTAL Omega.[32] Asterisks indicate positions with a single fully conserved residue, colons indicate conservation between groups with strongly similar properties, and periods indicate conservation between groups with weakly similar properties.

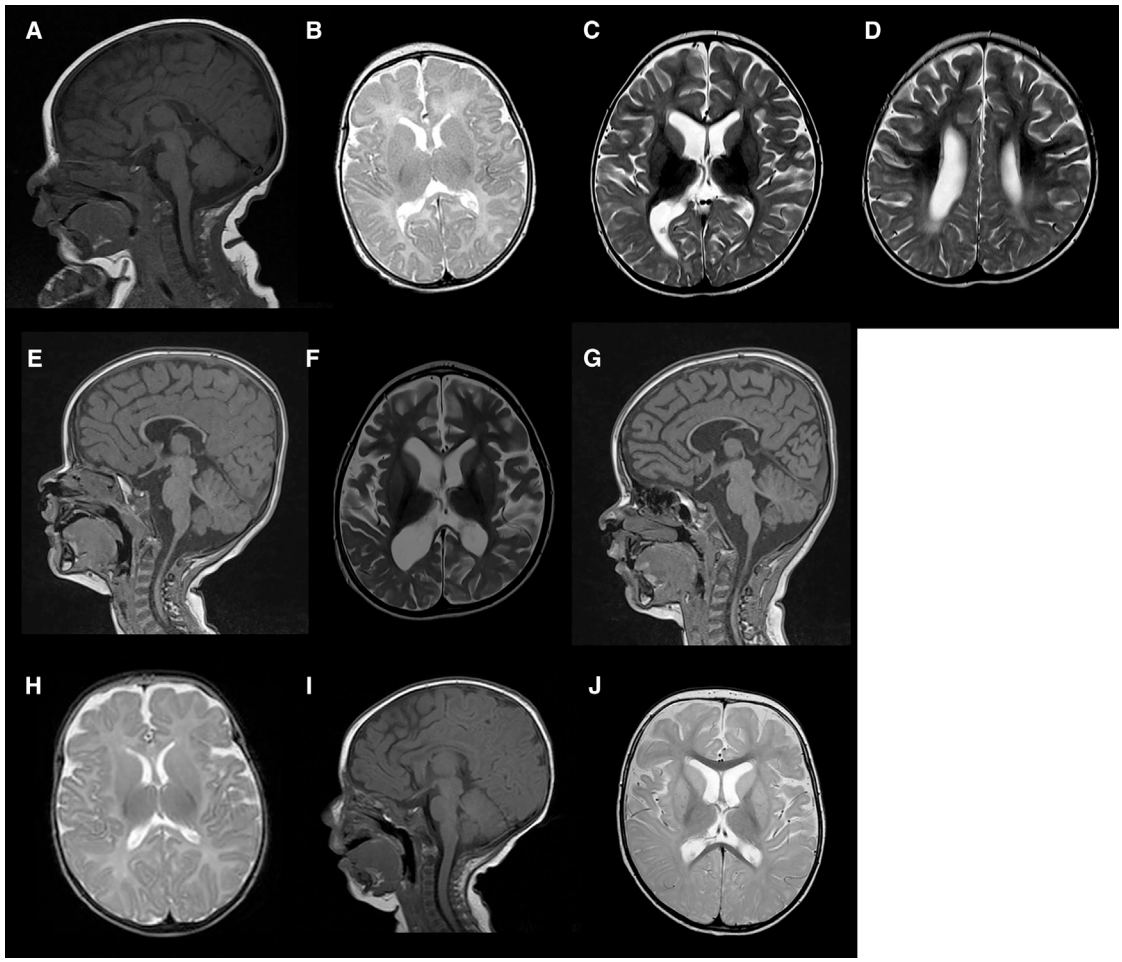

Figure 2. MRI Findings in Individuals with *SLC1A2* Mutations
(A–G) Subject A. Sagittal T1 (A) and axial T2 (B) at 6 weeks revealed a thin corpus callosum but within normal limits for age. Axial T2 (C and D) and sagittal T1 (E) at 2 years revealed volume loss and a thin corpus callosum, increased T2 white-matter signal intensity, delayed myelination, decreased T2 signal of thalami, and increased T2 signal of putamen and caudate. At 4 years, axial T2 (F) and sagittal T1 (G) showed progressive volume loss; persistent abnormal signal in thalami, putamen, and caudate; a focal area of increased T2 signal in the left lentiform nucleus; and a thin corpus callosum.
(H–J) Subject C. Axial T2 (H) and sagittal T1 (I) at 2 months were normal. At 2.5 years, axial T2 (J) revealed atrophy, delayed myelination, and bilateral T2 prolongation in caudate heads and putamina.

fever. Subject D's epilepsy is characterized by episodes of status epilepticus with or without fever. However, no subject had infantile spasms, which have been reported in some WBS individuals with atypical deletions encompassing *YWHAG*. The majority (three of four) have experienced seizure control on medication. All individuals have developmental delay with intellectual disability, and subject E was also diagnosed with autism spectrum disorder. Subjects B and E have tremor, coordination difficulties, or ataxia, and subjects E and F have scoliosis. Neuroimaging was unremarkable in subjects B, E, and F. The different and less severe clinical presentation of subject D might be explained by a partially different molecular mechanism associated with her specific mutation. Impairment of phosphopeptide binding is only a secondary consequence of impaired protein dimerization.

Empirically, we have demonstrated the validity of brain-specific co-expression for prioritizing EE candidate genes.[13,17] We have identified likely pathogenic mutations in 2 of 20 genes, which enabled us to make a genetic diagnosis in 5% (2/42) of our subjects. However, we do not rule out that mutations in the remaining 18 candidate genes have a role in EE, because more individuals must still be assessed. With the identification of both known and previously unreported mutations, our study provides further evidence for *SLC1A2* mutations in EE and suggests a gain-of-function mechanism for this rather severe presentation. Furthermore, our data indicate that *YWHAG* de novo mutations cause early-onset epilepsy, including EE and intellectual disability, and provide the rationale for therapeutic development to enhance 14-3-3γ dimerization and/or ligand binding. All identified de novo mutations are predicted to impair dimerization and phosphopeptide binding and were ultimately found in seven unrelated subjects, three of whom present with similar epilepsy phenotypes. Nevertheless,

it is important to consider both developmental and epileptic components, especially in *YWHAG* encephalopathy, given protein function and the observed phenotypic heterogeneity.

Accession Numbers

The ClinVar accession number for the data reported in this paper was not available from ClinVar as of the date this article was finalized for press; please contact the corresponding authors for the number.

Supplemental Data

Supplemental Data include a Supplemental Note and one table and can be found with this article online at http://dx.doi.org/10.1016/j.ajhg.2017.07.004.

Consortia

Investigators in the Epilepsy Genomics (EPGEN) Study include Shelin Adam, Cyrus Boelman, Corneliu Bolbocean, Tara Candido, Patrice Eydoux, Gabriella Horvath, Linda Huh, Tanya N. Nelson, Graham Sinclair, Clara van Karnebeek, and Suzanne Vercauteren.

Conflicts of Interest

M.B.C. received research grants and/or speakers honoraria from Union Chimique Belge, Novartis, Biocodex, Eisai, and Sage Therapeutics. All honoraria have been donated to the Epilepsy Research and Development Fund. She also received research grants from the Canadian Institute for Health Research (CIHR) and the Alva Foundation. She is co-chair of the Canadian Paediatric Epilepsy Network. M.J.F. received research support from the Canadian Federal Government (Canada Excellence Research Chair, Canada Foundation for Innovation, and CIHR) and the Cunhill and Alva Foundations. He is a founder and CEO of Neurocode Laboratories, which currently provides clinically accredited exome sequencing to the Province of British Columbia.

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Web Resources

AnnEX, https://annex.can.ubc.ca
ClinVar, https://www.ncbi.nlm.nih.gov/clinvar/
Combined Annotation Dependent Depletion (CADD), http://cadd.gs.washington.edu/
DECIPHER, https://decipher.sanger.ac.uk
ExAC Browser, http://exac.broadinstitute.org/
GenBank, https://www.ncbi.nlm.nih.gov/genbank/
GeneMatcher, http://genematcher.org
gnomAD, http://gnomad.broadinstitute.org/
OMIM, http://omim.org
PubMed, https://www.ncbi.nlm.nih.gov/pubmed/
RCSB Protein Data Bank, https://www.rcsb.org/pdb/home/home.do
REVEL: Rare Exome Variant Ensemble Learner, https://sites.google.com/site/revelgenomics/
UCSC Genome Browser, http://genome.ucsc.edu/

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Supplemental Data

De Novo Mutations in *YWHAG* Cause Early-Onset Epilepsy

Ilaria Guella, Marna B. McKenzie, Daniel M. Evans, Sarah E. Buerki, Eric B. Toyota, Margot I. Van Allen, Epilepsy Genomics Study, Mohnish Suri, Frances Elmslie, Deciphering Developmental Disorders Study, Marleen E.H. Simon, Koen L.I. van Gassen, Delphine Héron, Boris Keren, Caroline Nava, Mary B. Connolly, Michelle Demos, and Matthew J. Farrer

Supplemental Note: Case Reports

Subject-A (SCL1A2; c.244G>A, p.Gly82Arg)

A 6.5 year old boy with developmental and epileptic encephalopathy, profound global delay, early developmental arrest, cortical visual impairment, feeding difficulties and a hyperkinetic movement disorder. The pregnancy and delivery were uncomplicated. His parents were non-consanguinous, and had no family history of neurological disorders.

Seizure disorder and treatment

He presented at 6 weeks of age with prolonged focal motor seizures and reduced visual attentiveness. He initially developed left clonic leg jerks during wakefulness and sleep. This evolved to right or left limb involvement with impaired awareness or bilateral tonic-clonic seizures. His EEG at 2 months showed paroxysmal delta and frequent independent bilateral mid-central parietal spikes and sharp waves (right > left) and occasional right temporal spikes. His seizures initially responded partially to phenobarbital and levetiracetam, but he had seizures every 1 to 6 months. The longest seizure free period was 16 months, between 4 and 6 years. Seizures were often prolonged and/or occurred in cluster. Currently, he has been seizure free 5 months on phenobarbital, levetiracetam and clobazam. He did not tolerate topiramate. His EEG at 4 years revealed a slow dysrhythmic background with multifocal epileptiform discharges.

Development

Subject-A development never progressed past the infantile level; however, there was no definite regression until 4 years 9 months when he was hospitalized for lethargy, irritability and focal motor status epilepticus. Following seizure control, the lethargy and irritability improved, but he could no longer tolerate oral feeds and a G-tube was placed at 6 years. At 6.5 years, he is non-verbal, and unable to sit unsupported. His head circumference remains at the 50^{th} percentile. He has reduced visual attentiveness, sluggishly reactive pupils, axial hypotonia, mild limb spasticity and dyskinetic movements of his arms.

Neuroimaging

MRI of the brain at 1 month was normal. At 2 years, there was progressive atrophy, a thin corpus callosum, delayed myelination and abnormal white matter signal in the peritrigonal region. At 4 years there was further progressive generalized atrophy; abnormal signal in the basal ganglia and thalami; and periventricular white matter signal abnormalities (see Figure 2).

Other testing

A muscle biopsy at 5 years showed non-specific changes, including increased fibre size variability and type II fibre atrophy. Muscle mitochondrial respiratory enzymes were normal.

Subject-B (YWHAG; c.394C>T, p.Arg132Cys)

An 18 year old woman with developmental and epileptic encephalopathy, mild to moderate intellectual disability, attention deficient hyperactivity disorder (ADHD), anxiety, obsessive compulsive traits and an action tremor.

Seizure disorder and treatment

Seizures were first noted at 12 months but were undiagnosed until 27.5 months. Seizures were characterized by staring with eyelid fluttering with or without limb jerks. Video-EEG examination showed dysrhythmic background and frequent generalized atypical spike and wave, fragmentary spike and wave and frequent independent bilateral frontal spikes. She had multifocal myoclonic jerks some of which correlated with generalized spike and wave. Her seizures responded well to clonazepam, with only rare myoclonic jerks during sleep. However, clonazepam was discontinued at 8 years, and her EEG showed occasional fragmentary generalized spike and wave off medication. However, myoclonic seizures recurred and she also developed atypical absence and rare generalized tonic-clonic seizures. Lamotrigine proved ineffective. Seizures were stopped on Divalproex sodium and ethosuximide and she has been seizure free since 11 years. She has been on Divalproex sodium monotherapy since age 17 years. Her most recent EEG at 14 years revealed a dysrhythmic background and rare sharp waves in the bilateral anterior quadrants.

Development

Early development was normal except for language delay noted at 2 years, but with time, global developmental delay became more apparent. Neuropsychological assessment at 11 years confirmed mild to moderate intellectual disability and ADHD. Her ADHD symptoms improved on methylphenidate. She also has anxiety with obsessive compulsive traits, migraine and sleep difficulties. The maternal family history is significant for migraine and depression. Physical examination revealed normal head circumference, action tremor with mild coordination difficulties.

Neuroimaging

MRI of the brain performed at 3 years revealed an asymmetric brainstem of unlikely clinical significance.

Subject-C (SCL1A2; c.866C>G, p.P289R)

A 10-year old boy who presented with staring spells, who is the only child to healthy Caucasian parents who are first cousins. He has myoclonic jerks, absence and gelastic seizures and severe global developmental delay.

Seizure disorder and treatment

Subject-C developed twitching of his right foot in the first week of life followed by intermittent head turning to the right and twitching of his right arm in the third week and eye blinking with stiffening of the extremities at 4 weeks of age. From 7 weeks he had constant twitching of his legs..He was initially treated with phenobarbital which resulted in a seizure-free interval of 2 weeks. He then developed clusters of seizures which did not respond to phenytoin, rectal paraldehyde, midazolam, pyridoxine and clonazepam. An EEG at 3 months showed bilateral frontal sharp and slow wave discharges associated with arrest of activity and staring. Three weeks later he developed perioral twitches, myoclonic jerks involving the hands, feet and shoulders. His EEG showed irregular high-amplitude slow activity (delta) focal on the right and focal sharp waves in the left temporal area. Various combinations of anticonvulsants were tried with little response. There was a reduction in the frequency of his myoclonic jerks with some improvement in his development following a course of prednisolone, but there was no change in his EEG. At 6 years he continued to have 50 seizures daily on Rufinamide, Levetiracetam, Nitrazepam and the ketogenic diet.

Development

At 4 months, he had treatment resistant myoclonic seizures with loss of skills. He was unable to fix or follow. His head circumference was on the 91st percentile. There were no dysmorphic features nor neurocutaneous findings. There was hypotonia, with no head control, weak Moro and grasp reflexes, albeit with normal power and deep tendon reflexes in all limbs. He had a gastrostomy tube and fundoplication at 3 years. He required bilateral femoral osteotomies at 5 years. He has a tracheostomy and also developed severe scoliosis.

Neuroimaging

An initial MRI of the brain at 2 months was normal but at 2.5 years a second scan showed severely delayed myelination, non-progressive cerebral atrophy, and T2 prolongation in caudate heads and putamina bilaterally.

Other testing

Neurometabolic testing including analysis of CSF was normal. Standard karyotype and methylation analysis for Angelman syndrome were normal. Eye examination showed healthy maculae. No mutations

were identified in *SCN1A*, *MECP2*, *POLG*, *PEO1* or *ARX* genes. There was no evidence of the common mitochondrial DNA mutations causing MELAS (m.3243A>G) and MERRF (m.8344A>G) in lymphocyte DNA. He was entered into the DDD Study for trio whole exome sequencing. WES identified a *de novo* missense variant in the *SLC1A2* gene (c.866C>G; p.Pro289Arg) and he was compound heterozygous for two missense variant in the *SLX4* gene (c.1372A>G; p.Lys458Glu, maternally inherited, and c.2305G>C; p.Glu769Gln paternally inherited). These results were validated by Sanger sequencing in the proband and his parents. The *SLC1A2* variant was classified as likely pathogenic whereas the *SLX4* variants were classified as being of uncertain significance. Biallelic mutations in *SLX4* cause Fanconi anaemia Type P, but his full blood count showed no evidence of pancytopenia and his Mitomycin C test for Fanconi anaemia was normal. Chromosome microarray was normal.

Subject-D (YWHAG; c.44A>C, p.E15A)

A 10-year-old girl who is the only child of non-consanguineous parents. She has three paternal half-siblings. Pregnancy and delivery were uncomplicated. However, her mother has macrocephaly and reported a single febrile seizure.

Seizure disorder and treatment

Subject-D had two prolonged febrile seizures, at 6 and 12 months of age. After her first seizure, she showed developmental delay and hypotonia. At 2 years she had *status epilepticus* lasting about 6.5 hours, after which she developed a hemiparesis and regression of psychomotor development. At age 3, she had a second episode of *status epilepticus* followed by (reversible) cognitive deterioration. Currently, her epilepsy is relatively well controlled on divalproex sodium.

Development

She walked at 4 years but with splints because of her low muscular tone. She started to speak at 4.5 years. Her development is progressing slowly; her IQ is 55. On physical examination, her head circumference was normal (+1 SD), she had a broad nasal bridge with telecanthus, joint hyperlaxity, and a few café-au-lait spots at her back. *Neuroimaging*

MRI of the brain showed generalized atrophy.

Other testing

Ophthalmologic examination was normal with no Lisch nodules. Exome sequencing also identified a *de novo* variant in the NF1 gene of unknown significance.

Subject-E (YWHAG; c.394C>T, p.Arg132Cys)

A 22 year old female with a diagnosis of generalized epilepsy, the second child to healthy, unrelated Caucasian parents.

Seizure disorder and treatment

Subject-E parents' originally sought medical advice because they had witnessed jerks, predominantly of the upper limbs, in the first six months of life and on a few occasions they noticed their daughter's eyes rolling. At 18 months, she had a generalized tonic-clonic seizure which lasted about 45 minutes, associated with fever. Diazepam was required to terminate the seizure. At 20 months, she developed myoclonic jerks which occurred 30-40 times per week. An EEG showed bursts of generalized 3Hz spike and slow wave discharges associated with clinical absence seizures. Subject-E was started on sodium valproate and had a seizure free period of a month. Subsequently, she had upper limb jerks with absences, and had a further generalized tonic clonic seizure the following year. Various drug regimens were tried, all including sodium valproate, but without great effect. At 10 years, she was having up to 100 myoclonic seizures per day and video-telemetry at that time showed frequent bursts of very large amplitude multiple sharp, polyspike and slow wave activity lasting 1-6 seconds. During sleep there was almost continuous multiple spike and slow wave complexes at 1-3Hz. At 14 years, a trial of stiripentol was initiated in conjunction with sodium valproate and topiramate and there was a marked improvement in her seizure control. She had one normal EEG six months after starting stiripentol but a subsequent EEG showed occasional spike and wave activity. Currently she has occasional absences usually lasting a couple of seconds and occasional myoclonic jerks which tend to occur in clusters. Topiramate has been discontinued.

Development

In early life there were some minor feeding difficulties, and her tongue protruded. On examination, she was not dysmorphic. Nevertheless, her head circumference was on the 20^{th} percentile. It became evident by 8 months that she was not achieving her developmental milestones, as she was unable to sit unsupported. However, she did make slow developmental progress and walked at 16 months. She had a wide-based gait, poor coordination and ataxia. While there was evidence of motor developmental delay prior to the onset of seizures, her progress slowed at the onset of seizures. She attended a special school for children with moderate learning difficulties and she now attends a specialist boarding college for individuals with complex epilepsy. She has relatively good expressive language and is able to read words but her numeracy is poor. She has a recent diagnosis of autism spectrum disorder. At 16 years she was noted to have scoliosis that progressed rapidly to 45^{o} over a period of months.

Neuroimaging

Her brain MRI was normal at 10 years.

Other testing

Karyotype and Comparative Genomic Hybridization (CGH) array were normal, and no pathogenic variants were identified in *SCN1A* or *PCDH19*. She was entered into the DDD study for trio whole exome sequencing and a *de novo* missense heterozygous variant in *YWHAG* was identified.

Subject-F (YWHAG; c.394C>T, p.Arg132Cys)

This 15-year-old girl is the only child of non-consanguineous French parents. Pregnancy and delivery were uncomplicated. She has one paternal elder half-brother. A maternal aunt has epilepsy and there is no family history of other neurological problems.

Seizure disorder and treatment

At 6 years, she had multiple generalized seizure types: absence, eyelid myoclonia, myoclonic and probable myoclonic-atonic seizures. Her parents were unable to recall the age at seizure onset. Her seizures are relatively well controlled on divalproex sodium and lamotrigine (except for occasional absence seizures).

Development

At 2 years, she was referred for assessment of motor and speech (she walked at 19 months). She had mild dysmorphic features. At 6 years IQ was evaluated (Verbal IQ 73, Performance IQ 58). On physical examination at 15 years, she has a normal head circumference (+0 SD), facial dysmorphism and thoraco-lumbar kyphoscoliosis.

Neuroimaging

Brain MRI was normal.

Table S1: list of analyzed genes, prioritized based on brain-specific co-expression with established EE genes

GENE	RefSeq	Genomic coordinates (hg19)	Coverage 10x (%)
ACOT4[a]	NM_152331	chr14:74058663-74062358	84%
AKAP6	NM_004274	chr14:32902699-33293979	100%
ANK3	NM_001204403	chr10:61802448-62493077	100%
CRTAC1	NM_018058	chr10:99625304-99790229	99%
DAO	NM_001917	chr12:109278782-109294311	100%
FBXO41[a]	NM_001080410	chr2:73486109-73496758	83%
GABRB1	NM_000812	chr4:47033668-47428035	100%
GABBR2[a]*	NM_005458	chr9:101052865-101471019	86%
IQSEC2	NM_001111125	chrX:53263400-53350321	92%
KNDC1[a]	NM_152643	chr10:134973971-135038394	86%
MAST1	NM_014975	chr19:12949386-12985684	94%
NBEA[a]	NM_015678	chr13:35516957-36245128	97%
PACS2	NM_001100913	chr14:105781255-105861009	96%
PAK6	NM_001128628	chr15:40556986-40568295	100%
PLXNA1	NM_032242	chr3:126707436-126752860	100%
RALGPS1	NM_014636	chr9:129724568-129981048	100%
SELRC1[a]	NM_023077	chr1:53153391-53163998	100%
SLC1A2	NM_004171	chr11:35282440-35440513	100%
TRIO	NM_007118	chr5:14143834-14508531	94%
YWHAG	NM_012479	chr7:75958893-75988125	100%

[a] denotes 6 additional genes prioritized by Oliver et al. [13].