Analysis of rare copy number variation in absence epilepsies

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ABSTRACT

Objective: To identify shared genes and pathways between common absence epilepsy (AE) subtypes (childhood absence epilepsy [CAE], juvenile absence epilepsy [JAE], and unclassified absence epilepsy [UAE]) that may indicate common mechanisms for absence seizure generation and potentially a diagnostic continuum.

Methods: We used high-density single-nucleotide polymorphism arrays to analyze genome-wide rare copy number variation (CNV) in a cohort of 144 children with AEs (95 CAE, 26 UAE, and 23 JAE).

Results: We identified CNVs that are known risk factors for AE in 4 patients, including 3x 15q11.2 deletion. We also expanded the phenotype at 4 regions more commonly identified in other neurodevelopmental disorders: 1p36.33 duplication, 1q21.1 deletion, 22q11.2 duplication, and Xp22.31 deletion and duplication. Fifteen patients (10.5%) were found to carry rare CNVs that disrupt genes associated with neuronal development and function (8 CAE, 2 JAE, and 5 UAE). Four categories of protein are each disrupted by several CNVs: (1) synaptic vesicle membrane or vesicle endocytosis, (2) synaptic cell adhesion, (3) synapse organization and motility via actin, and (4) gap junctions. CNVs within these categories are shared across the AE subtypes.

Conclusions: Our results have reinforced the complex and heterogeneous nature of the AEs and their potential for shared genetic mechanisms and have highlighted several pathways that may be important in epileptogenesis of absence seizures. ***Neurol Genet*** **2016;2:e56; doi: 10.1212/NXG.0000000000000056**

GLOSSARY

AE = absence epilepsy; **BDNF** = brain-derived neurotrophic factor; **CAE** = childhood absence epilepsy; **CNV** = copy number variation; **GGE** = genetic generalized epilepsy; **GO** = Gene Ontology; **GOI** = gene of interest; **ID** = intellectual disability; **JAE** = juvenile absence epilepsy; **LTP** = long-term potentiation; **MR** = mental retardation; **SNP** = single-nucleotide polymorphism; **UAE** = unclassified absence epilepsy.

Absence seizures, abrupt and brief epileptic disruptions of consciousness associated with spike-and-wave discharges on EEG, are predominant in 2 pediatric genetic generalized epilepsies (GGEs): childhood absence epilepsy (CAE) and juvenile absence epilepsy (JAE). Debate concerning the most appropriate diagnostic criteria means many patients receive unclassified absence epilepsy (UAE) diagnosis.[1] UAE could reflect a syndromic continuum between CAE and JAE or could be a distinct group (or groups) with different prognoses and potentially distinct pathophysiologic mechanisms.

The complex genetic basis of CAE and JAE remains largely undiscovered; rare mutations and susceptibility alleles in predominantly $GABA_A$ receptors and voltage-activated calcium channels

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Funding information and disclosures are provided at the end of the article. Go to Neurology.org/ng for full disclosure forms. The Article Processing Charge was paid by the Lilly Innovation Fellowship Grant to Laura Addis at King's College London.

have been identified to date.[2,3] Multiple GGE subsyndromes can occur in one family, which indicates a close genetic relationship between the AEs, consistent with an oligogenic model of inheritance.[4] AEs are often studied apart, but some investigations show common genetic causality,[5] whereas others identify factors that are not shared.[6] Recurrent microdeletions at 15q11.2, 15q13.3, and 16p13.11 are strongly associated risk factors for GGEs, occurring in 0.5% to 1% of patients.[7] Around 8% of patients with GGE also carry rare gene-disrupting copy number variations (CNVs), with enrichment for genes previously implicated in neurodevelopmental disorders, including deletions of *RBFOX1* and *NRXN1*.[8–11]

We identify novel rare CNVs in a cohort of classical CAE and JAE cases, as well as UAEs with atypical seizure patterns or age at onset. The identification of shared genes and pathways could indicate common mechanisms for absence seizure generation.

METHODS

Study participants and phenotyping. Unrelated patients of European ancestry, previously recruited for 2 studies of GGEs from 1997 to 2007, were ascertained from hospitals across Europe: United Kingdom, Greece, France, Germany, Austria, the Netherlands, Denmark, Sweden, Finland, and Italy, as reported previously.[1,12] In patients with absences but without notable myoclonus, we used an adapted version of the International League Against Epilepsy 1989 Criteria to classify as CAE, JAE, and UAE (table e-1 at Neurology.org/ng) based on age at onset, seizure frequency, and EEG findings.[1,13] The criteria require "normal background" EEG, which was interpreted as age-appropriate normal posterior dominant rhythm during wakefulness (sleep EEG was not specifically evaluated for this study). Interictal fragments of generalized or bilateral symmetric spike-and-wave discharges and some isolated focal discharges are commonly seen in AEs and were not considered an exclusion criterion for our study.

To maximize inclusion of relevant patients, criteria were adapted as follows: (1) very frequent absences (several times a day, pyknolepsy) were considered an inclusion criterion for CAE and an exclusion criterion for JAE, which aided classification of children in the intermediate age range; (2) patients <4 years who would otherwise meet the definition for CAE were included as CAE in our analysis, as their clinical course often resembles that of classical CAE[14]; and (3) patients with focal neurologic deficits were excluded from analysis. Although grossly normal cognitive development is presumed in CAE and JAE, patients with comorbid developmental delay were included as UAE.

This study was a retrospective analysis of previously recruited cohorts; full reports of MRIs and EEGs of some patients were not available for reanalysis in this study (table 1). Patients underwent EEG in their clinical workup and had syndromic diagnoses of a GGE with predominant absences based on these[1,12]; further analysis of phenotypic information presented in this study was used to consistently classify patients into subtypes, rather than question the GGE diagnosis. Any genetic testing performed as part of clinical care was not accessed.

Standard protocol approvals, registrations, and patient consents. Individuals from the United Kingdom were recruited into a previous study as detailed in references 1 and 15 (ethics approval numbers 835/5/97 and 98-334, respectively). Written informed consent was obtained from all participants and/or their parents. Other UK individuals and those with European ancestry from Greece, France, Germany, Austria, the Netherlands, Denmark, Sweden, Finland, and Italy, collected as part of the second study detailed here,[12] had age-appropriate written informed consent obtained. Full protocol approval was obtained from local research ethics committees and/or participating institutions as appropriate.

Genotyping and copy number variant detection. High-density single-nucleotide polymorphism (SNP) genotyping arrays (HumanCoreExome-12 v1-0; Illumina, San Diego, CA) were used to detect the presence of CNVs from genomic DNA. Arrays were processed according to the manufacturer's instructions. To minimize false-positives, CNVs were called using the Nexus Copy Number package (BioDiscovery Inc, Hawthorne, CA) from signal intensity data after preprocessing in Illumina GenomeStudio Software. In Nexus, systematic array correction files were used with the linear correction model to correct for GC bias, and a significance threshold of 1×10^{-7} was applied. The SNP-FAST2 Segmentation algorithm was used for analysis, with homozygous frequency threshold at 0.95, hemizygous loss threshold at −0.23, and single copy gain at 0.13 for the log R ratio. A total of 184 samples were processed on the arrays. Samples were removed from the project if they had 1 or more of the following: a <95% call rate (0 samples), a probe-to-probe variability (quality) of >0.1 (15 samples), a sex mismatch (0 samples), and >100 CNVs (34 samples), leaving n = 144. To avoid false-positives, only variants that contained >12 consecutive altered SNP probes and that were >20 kb in size were analyzed. CNVs showing >90% coverage of variants of a frequency of ≥0.1% of the same type, reported in the Database of Genomic Variants (http://dgv.tcag.ca/dgv/app/home), using array comparative genomic hybridization or SNP arrays, were considered copy number polymorphisms and were excluded from further analysis (i.e., CNVs reported in this study are designated as "rare"). CNVs that did not overlap exons of a gene were also excluded. The potential for pathogenicity was based on gene content/disruption, CNV size, frequency, and previous association of genes or regions with epilepsy and related neurologic conditions. Gene products were annotated for Gene Ontology (GO) categories within biological processes and molecular functions using the Gene Ontology Consortium Web tool at http://geneontology.org/.

Validation of copy number variants. CNV validation was performed with real-time quantitative PCR using the Qiagen (Hilden, Germany) Type-it CNV Sybr Green Kit according to the manufacturer's instructions. Reactions were performed in 10.4-μL volumes in the ABI PRISM 7900 system (Applied Biosystems, Foster City, CA). PCR conditions were 5 minutes at 95°C followed by 35 cycles of 30 seconds of denaturation at 95°C and 30 seconds of annealing/extension at 60°C. All samples were run in triplicate. The PCR efficiency of each primer pair was checked over a dilution series of DNA for comparability with the proprietary reference assay of a multicopy gene. The $\Delta\Delta C_T$ method of relative quantification was used, and the ratio (R) of the copy number change of the gene of interest (GOI) in the case sample was compared with the control sample calculated using $R = 2^{-\Delta\Delta CT}$. If $R > 1$, the copy number of the GOI was higher in

Table 1 Clinical characteristics of patients with absence epilepsy with recurrent risk factor or rare potential risk factor CNVs

Patient ID (sex)	Diagnosis (age at onset, y)	Seizures	Family history (degree)	Interictal epileptiform abnormalities	Ictal EEG and activation procedures
C382 (F)	JAE (9-GCTS)	GTCS, absences, very rare myoclonic jerks	None	PSW	Response to hyperventilation not listed
361202 (M)	CAE (8)	Typical absences	NA	None seen	3-Hz GSW on hyperventilation
C626 (F)	JAE (14)	Absences; initially GTCS, nonepileptic seizures[a]	None	Atypical GSW: irregular spikes and sharp waves	No ictal EEG, no response to hyperventilation; PPR unknown
371201 (F)	CAE (7)	Typical absences	NA	Brief generalized PSW	3-Hz GSW on hyperventilation
367202 (F)	UAE (7)	Atypical absences (>1-min duration)	NA	None seen	No ictal EEG
C215 (M)	CAE (7)	Typical absences	Yes (2nd)	None seen	3-Hz GSW on hyperventilation
C457 (F)	CAE (7)	Typical absences	None	None seen	3-Hz GSW on hyperventilation, several spontaneous absences
C485 (F)	CAE (8)	Typical absences, pyknoleptic presentation; GTCS onset 14 y	None	GSW with right emphasis	GSW with right emphasis; GSW on hyperventilation; absence during EEG
424004 (F)	UAE (7)	Atypical absences (degree of postural tone loss)	NA	NA for review	NA for review
C516 (M)	JAE (9-GTCS)	GTCS; absence onset in teens	None	Atypical GSW	PPR present; no absence during EEG
667201 (M)	CAE (9)	Typical absences	NA	None seen	3-Hz GSW and PSW on hyperventilation
369201 (F)	CAE (6)	Typical absences	NA	None seen	3-Hz GSW
341203 (F)	UAE (4)	Typical absences[b]	NA	None seen	3-Hz GSW on hyperventilation
357201 (M)	UAE (1)	Typical absences[c]	Yes (1st)	GSW	3-Hz GSW on hyperventilation
397201 (M)	CAE (3)	Typical absences, GTCS	NA	GSW	NA for review
C329 (M)	CAE (7)	Typical absences (until 21 y), GTCS in teens	None	NA for review	"Typical centrencephalic petit mal epilepsy" on EEG report
C8 (F)	UAE (7)	Atypical absences, initially 2–3/day; aura later reported	Yes (2nd)	Generalized PSW, bilateral temporal sharp waves	PSW with aura later reported; no absence during EEG
717201 (F)	CAE (10)	Typical absences	NA	None seen	3-Hz GSW
C72 (M)	UAE (6)	Atypical absences (atypical spike-wave on hyperventilation pause)	NA	Atypical spike-wave	Bursts of slow waves with few associated spikes with pause in hyperventilation
349201 (M)	CAE (6)	Typical absences	NA	None seen	2.5-Hz SW on hyperventilation and photosensitive
C451 (M)	CAE (4)	Atypical absences	Yes (1st)	GSW	No ictal EEG, no response to hyperventilation
830201 (F)	CAE (9)	Typical absences	NA	Reported focal abnormality, NA for review	3-Hz GSW on hyperventilation
C454 (M)	JAE (11)	Typical absences daily and in runs; 1 GTCS age 11y	None	PSW; focal right-sided discharge	PSW; right-sided discharge

Abbreviations: CAE = childhood absence epilepsy; CNV = copy number variation; GCTS = generalized tonic-clonic seizures; GSW = generalized spike-wave discharges; JAE = juvenile absence epilepsy; NA = not available; PSW = polyspike-wave discharges; UAE = unclassified absence epilepsy.
Typical absences: brief interruptions of consciousness (4–20 seconds) with EEG ictal GSW at 3 Hz. Family history refers to family history of any epileptic seizure disorder but does not include febrile seizures. Under the "Seizures" and "Ictal EEG" columns, no GTCS, no myoclonus, no febrile seizures (FS), and no photoparoxysmal response (PPR) is implied unless stated otherwise. Although patients underwent EEG as part of their initial clinical workup and diagnosis, not all full EEGs or EEG reports were available for review; this is indicated in the table above.
[a] MRI: no epileptogenic lesion but scattered white matter change and cerebellar atrophy.
[b] Also has developmental delay and FS.
[c] MRI reported abnormal but no epileptogenic lesion; measles at 10 months; FS provoked by measles, mumps, and rubella vaccine; developmental delay.

the case than in the control; if $R < 1$, the copy number was lower in the case.

RESULTS We studied genome-wide CNV in a cohort of 144 European patients with AEs. Of these, 95 (66%) had CAE, 26 (18%) had UAE, and 23 (16%) had JAE. All CNVs called are listed in table e-2. We identified recurrent CNV hotspots that are known risk factors for GGEs in 4 individuals (tables 1 and 2). At the GGE hotspot 15q11.2, there were 3 deletions: 1 in a patient with CAE, 1 in a patient with JAE, and 1 in a patient with UAE. We also noted a 15q11.2 duplication in a patient with CAE. We recorded a smaller duplication

Table 2 Genetic characteristics of patients with absence epilepsy with recurrent risk factor or rare potential risk factor CNVs

Patient ID (sex)	Absence epilepsy	CNV coordinates (hg19/B37); cytoband	Size (kb) and type	UCSC gene content[a]
Cases with recurrent risk factor CNVs				
C382 (F)	JAE	chr1:0-914,659; 1p36.33	914, Dup	*OR4F5, OR4F29 SAMD11, NOC2L,* ***KLHL17****, PLEKHN1, PERM1*
361202 (M)	CAE	chr1:146,295,308-147,826,789; 1q21.1	1531, Del	*LOC100288142, PRKAB2, PDIA3P,* ***GJA5****,* ***GJA8*** (+7 others)
C626 (F)	JAE	chr15:21,903,815-23,103,405; 15q11.2	1199, Del	*CXADRP2, POTEB, OR4M2, OR4N4* (+7 others including ***NIPA2***)
		chr15:31,991,226-32,567,234; 15q13.3	576, Dup	***CHRNA7***
		chrX:6,457,553-8,123,447; Xp22.31	1666, Dup	*HDHD1, STS, VCX, PNPLA, VCX2*
371201 (F)	CAE	chr15:21,903,815-23,103,405; 15q11.2	1199, Dup	As above
367202 (F)	UAE	chr15:21,903,815-23,103,405; 15q11.2	1199, Del	As above
C485 (F)	CAE	chr22:21,803,945-24,654,974; 22q11.21-q11.23	2851, Dup	*HIC2, TMEM191C, PI4KAP2, UBE2L3, YDJC* (+ 42 others)
C215 (M)	CAE	chr15:22,522,310-23,249,493; 15q11.2	727, Del	*GOLGA8DP, GOLGA6L1, TUBGCP5, CYFIP1,* ***NIPA2****, NIPA1*
C457 (F)	CAE	chrX:6,449,682-8,138,035; Xp22.31	1688, Del	*VCX3A, HDHD1, STS, VCX, PNPLA, VCX2*
Cases with potential risk factor CNVs				
424004 (F)	UAE	chr1:240,509,364-240,536,152; 1q43	26, Dup	***FMN2***
C516 (M)	JAE	chr4:20,979,028-21,177,688; 4p15.32	199. Del	***KCNIP4***
667201 (M)	CAE	chr4:183,599,915-184,966,267; 4q35.1	1366, Dup	***TENM3****, DCTD, FAM92A1P2, WWC2-AS2, WWC2* (+7 others)
369201 (F)	CAE	chr5:75,611,865-75,640,024; 5q13.3	28, Dup	***SV2C***
341203 (F)	UAE	chr6:38,360,355-38,455,141; 6p21.2	95, Del	***BTBD9***
357201 (M)	UAE	chr7:37,703,928-39,432,281; 7p14.1	1728, Del	*GPR141, NME8, SFRP4, EPDR1, STARD3NL* (+5 others including ***AMPH***)
		chr3:96,735,452-97,332,506; 3q11.2	597, Del	***EPHA6***
397201 (M)	CAE	chr9:12,418,777-13,258,831; 9p23	840, Dup	*TYRP1, LURAP1L,* ***MPDZ***
C329 (M)	CAE	chr9:87,412,328-88,425,972; 9q21.33	1013, Dup	***NTRK2****, AGTPBP1*
C8 (F)	UAE	chr10:56,195,290-56,465,459; 10q21.1	270, Del	***PCDH15***
717201 (F)	CAE	chr11:93,772,465-93,903,781; 11q21	131, Dup	*HEPHL1,* ***PANX1***
C72 (M)	UAE	chr14:103,402,254-103,462,143; 14q32.3	60, Del	***CDC42BPB***
349201 (M)	CAE	chr16:77,768,588-78,186,513; 16q23.1	417, Del	*NUDT7, VAT1L, CLEC3A,* ***WWOX***
C454 (M)	JAE	chr16:78,404,208-78,431,974; 16q23.1	27, Del	***WWOX***
C451 (M)	CAE	chr20:12,662,517-14,147,317; 20p12.1	1484, Dup	*SPTLC3, ISM1, TASP1, ESF1, NDUFAF5, SEL1L2,* ***MACROD2***
830201 (F)	CAE	chrX:70,026,229-71,094,940; Xq13.1	1069, Del	*TEX11, SLC7A3, SNX12, FOXO4* (+13 others including ***NLGN3****,* ***GJB1***)

Abbreviations: CAE = childhood absence epilepsy; CNV = copy number variation; Del = deletion; Dup = duplication; JAE = juvenile absence epilepsy; UAE = unclassified absence epilepsy.
Eight patients with absence epilepsy carry 10 recurrent CNVs classified as risk factors for their epilepsy, and 15 patients carry CNVs classified as potential risk factors.
[a] Boldface indicates candidate gene.

in the patient with JAE (C626) within a second GGE hotspot, 15q13.3, including the candidate gene *CHRNA7*. CNVs at 4 further recurrent CNV hotspots, more commonly recorded in other neurodevelopmental disorders, were also identified in this study: a distal 1p36.3 duplication including the infantile spasms candidate gene *KLHL17*[16] (JAE), a 1q21.1 deletion (CAE), a 2.8-Mb duplication at 22q11.2 (CAE), and 1 deletion and 1 duplication at Xp22.31 (CAE/JAE). Of note, 1 patient with JAE, C626, carries 3 of these recurrent CNVs (tables 1 and 2).

Fifteen patients (11%) were found to carry rare CNVs that disrupt genes associated with neuronal

development and function (tables 1 and 2). One patient with UAE carries 2 of these CNVs. Although the numbers are too small to make population-level inference, it seems that the patients with UAE are more likely to have a CNV in this category than are the patients with JAE and CAE (5/23 UAE, 2/22 JAE, and 8/91 CAE). The assumption of potential pathogenicity is detailed in the Methods section. Of these 15 patients, 4 patients with CAE and 1 patient with UAE carry large novel CNVs of >1 Mb in size. The other 11 CNVs range from 26 to 840 kb. For patients with multigene CNVs, several genes may contribute to the phenotype, depending on the function.

Four categories of protein are each disrupted by several CNVs: synaptic vesicle membrane or vesicle endocytosis (GO:0030672/GO:1900242), synaptic cell adhesion (GO:0007155), synapse organization and neuronal migration via actin (actin binding GO:0003779 and actin cytoskeletal reorganization GO:0031532), and gap junctions (GO:0005921); these are shared across the AE subtypes. We also report 2 individuals with CAE (C454 and 349201) and deletions disrupting the WW domain-containing oxidoreductase (*WWOX*), known to cause epilepsy, cerebellar ataxia, and mental retardation (MR) as well as infantile epileptic encephalopathies.[17]

In 2 individuals with UAE, genes involved with synaptic vesicles are disrupted: the vesicle surface protein amphiphysin 1 (*AMPH*) (357201) and *BTBD9*, which controls vesicle recycling (341203). *SV2C*, encoding synaptic vesicle glycoprotein 2C, is also disrupted in individual 369201 with CAE.

Synaptic cell adhesion genes are disrupted by CNVs in 4 individuals: 2 with CAE and 2 with UAE. Individual 830201 with CAE carries a large novel deletion of 17 genes, including *NLGN3* (neuroligin3), a postsynaptic cell adhesion molecule; individual 357201 with UAE (also with the *AMPH* deletion described above) has a breakpoint within the Eph receptor tyrosine kinase *EPHA6*, signaling through which neuronal adhesion and development are regulated. *TENM3*, encoding a teneurin transmembrane protein that promotes cell adhesion, is disrupted by a duplication breakpoint in patient 667201 with CAE. Lastly, 3 exons of the protocadherin *PCDH15* are deleted in C8 with UAE.

Several AE CNVs reported in this study also disrupt proteins that act to organize the synapse or promote neuronal migration via interactions with the actin cytoskeleton: the serine/threonine protein kinase *CDC42BPB* (C72, UAE), *FMN2* encoding formin 2 (424004, UAE), and *MPDZ* (previously *MUPP1*), which contains multiple protein interaction PDZ domains for controlling large synaptic complexes (397201, CAE). *EPHA6*, mentioned above, also regulates cell–matrix interactions and migration, which indicates the complex interplay between these pathways.

Two patients with CAE carry disrupted gap junction genes: *GJB1*, encoding *CX32* (830201), a brain and peripheral myelin connexin family member, and *PANX1*, encoding Pannexin1. Of note, the hotspot deletion at 1q21.1 in individual 361202 also contains 2 further connexin gap junction genes, *GJA5* and *GJA8*, although they seem not to be expressed in neurons.

CNVs in the final 3 patients, although disrupting genes that have known functions in neuronal development and activity, do not share common features with the others. Individual C451 with CAE has a 1.5-kb duplication of 7 genes at 20p12.7, including a breakpoint in *MACROD2*, an enzyme that removes ADP-ribose from proteins and a well-known risk factor for autistic traits.[18] The potassium channel interacting protein gene *KCNIP4* is disrupted by a deletion breakpoint in JAE patient C516. Lastly, C329, diagnosed with CAE, carries a duplication with a breakpoint in *NTRK2* (previously *TRKB*), a neurotrophic tyrosine kinase receptor and brain-derived neurotrophic factor (BDNF) receptor.

DISCUSSION The genetic basis of the AEs is complex, with individuals carrying different patterns of genetic variants that determine their risk for seizures, some of which may be shared between the different types of epilepsy.[3] Even in large families, it is difficult to establish genotype–phenotype relationships because different members may carry the same genetic variants but have different phenotypic manifestations of AE. The search for susceptibility variants has now moved to whole-genome studies of CNV, epigenetic analysis, and genome sequencing.

Recurrent deletions at 15q11.2, 15q13.3, and 16p13.11 are consistently identified rare risk factors for GGEs including AEs,[7] and indeed, we found 3 deletions at 15q11.2 in our cohort. We also noted a microduplication within the 15q13.3 hotspot of the candidate gene *CHRNA7* in a patient also carrying a 15q11.2 deletion. Although deletions at 15q11.2 are robustly associated with GGEs and developmental disorders, duplications at this locus, seen in 1 patient in our cohort, were initially reported as variants of unknown significance. However, more recent studies of the region indicate that mild intellectual disability (ID), autism, and seizures are common features in individuals carrying these duplications,[19] providing some evidence that this CNV may be a risk factor for the epilepsy in the individual described here. We also uncovered CNVs that are more commonly recorded in other neurodevelopmental disorders at 4 further recurrent regions. Only 1 patient with JAE and none with CAE have been previously reported with the 1q21.1 deletion.[20] The deletion leads to a variable

phenotype, and seizures are seen infrequently, indicating the novelty of this region in a patient with CAE. We also identified 1 deletion and 1 duplication at Xp22.31, previously associated with MR and ichthyosis. Although seizures are now becoming a more widely reported phenotype,[21] absence seizures are not. 22q11.2 duplication syndrome has a variable phenotype, with MR and motor delay being the most common features. Seizures are reported rarely but are not well described apart from a recent case with continuous spikes and waves during sleep.[22] Lastly, we observed a 914-kb duplication of the distal end of Chr1 (1p36.33), which included the infantile spasm candidate *KLHL17*.[16] Duplications of 1p36.3 are less frequently recorded, are of variable size, and include developmental delay, seizures, and hypotonia with wide phenotypic heterogeneity and an overall "milder" phenotype than the reciprocal deletions.[23] It may be that the smaller number of duplicated genes in the patient described here, including *KLHL17*, could predispose to her JAE, but it is difficult to ascribe pathogenicity in such an isolated case. It seems from previous large-scale studies of the GGEs that variation at these recurrent regions is indeed rare within the AEs, but targeted studies in more patients could help to resolve this.

The identification of 2 patients with CAE with deletions that disrupt coding regions of *WWOX*, known to cause infantile epileptic encephalopathies as well as epilepsy, cerebellar ataxia, and MR, is intriguing.[17] These severe phenotypes are caused by biallelic mutations or CNV within the gene. The rare heterozygous CNVs seen here may cause the less severe syndrome of CAE, although very rare exonic deletions (0.04%) have been reported in the Database of Genomic Variants. Screening for mutations and CNV as well as protein function work in other patients with AE may help to answer this question.

In this investigation we also show that patients from all 3 subsyndromes carry rare CNVs that disrupt genes shared largely within 4 categories of function, involved in developing neural circuitry and at the mature synapse. All of these CNVs are unique to a given individual and confirm the strong genetic heterogeneity in the AEs.

Synaptic vesicles store and move neurotransmitters for release at the presynaptic membrane, and several proteins involved in vesicle release and recycling have been related directly to epileptogenesis[24] and are also enriched in CNVs from patients with infantile spasms.[16] In our investigation we identified 3 individuals with disrupted synaptic vesicle genes: *AMPH*, *SV2C*, and *BTBD9*. AMPH is involved in neuronal transmission and development through clathrin-mediated endocytosis of synaptic vesicles. Amphiphysin 1 is also a substrate for CDKL5, a kinase associated with neurodevelopmental disorders such as X-linked West (infantile spasms) syndrome and Rett syndrome.[25] SV2C acts via presynaptic calcium to regulate neurotransmitter release from vesicles in glutamatergic synapses. SV2C shows altered brain expression patterns in patients with temporal lobe epilepsy,[26] and all 3 SV2 family members (A, B, and C) are candidates for epilepsy.[27] Lastly, *BTBD9*, a gene associated with restless legs syndrome and Tourette syndrome, may regulate synaptic plasticity via altered synaptic vesicle recycling.[28]

Several mechanisms believed to contribute to epileptogenesis are likely to be regulated by cell adhesion, such as the dysregulation of GABAergic transmission, the guidance of axonal growth, and the stabilization of synaptic contacts and long-term potentiation (LTP).[29] Synaptic cell adhesion genes *PCDH15*, *NLNG3*, *TENM3*, and *EPHA6* are disrupted by CNV in our study. The protocadherin PCDH15 mediates the formation, maturation, and specification of synapses and is a determinant of brain serotonin transporter expression.[30] Mutations of *PCDH15* are known to cause Usher syndrome 1F, in which ID and psychiatric disturbances are common, and deletions are found in patients with epileptic encephalopathies.[31] The family member *PCDH19* also causes X-linked infantile epileptic encephalopathy.[32] The postsynaptic cell adhesion molecule NLGN3 functions in synaptogenesis and neuron–glia communications and is a candidate for autism with comorbid epilepsy, in which it may influence seizure susceptibility.[33] The teneurin member TENM3 promotes cell adhesion and synaptic organization, similar to the role of neuroligins, and may also regulate excitatory synaptic strength via latrophilin binding.[34] Lastly, the Eph receptor tyrosine kinase EPHA6 also regulates neuronal cell adhesion, cell–matrix interactions (below), and migration, with clear roles in modulating synapse formation and plasticity and axon guidance.[35]

A third category of genes that are disrupted in 3 patients with AE are those that organize the synapse and neuronal migration via the actin cytoskeleton. MPDZ controls large complexes at the synapse and is involved in learning- and memory-related synaptic plasticity. Its dysfunction has pleiotropic effects on vulnerability to seizures through interactions with many types of synaptic receptors.[36] The kinase CDC42BPB regulates cytoskeletal remodeling and cell migration[37] and is involved in hippocampal LTP. Lastly, FMN2 mediates synaptic spine density and is highly expressed in the developing brain. *FMN2* mutations can cause nonsyndromic ID.[38]

Gap junctions, both between dendrites and between axons and glia, are highly implicated in synchronous seizure activity, and blocking communication at gap junctions is anticonvulsant.[39] Two gap

junction proteins disrupted here, connexin Cxs32 (encoded by *GJB1*) and Pannexin1 (encoded by *PANX1*), are good AE candidates. Mutations in *GJB1* cause X-linked Charcot-Marie-Tooth disease, with some patients also showing CNS symptoms.[40] Cxs32 expression is also altered in the hippocampi of patients with mesial temporal lobe epilepsy.[41] Pannexin1 is upregulated in epileptic brain tissue and may contribute to seizures by increasing the levels of extracellular ATP. Targeting Pannexin1 improves seizure outcome in animal models.[42]

Other GOIs that were disrupted by rare CNVs in patients with AE include *NTRK2*, a BDNF receptor that modulates excitatory transmission, synaptic plasticity, and hippocampal LTP and is required for epileptogenesis in animal models.[43] Patients with mesial temporal lobe epilepsy show altered NTRK2 expression,[44] and dysregulated NTRK2-BDNF signaling is implicated in several neurodevelopmental disorders, indicating its excellent candidacy for AE. The potassium channel interacting protein gene *KCNIP4*, deleted in a patient with JAE, forms part of a negative feedback loop in the Wnt/β-catenin pathway that regulates neuronal development and is a candidate for attention-deficit/hyperactivity disorder.[45] Lastly, the autism risk factor gene *MACROD2* was also disrupted in a patient with CAE.[18]

A major limitation of our study is that it is a retrospective analysis of cohorts collected for previous genetic studies of AEs. This meant that we did not have useable DNA from family members to assess the inheritance of the CNVs and we were not able to recontact the families for collection of new DNA. Analysis of CNV inheritance would have aided in our putative assignment of pathogenicity to the CNVs, as those that were inherited with the disorder, or de novo in the probands, would be more likely to predispose to the epilepsy. We were also unable to access the original EEGs and the MRI reports of some patients, which would have allowed us to provide more detailed phenotypes in table 1. However, patients' diagnoses were ascertained both through the clinical services from which they were recruited and by experts in childhood epilepsies collating the cohorts for the initial studies; we therefore believe that other diagnoses have been sufficiently excluded, and further classification based on available data for our cohort is robust.

Our study of CNV across the spectrum of AEs has reinforced both the complex and heterogeneous nature of these disorders and their potential for shared genetic mechanisms. We have strengthened the evidence for the role of recurrent CNVs and added AEs as disorders potentially associated with CNV at 1q21.1 and Xp22.31. Through the study of rare CNV, we indicate pathways that may be disrupted across AE subtypes and open the door for investigations of neural network behavior in future large-scale studies of broad category patients with AE and their families. This, as well as functional work of the disrupted genes, will help in understanding the role of these potential new pathways in seizure generation.

AUTHOR CONTRIBUTIONS

L.A. and D.K.P. conceptualized the study. L.A. designed the study. L.A. and R.E.R. carried out the laboratory work and analysis of CNV data. A.V., R.R., K.V.E., A.M., and L.N. carried out the collection, phenotyping, and databasing of the patient samples. All authors contributed to drafting and revising the manuscript.

ACKNOWLEDGMENT

The authors thank the participants in this study and their families, without whom this research would not be possible.

STUDY FUNDING

This work was supported by a Lilly Innovation Fellowship award (L.A.); The Waterloo Foundation (L.A., D.K.P.); European Union Marie Curie International Reintegration Award of the Seventh Framework Programme (D.K.P.); Charles Sykes Epilepsy Research Trust (D.K.P.); NIHR Specialist Biomedical Research Centre for Mental Health of South London and Maudsley NHS Foundation Trust (D.K.P.); Isaak Schapera Trust for Medical Research (R.R.). The original patient collections were funded by the Fund for Epilepsy, the Epilepsy Research Foundation, Action Research, the Wellcome Trust, the MRC, and the European Commission.

DISCLOSURE

Laura Addis is a contractor for Eli Lilly and has received research support from Eli Lilly and the Waterloo Foundation. Richard E. Rosch reports no disclosures. Antonio Valentin has received speaker honoraria from Eisai Co.; has received publishing royalties for *Introduction to Epilepsy*, Cambridge University Press, 2012; and has been a tutor for the nonprofit VIREPA Distance Education Program (International League Against Epilepsy, ILAE). Andrew Makoff, Robert Robinson, and Kate V. Everett report no disclosures. Lina Nashef has served on the scientific advisory boards of SUDEP Action (formerly Epilepsy Bereaved UK), Epilepsy Research UK, and FQMS; has received speaker honoraria/travel funding from Eisai and Bial; has served on the editorial board of *Epilepsia*; has received publishing royalties from *Oxford Specialist Handbooks in Neurology: Epilepsy*, Oxford University Press, 2009 and *Neurology and Pregnancy*, Informa 2013; has been an employee of the National Health Service (UK) and King's College Hospital; and has received the eTNS system free for use by some patients under her care from NeuroSigma. Deb K. Pal has served on the scientific advisory board of Amplexa Genetics, holds a patent for Biomarker for centrotemporal spikes, and has received research support from the Canadian Institutes for Health Research, the European Research Commission, and the Medical Research Council. Go to Neurology.org/ng for full disclosure forms.

Received August 31, 2015. Accepted in final form January 4, 2016.

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