GRIN2A mutations cause epilepsy-aphasia spectrum disorders

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Epilepsy-aphasia syndromes (EAS) are a group of rare, severe epileptic encephalopathies of unknown etiology with a characteristic electroencephalogram (EEG) pattern and developmental regression particularly affecting language. Rare pathogenic deletions that include GRIN2A have been implicated in neurodevelopmental disorders. We sought to delineate the pathogenic role of GRIN2A in 519 probands with epileptic encephalopathies with diverse epilepsy syndromes. We identified four probands with GRIN2A variants segregated with the disorder in their families. Notably, all four families presented with EAS, accounting for 9% of epilepsy-aphasia cases. We did not detect pathogenic variants in GRIN2A in other epileptic encephalopathies (n = 475) nor in probands with benign childhood epilepsy with centrotemporal spikes (n = 81). We report the first monogenic cause, to our knowledge, for EAS. GRIN2A mutations are restricted to this group of cases, which has important ramifications for diagnostic testing and treatment and provides new insights into the pathogenesis of this debilitating group of conditions.

Epileptic encephalopathies are a group of severe disorders characterized by seizures and abundant epileptiform activity that contribute to cognitive and behavioral impairment1. Epileptic encephalopathies comprise a range of electroclinical syndromes with characteristic ages of onset and clinical and EEG manifestations. Two syndromes with overlapping manifestations have a notable EEG signature of continuous spike and wave during slow-wave sleep (CSWS) in which the non-REM (rapid eye movement) sleep EEG shows virtually continuous (285%) high-voltage bilateral slow spike-and-wave activity that largely remits on awakening. In Landau-Kleffner syndrome (LKS), children who were previously normal or had isolated language delay present with acquired epileptic aphasia; focal motor seizures occur in 70% of cases and are usually easily controlled. In contrast, in the syndrome of epileptic encephalopathy with continuous spike and wave during slow-wave sleep (ECSWS), previous development is delayed in half of affected children, and refractory epilepsy with multiple seizure types is usual. Regression is more global, with language, behavior and motor impairment2. Magnetic resonance imaging (MRI) brain studies are often normal or may show a malformation of cortical development such as perisylvian polymicrogyria.

In clinical practice, there are patients who do not meet the criteria for LKS and ECSWS on the basis of EEG or clinical characteristics, usually because their EEG abnormalities do not occur for ≥85% of slow sleep, yet they have substantial language or learning difficulties that may fluctuate in severity. There is debate about whether bilateral epileptiform activity during <85% of non-REM sleep is diagnosable as CSWS or whether it should be regarded as indicative of an intermediate epilepsy-aphasia disorder (IEAD)3. These disorders can be conceptualized as falling along a spectrum with LKS, with ECSWS at the severe end, IEAD in the middle and benign childhood epilepsy with centrotemporal spikes (BECTS) at the mild end of the spectrum. BECTS is the most common focal epilepsy syndrome in childhood and occurs in normal children who present with focal motor Rolandic seizures. The typical EEG shows unilateral or bilateral centrotemporal spikes that are activated by sleep but do not show the almost continuous bilaterally synchronous pattern of CSWS, and affected children do not show cognitive decline. The presence of subtle oral dyspraxia has been noted in some children with BECTS4.

Until recently, there has been scant evidence for a genetic etiology for the disorders of the epilepsy-aphasia spectrum. So far, only four families have been reported with monogenic inheritance of Rolandic epilepsy and speech or language difficulties. In 1995, we reported a family with autosomal dominant transmission with the syndrome of autosomal dominant Rolandic epilepsy with speech dyspraxia.
An additional three-generation family with a notably similar phenotype was reported more recently. Finally, a family with dysphasia and epilepsy with generalized and focal manifestations was reported. A causal gene has not been implicated in these families. Conversely, a fourth family, presenting with X-linked rolandic epilepsy, oral and speech dyspraxia and intellectual disability, was identified with a gain-of-glycosylation SRPX2 mutation. Besides an SRPX2 mutation in an unrelated proband with perisylvian polymicrogyria and rolandic seizures who had female relatives with mild intellectual disability, no additional SRPX2 variants in epilepsy-aphasia phenotypes have been described.

Clinical genetic studies of probands with BECTS or EAS provide little support for genes of major effect. Investigation of family members with up to three degrees of relatedness with probands with BECTS or epilepsy-aphasia spectrum disorders suggests that complex inheritance is most likely, with febrile seizures being the most common phenotype in relatives of probands.

Although there has been strong contention that epilepsy-aphasia syndromes have an immune basis, partly based on their resolution with high-dose steroids, a genetic etiology is supported by the rare familial forms described. Furthermore, recent evidence for a genetic etiology has come from copy number variant (CNV) studies. An excess of rare CNVs was noted in a cohort of probands with LKS and ECSWS, including in a single proband with LKS with a 16p13 deletion containing one gene, GRIN2A (NM_000833.3). Furthermore, three children with complex dysmorphic phenotypes were reported with 16p13 deletions that included GRIN2A. GRIN2A encodes the NR2A (GluN2A) subunit of the N-methyl-D-aspartate (NMDA) receptor, a neurotransmitter-gated ion channel that mediates excitatory transmission in the mammalian brain, making it an attractive candidate to have a role in epileptogenesis. GRIN2A mutation screening in 127 probands with epilepsy or abnormal EEG and/or intellectual disability detected 2 pathogenic mutations: a nonsense mutation segregating with epilepsy or abnormal EEG in three family members and a de novo missense mutation in an individual with severe early-onset epileptic encephalopathies.

Furthermore, two de novo missense mutations were recently reported in a large exome sequencing cohort of individuals with intellectual disability. Although these observations strongly support a role for GRIN2A in epilepsy and intellectual disability, no clear genotype-phenotype correlations have emerged. Therefore, we sought to delineate the phenotypic spectrum of GRIN2A mutations by screening a large cohort of individuals with epileptic encephalopathy.

We performed high-throughput sequence analysis of GRIN2A in 519 probands with a range of epileptic encephalopathies (Table 1).

As part of a larger study, we performed targeted gene capture of 13 genes associated with epilepsy, including GRIN2A. Briefly, we resequenced all exons and 5 bp of flanking sequence using molecular inversion probes (MIPs), highly multiplex PCR and next-generation sequencing as described previously with minor exceptions (Online Methods). Using this approach, we achieved, on average, 98% coverage (25x) across GRIN2A for all probands.

We identified four probands with GRIN2A mutations, each of which was confirmed by Sanger sequencing. Segregation analysis in additional family members showed that each variant segregated in an autosomal dominant manner (Fig. 1 and Table 2). These GRIN2A variants were not present in 6,500 control exomes (see URLs). Two families (A and C) carried the same c.1007+1G>A variant affecting a highly conserved splice donor site. Genotyping of microsatellite markers and a rare single-nucleotide variant (SNV) flanking this GRIN2A mutation identified an identical haplotype in these families, suggesting a common founder mutation (Supplementary Fig. 1). The c.1007+1G>A variant was predicted in silico to cause skipping of exon 4 during pre-mRNA splicing, resulting in the removal of 593 exonic nucleotides from the mature transcript and thus representing a frameshift alteration, p. Phe139Ilefs*15 (predicted) (Supplementary Table 1). We tested for the presence of a rare exonic SNV (rs61753382), encompassed by the common haplotype in affected individuals, in the RNA transcripts of three affected individuals, two from family A (II-3 and III-3) and one from family C (III-1). We detected monoallelic expression of the wild-type variant, suggesting nonsense-mediated decay of the mutant transcript (Supplementary Fig. 2).

We detected a p.Met1Thr variant in family B. The alteration of the translation start codon is likely to have detrimental effects on...
Table 2 Pathogenic GRIN2A mutations in four families with EAS

| Family | Chr. | Position (bp) | cDNA change | GERP | Grantham score | PolyPhen score | SIFT | Protein change | Phenotype        |
|--------|------|---------------|-------------|------|----------------|----------------|------|----------------|------------------|
| A      | 16   | 10031815      | c.1007+1G>A | 5.2  | NA             | NA             | NA   | p.Phе139Ilefs*15 (predicted) | ADRESD          |
| B      | 16   | 10274267      | c.2T>C      | 4.5  | 81             | 0.213 (benign) | 0 (damaging) | p.Met1Thr       | LKS              |
| C      | 16   | 10038185      | c.1007+1G>A | 5.2  | NA             | NA             | NA   | p.Phe139Ilefs*15 (predicted) | ECSWS           |
| D      | 16   | 9934563       | c.1592C>T   | 5.1  | 81             | 1.000 (probably damaging) | 0 (damaging) | p.Thr531Met     | ECSWS, IEAD      |

Chromosomal coordinates are given relative to the hg19 genome build; cDNA positions are given relative to transcript variant 2 (NM_000833.3), and protein positions are given relative to isoform 1 (NP_000824.1). Chr., chromosome; GERP, genomic evolutionary rate profiling; SIFT, sorting intolerant from tolerant; ADRESD, autosomal dominant Rolandic epilepsy with speech dyspraxia; LKS, Landau-Kleffner syndrome; ECSWS, epileptic encephalopathy with continuous spike and wave in slow-wave sleep; IEAD, intermediate epilepsy aphasia disorder; NA, not available.

GRIN2A protein synthesis, resulting in either complete absence of product due to failure of translation initiation at the start codon or a truncated protein stemming from translation initiation at an alternate start codon. We were unable to test these possibilities, as RNA from the proband was unavailable.

Finally, we describe a p.Thr531Met variant that affects a highly conserved residue (as predicted by high GERP and Grantham scores) that is predicted to be probably damaging by PolyPhen-2 and SIFT (Table 2). This variant is located in the extracellular ligand-binding domain of NR2A. Specific sites within this domain are known to influence the gating and kinetic properties of NMDA receptors16,17. We assessed the effect of the p.Thr531Met alteration on NR2A function by coexpressing mutant NR2A with wild-type NR1 in COS-7 cells to form a mutant heteromorphic NMDA receptor. A resultant shift in NMDA receptor kinetics was observed by single-channel recordings, with a fourfold increase in the mean duration of the open state that is predicted to damage (p value = 0.953, Mann-Whitney, two-tailed) (Fig. 2). This newly identified variant had clinical and functional consequences similar to those reported for missense mutations affecting the same domain in a parallel study by Lesca and colleagues18.

The c.1007+1G>A and p.Met1Thr variants likely cause disease as a result of haploinsufficiency for the NR2 subunit of the NMDA receptor, possibly because of aberrant NMDA receptor composition or distribution in the brain. Furthermore, we show that the p.Thr531Met variant has a profound effect on NMDA receptor kinetics. Given that the pathogenic effect of these variants and their segregation with EAS, we conclude that GRIN2A mutations are causal in these families.

Notably, all four families positive for GRIN2A mutations presented with EAS, yielding a 9% (4/44) mutation rate in individuals with this group of epileptic encephalopathy disorders. No additional pathogenic variants were detected in the remaining epilepsy encephalopathy phenotypes (Table 1). In the 40 remaining individuals with EAS, we performed array-based comparative genomic hybridization (aCGH) using a custom microarray with probes spanning GRIN2A at an average density of one probe every ~350 bp. No copy number alterations were detected.

Given that BECTS lies at the mild end of the epilepsy-aphasia spectrum, we next screened 81 probands with BECTS for GRIN2A variants using Sanger sequencing. No additional pathogenic variants were identified.

In total, we identified 16 subjects with GRIN2A mutations. Segregation was perfect in the seven affected members of the original family with ADRESD (family A; Fig. 1). The same mutation was found in a father-son pair with ECSWS (family C). Notably, GRIN2A mutations were associated with a range of epilepsy-aphasia spectrum phenotypes, including LKS, ECSWS and IEAD (Table 2). All individuals with LKS and ECSWS showed CSWS in EEG studies. Individuals with IEAD had not had a sleep EEG performed to detect CSWS. Affected family members had a complex phenotype, including epilepsy (14/16) and speech and language difficulties (16/16). Intellectual disability occurred in 6 of 16 mutation carriers, and a further 2 were of borderline intellect (Supplementary Table 2).

Previous cases implicating mutations in GRIN2A have not identified a consistent epilepsy phenotype but have shared features with our cases. Four cases with 16p13 microdeletions including GRIN2A have been reported, one of which had LKS10. The remaining three cases had seizures in the setting of more complex phenotypes, including dysmorphic features and moderate-to-severe intellectual disability11. Although two cases had EEG patterns suggestive of CSWS, only...
one fell clearly along the epilepsy-aphasia spectrum with the syndrome of atypical partial epilepsy\(^3\). In another study, a three-generation family had a translocation disrupting \textit{GRIN2A} that was associated with childhood- and adolescent-onset convulsions in the setting of learning difficulties or intellectual disability. There was no suggestion of CSWS in EEG studies of this family, and no epilepsy syndrome was determined.

We conclude that \textit{GRIN2A} mutations are highly predictive of the epilepsy-aphasia spectrum disorders that include LKS, ECSWS and IEAD. Furthermore, in a separate study, Lesca and colleagues report \textit{GRIN2A} mutations in 20\% of cases with LKS, ECSWS (also called CSWS syndrome, CSWSS) and atypical rolandic epilepsy with speech impairment\(^{15,16}\), confirming the importance of \textit{GRIN2A} to EAS. Of note, we did not detect any \textit{GRIN2A} variants in 475 probands with other epileptic encephalopathy phenotypes or in 81 probands with BECTS. Furthermore, in a large series of autism probands (\(n = 1,703\)), no \textit{GRIN2A} mutations were identified\(^{19}\). These results demonstrate that the genetic etiology of EAS may well be distinct, an observation that balks the current trend toward an overlapping etiology for neurodevelopmental disorders. We hypothesize that altered NMDA receptor activity due to \textit{GRIN2A} haploinsufficiency or missense mutations results in aberrant ion flux and disruption of the downstream signaling cascade. NMDA receptor aberration and its potential role in disrupting the corticothalamic network during slow sleep will be an important area of future research. This study is the first, to our knowledge, to detect a monogenic cause for EAS with a mutational rate of 9\%. These results strongly suggest that \textit{GRIN2A} diagnostic testing is warranted in individuals with epilepsy-aphasia spectrum disorders and will enhance prognostic and genetic counseling for families.

**METHODS**

Methods and any associated references are available in the online version of the paper.

Note: Any Supplementary Information and Source Data files are available in the online version of the paper.

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**AUTHOR CONTRIBUTIONS**

G.L.C., H.C.M. and I.E.S. designed the study and wrote the manuscript. H.C.M. and I.E.S. supervised the study. G.L.C. constructed libraries, developed the variant calling pipeline (assisted by J.C.), analyzed sequence data, conducted RNA transcript analysis (assisted by E.G.) and performed haplotypeing. J.C. and G.L.C. performed aCGH. A.K. performed mutation segregation analysis. B.J.O. and S.F.B. developed the computational methodologies and analysis pipeline. B.M.R., S.C.Y., L.G.S., S.J.T., M.-H.T. and R.W. performed phenotypic analysis. R.O., J.A.D. and M.S.H. conducted mutation screening in the BECTS cohort. B.M.R., S.F.B. and I.E.S. critically reviewed the manuscript. N.L., N. Bruneau, N. Burnashev and P.S. generated mutant transcripts and performed single-channel recordings and analysis.

**COMPETING FINANCIAL INTERESTS**

The authors declare no competing financial interests.

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ONLINE METHODS

Human subject recruitment and diagnosis. This study was approved by the Human Research Ethics Committees of Austin Health and the University of Washington. Probands with epileptic encephalopathies were recruited from the epilepsy clinic at Austin Health, the practices of the investigators and by referral for epilepsy genetics research from around Australia and internationally after informed consent. The cohort consisted of 519 individuals with a diverse range of epileptic encephalopathy phenotypes. An epileptic encephalopathy was defined as refractory seizures and cognitive slowing or regression associated with frequent, ongoing epileptiform activity 1. Detailed epilepsy and medical histories were obtained together with the results of investigations including EEG and MRI studies. Epilepsy syndromes were classified according to the Organization for the International League Against Epilepsy Commission on Classification 1.

Data analysis and variant calling. Raw read processing and alignment was performed as previously14. However, variant (single nucleotide and indel) calling and filtering was performed using the Genome Analysis Tool Kit (GATK; see URLs). Variants that did not adhere to the following criteria were excluded from further analysis: allele balance of >0.70, QUAL of <30, QD of <5, coverage of <25×, clustered variants (window size of 10) and variants in homopolymer runs (5 bp). Variants were annotated with SeattleSeq (see URLs), and the ESP6500 data set (see URLs) was used to assess variant frequency in the control population. PCR and Sanger sequencing were conducted according to standard methods as described previously.

aCGH. We performed aCGH using a custom-designed 8-plex microarray (Agilent Technologies) designed to detect copy number alterations in known epilepsy-related genes. 

GRIN2A was covered at a density of one probe for every ~350 bp. All experiments were performed according to the manufacturer’s instructions, and data analysis was conducted using Genomic Workbench (Agilent Technologies).

Genotyping. We performed genotyping in all available affected and unaffected members of families A and B who carried the c.1007+1G>A variant. We selected three microsatellite markers, D16S404, D16S3126 and D16S407, spanning a 0.56-Mb interval across GRIN2A. Fluorescently labeled PCR products were analyzed on an ABI3100 genetic analyzer, and allele size ranges were determined with the GS500LIZ size standard (Applied Biosystems) using PeakScanner V2.0 software (Applied Biosystems). Furthermore, we genotyped all family members for the rare exonic GRIN2A variant (rs61753382) using Sanger DNA sequencing.

RNA transcript analysis. RNA was isolated from the whole blood of affected family members and controls using the PAXgene Blood RNA kit (PreAnalytiX). cDNA synthesis was performed using 1 µg of DNA with the iScript Reverse Transcription Supermix kit (Bio-Rad). Nested PCR and Sanger sequencing were performed for GRIN2A RNA transcript analysis in three affected family members from families A (II-5 and III-5) and C (III-1) with the c.1007+1G>A mutation. We assessed the presence of the rs6173382 variant; the minor allele was linked to the c.1007+1G>A mutation (see Supplementary Table 3 for the sequences of primer pairs).

Constructs and transfections. NR1 and NR2A constructs were purchased (Genecopia). Site-directed mutagenesis (Agilent Technologies) was used to generate the mutant NR2A (Thr531Met) construct (Supplementary Table 3). All wild-type and mutant constructs were verified by Sanger sequencing.

Monkey kidney fibroblast-like COS-7 cells were seeded in 6-well plates (1 x 10^5 cells/well) 1 d before transfection. Magnetofection of cells with NR1 and NR2A constructs (1:3 ratio) was performed using the Magnetofectamine transfection kit (Oz Biosciences). The presence of the NR1 and NR2A (wild type and mutant) subunits at the plasma membrane was verified by immunocytochemistry experiments (data not shown).

Single-channel recordings and analysis. Single-channel recordings were made from transfected COS-7 cells (ATCC, CRL-1651) in cell-attached patches at holding potential of +100 mV using an EPC-10 amplifier (HEKA Elektronik). Cells were negative for mycoplasma contamination using the MycoTrace Mycoplasma PCR detection kit (PAA). No authentication was made. Cells in the recording chamber were perfused with oxygenated ACSF containing 126 mM NaCl, 3.5 mM KCl, 1 mM MgCl2, 2 mM CaCl2, 10 mM (±)-glucose, 1.20 mM NaH2PO4 and 26 mM NaHCO3 (oxygenated with 5% CO2/95% O2). Recording patch pipettes were pulled from borosilicate glass capillaries (World Precision Instruments) and had resistances of 4 to 7 MΩ when filled with the solution of the following composition: 140 mM NaCl, 2.8 mM KCl, 2 mM CaCl2, 10 mM (±)-glucose, 1.20 mM NaH2PO4 and 26 mM NaHCO3 (oxygenated with 5% CO2/95% O2). Recoding patch pipettes were filled with the solution of the following composition: 140 mM NaCl, 2.8 mM KCl, 2 mM CaCl2, 10 mM (±)-glucose and 20 mM HEPES/NaOH (pH 7.5, 320–330 mOsm). Channels were activated with 50 µM glycine and 1 mM glutamate in the pipette solution. Recordings were performed at room temperature (22–24 °C). For analysis, recordings were filtered at 2 kHz (~3 dB) and digitized at 20 kHz. Lifetime analysis was performed using Clampfit 10.2 (Molecular Devices) and Origin 8.5 (Origin-Lab) software. For data analysis by Clampfit, a digital eight-pole Bessel low-pass filter was set at 1 kHz. Average values were expressed as means ± s.e.m. The statistical significance of the differences was evaluated by nonparametric Mann-Whitney test (two-tailed).