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Mutations in *GRIN2A* cause idiopathic focal epilepsy with rolandic spikes

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Idiopathic focal epilepsy (IFE) with rolandic spikes is the most common childhood epilepsy, comprising a phenotypic spectrum from rolandic epilepsy (also benign epilepsy with centrotemporal spikes, BECTS) to atypical benign partial epilepsy (ABPE), Landau-Kleffner syndrome (LKS) and epileptic encephalopathy with continuous spike and waves during slow-wave sleep (CSWS)[1,2]. The genetic basis is largely unknown. We detected new heterozygous mutations in *GRIN2A* in 27 of 359 affected individuals from 2 independent cohorts with IFE (7.5%; $P = 4.83 \times 10^{-18}$, Fisher's exact test). Mutations occurred significantly more frequently in the more severe phenotypes, with mutation detection rates ranging from 12/245 (4.9%) in individuals with BECTS to 9/51 (17.6%) in individuals with CSWS ($P = 0.009$, Cochran-Armitage test for trend). In addition, exon-disrupting microdeletions were found in 3 of 286 individuals (1.0%; $P = 0.004$, Fisher's exact test). These results establish alterations of the gene encoding the NMDA receptor NR2A subunit as a major genetic risk factor for IFE.

Within the spectrum of idiopathic (genetic) focal epilepsy (IFE) with rolandic spikes, BECTS is characterized by focal and secondarily generalized seizures, which usually remit by puberty. Electroencephalograms (EEGs) show rolandic spike-and-wave discharges (mainly centrotemporal spikes, CTS) as a hallmark[3]. ABPE, CSWS and LKS represent more severe disorders with various seizure types and/or a highly pathological (sleep) EEG[2,4] as well as cognitive, language and behavioral deficits.

The genetic causes of IFE are largely unknown, although family studies have provided evidence for presumed autosomal dominant inheritance of CTS[5,6]. Linkage studies identified loci for CTS on chromosomes 15q14 (ref. 7) and 11p13 (ref. 8). Association of CTS with markers in *ELP4* has been replicated, but no causative mutation has yet been identified[9]. Following hints from a microdeletion study published by our group that *GRIN2A*, encoding the α2 subunit (NR2A; also known as GluNR2A) of the *N*-methyl-D-aspartate (NMDA)-selective glutamate receptor, could be an interesting candidate gene in IFE[10], we here identify various mutations in *GRIN2A* in two large independent cohorts comprising the whole spectrum of IFE.

We first performed a mutation analysis in cohort I (screening cohort) comprising 39 individuals with IFE and CTS (for details on all cohorts, see **Supplementary Table 1**). One subject (index subject 1; 1/39; 2.6%) with BECTS and learning difficulties was found who carried a newly identified missense mutation (c.728C>T; p.Ala243Val; family history and segregation unknown, parents unavailable) in *GRIN2A* predicted to be located in the Zn^{2+}-binding domain of the glutamate-gated NR2A subunit (**Fig. 1a**,**b**). Maximal inducible currents, agonist affinities and relative open-state probabilities of mutant NR1–NR2A Ala243Val receptors were not significantly different, upon heterologous expression, from those of the respective receptors

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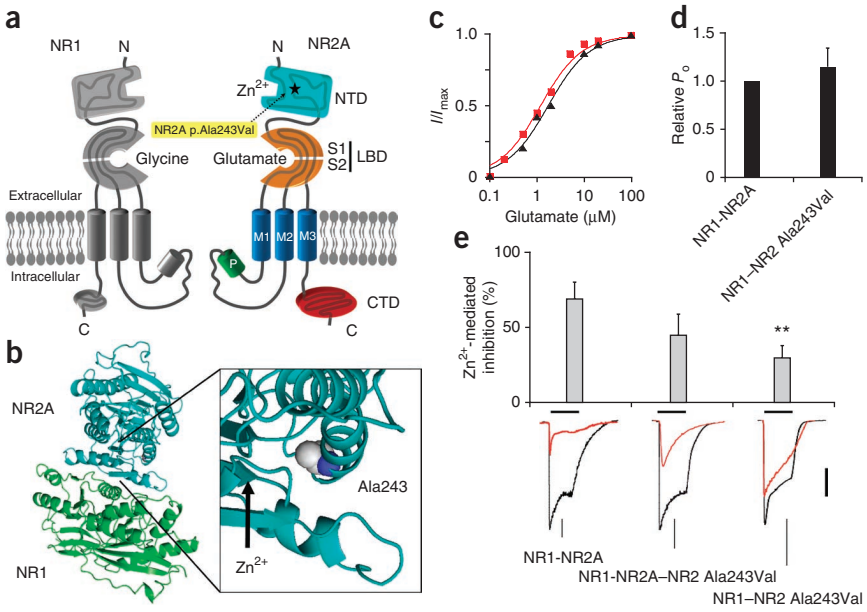

Figure 1 Structural and functional consequences of the missense mutation in *GRIN2A* encoding p.Ala243Val. Functional analysis of the missense alteration p.Ala243Val (index subject 1) showed a significant reduction in high-affinity Zn^{2+} inhibition, whereas current amplitude, glutamate and glycine affinities and relative open-state probability remained unchanged. (**a**) Topology model of an NR1 and an NR2A subunit. The position of p.Ala243Val is indicated by a star in the NR2A subunit consisting of an N-terminal domain (NTD), the ligand-binding domain (LBD) including the S1 and S2 peptide segments, three transmembrane segments (M1–M3), a re-entrant pore loop (P) and an intracellular C-terminal domain (CTD). Ala243 lies within the Zn^{2+}-binding NTD in NR2A. (**b**) Model of the NR2A NTD (cyan) together with an adjacent NR1 NTD (green). An enlarged view shows Ala243 within the Zn^{2+}-binding NR2A NTD. (**c**) Pharmacological characterization of the apparent agonist affinities of wild-type and mutant NMDA receptors. Glutamate dose-response curves of wild-type NR1-NR2A (black triangles) and mutant NR1–NR2A Ala243Val (red squares) NMDA receptors were measured upon heterologous expression in *Xenopus laevis* oocytes by two-electrode voltage clamping (TEVC; $n = 5$). Similar glutamate and glycine (data not shown) concentrations were required for a half-maximal response (EC_{50}); representative dose-response curves are shown for wild-type and mutant receptors. I/I_{max} is the relative current, normalized to the maximal inducible current (in μA). (**d**) Maximal current responses and kinetics of the open-channel blocker MK-801 for wild-type and mutant NMDA receptors show similar channel activity. The maximal agonist-inducible currents and rate kinetics of MK-801–mediated inhibition were used to determine the relative open-state probability (P_o) of wild-type NR1-NR2A compared to mutant NR1–NR2A Ala243Val NMDA receptors. Data are shown as mean + s.d. (**e**) Inhibition of agonist-evoked currents by low concentrations of Zn^{2+} at wild-type and mutant NMDA receptors. Currents for NR1-NR2A, NR1-NR2A–NR2A Ala243Val and NR1–NR2A Ala243Val receptors show a gradual loss of high-affinity inhibition by 0.1 μM Zn^{2+} ($n = 5$; $P < 0.01$, Student's t test). Traces show currents for the NMDA receptors in the absence (black) and presence (red) of 0.1 μM Zn^{2+}.

containing the wild-type NR2A subunit (**Fig. 1c**,**d**). In contrast, currents for NR1-NR2A, NR1-NR2A–NR2A Ala243Val and NR1–NR2A Ala243Val receptors showed a gradual loss of high-affinity inhibition by 0.1 μM Zn^{2+} ($P < 0.01$; **Fig. 1e**), suggesting increased activation *in vivo* due to impaired tonic inhibition of NR1–NR2A Ala243Val receptors at physiological concentrations of Zn^{2+}. Furthermore, 2 different mutations in *GRIN2A* were identified by next-generation sequencing of >300 known and suggested epilepsy genes for diagnostic purposes[11] in 2 individuals with LKS not included in cohort I. Index subject 2 carried a new truncating mutation, c.2041C>T, encoding p.Arg681* (pedigree shown in **Fig. 2**), and index subject 3 carried a new splice-site mutation, c.1007+1G>A (positive family history, segregation unknown, parents unavailable), both predicted to result in non-functional proteins.

Motivated by these findings and to evaluate the relevance of these preliminary results, we recruited cohort II (validation cohort) comprising 119 additional independent individuals with IFE. Sanger sequencing of *GRIN2A* identified mutations in 8 of 119 subjects (6.7%) that were not listed in dbSNP, the 1000 Genomes Project database or the Exome Variant Server (EVS). With respect to the different subentities, mutations were found in 0 of 3 subjects with isolated CTS, 1 of 48 subjects with BECTS (2.1%), 1 of 17 subjects with ABPE (5.9%), 0 of 17 subjects with LKS and 6 of 34 subjects with CSWS (17.6%) (**Table 1**, **Supplementary Fig. 1** and **Supplementary Tables 1–3**). Finally, we replicated these findings in cohort III (replication cohort) comprising 240 additional individuals who underwent whole-exome sequencing and subsequent validation by Sanger sequencing. The proportion of *GRIN2A* mutation carriers in the replication cohort was similar to that in the validation cohort, with an overall mutation rate of 19/240 (7.9%). Mutations were identified in 0 of 2 subjects with isolated CTS, 11 of 197 subjects with BECTS (5.6%), 4 of 20 subjects with ABPE (10.0%), 1 of 4 subjects with LKS (25.0%) and 3 of 17 subjects with CSWS (17.6%) (**Table 1**, **Supplementary Fig. 1** and **Supplementary Tables 1–3**).

We then combined cohorts II and III for subsequent statistical analysis, including a total of 359 subjects. Mutations occurred at significantly higher frequency in our cohort of affected individuals (27/359; 7.5%) than in EVS (37/6,503; 0.6%), which was used as a reference panel of unaffected controls ($P = 4.83 \times 10^{-18}$, Fisher's exact test). Restricting the comparison to affected individuals and controls of European ancestry yielded similar results (26/315 versus 27/4,300; $P = 1.18 \times 10^{-16}$, Fisher's exact test). The frequency of mutations significantly increased with more severe phenotypes ($P = 0.009$, Cochran-Armitage test for trend; **Supplementary Fig. 2**). Furthermore, severity of phenotypes showed substantial association with the type of mutation (Pearson's corrected contingency coefficient $C_{corr} = 0.52$; **Supplementary Fig. 3**). For index subject 2 and 19 cases from cohorts II and III, additional family information was available. Of these cases, two were found to have different *de novo* mutations. The remaining 17 cases each had a newly identified mutation that cosegregated with a phenotype of different epileptic disorders (often but not exclusively associated with CTS) and various degrees of intellectual disability within the family (**Fig. 2**). The *GRIN2A* locus showed significant linkage to phenotype in these 17 families (2-point parametric logarithm of odds (LOD) score of 3.55 under a dominant risk model with reduced penetrance of 80% and complete linkage).

In addition, 286 individuals with IFE were screened for copy number variations (CNVs) in *GRIN2A* using the Illumina HumanOmniExpress BeadChip (cohort IV, CNV cohort). This cohort included all affected individuals from cohort III (an overlap

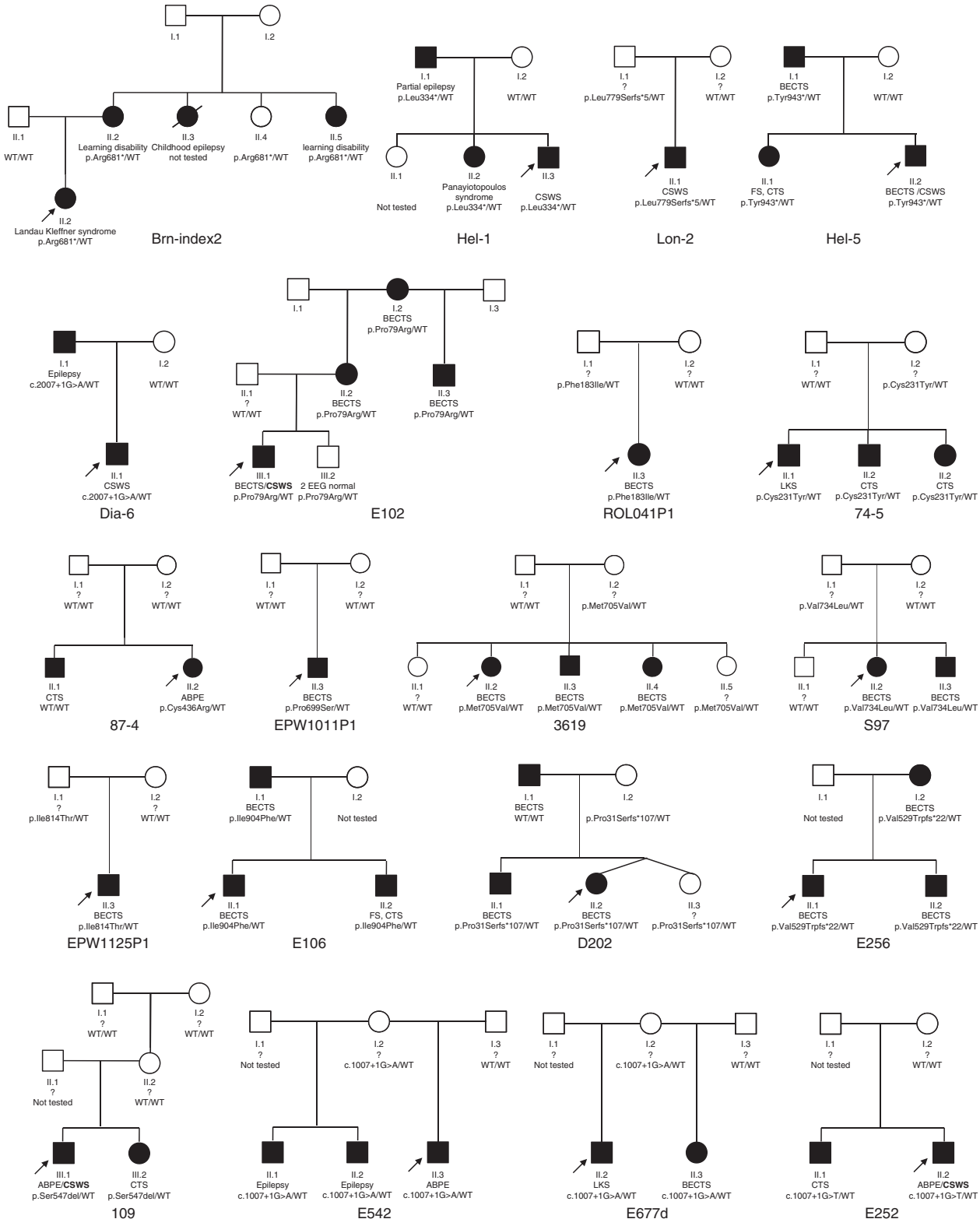

Figure 2 Pedigrees of affected individuals with available family information. Analysis of possible segregation of the respective mutation in family members could be performed for index subject 2 and for 19 of 27 mutation carriers in cohorts II and III where DNA samples were available from family members. The respective *GRIN2A* mutation segregated with a variable phenotype of seizures, pathologic EEG patterns and/or intellectual disability in family members. A few individuals carried the familial mutation but did not present any clinical features, indicating incomplete penetrance of the mutations or mosaicism. However, subclinical phenotypes (for example, EEG patterns) have not been investigated in these individuals. Pedigree 87-4 suggests phenocopy in the proband's brother. WT, wild type.

Table 1 Newly identified mutations detected in *GRIN2A*

ID	Case	Epilepsy syndrome	DNA mutation	Protein alteration
	K_i-index1	BECTS	c.728C>T	p.Ala243Val
	Brn-index2	LKS	c.2041C>T	p.Arg681*
	Brn-index3	LKS	c.1007+1G>A	IVS4, p.?
1	Ant-18	BECTS	c.1108C>T	p.Arg370Trp
2	Ant-11	CSWS	c.2140G>A	p.Glu714Lys
3	K_i-11	CSWS	c.2927A>G	p.Asn976Ser
4	K_i-40[a]	ABPE	c.594G>A	p.Trp198*
5	Hel-1	Panayiotopoulos/**CSWS**	c.1001T>A	p.Leu334*
6	Lon-2[b]	ABPE/**CSWS**	c.2334_2338delCTTGC	p.Leu779Serfs*5
7	Hel-5	CSWS	c.2829C>G	p.Tyr943*
8	Dia-6	CSWS	c.2007+1G>A	IVS7, p.?
9	E102	BECTS/**CSWS**	c.236C>G	p.Pro79Arg
10	ROL 041P1	BECTS	c.547T>A	p.Phe183Ile
11	74-5	LKS	c.692G>A	p.Cys231Tyr
12	EPW 1109P1	BECTS	c.869C>T	p.Ala290Val
13	87-4	ABPE	c.1306T>C	p.Cys436Arg
14	EPW 1011P1	BECTS	c.2095C>T	p.Pro699Ser
15	3619	BECTS	c.2113A>G	p.Met705Val
16	EPW 1083P1	BECTS	c.2179G>A	p.Ala727Thr
17	S97	BECTS	c.2200G>C	p.Val734Leu
18	EPW 1128P1	ABPE	c.2314A>G	p.Lys772Glu
19	EPW 1125P1	BECTS	c.2441T>C	p.Ile814Thr
20	E106	BECTS	c.2710A>T	p.Ile904Phe
21	ROL 057P1	ABPE	c.2927A>G	p.Asn976Ser
22	D202	BECTS	c.90delTins(T)2	p.Pro31Serfs*107
23	E256	BECTS	c.1585delG	p.Val529Trpfs*22
24	109-4	ABPE/**CSWS**	c.1637_1639delCTT	p.Ser547del
25	E542	ABPE	c.1007+1G>A	IVS4, p.?
26	E677d	BECTS	c.1007+1G>A	IVS4, p.?
27	E252	ABPE/**CSWS**	c.1007+1G>T	IVS4, p.?
28	NB3	ABPE	CNV deletion	CNV deletion
29	72-3	BECTS	CNV deletion	CNV deletion
30	EPW 1111P1	BECTS	CNV deletion	CNV deletion
31	145	CSWS	CNV duplication	CNV duplication

BECTS, benign epilepsy with centrotemporal spikes (rolandic epilepsy); Panayiotopoulos, Panayiotopoulos syndrome; ABPE, atypical benign partial epilepsy; LKS, Landau-Kleffner syndrome; CSWS, epileptic encephalopathy with continuous spike and wave during sleep. Phenotype appears in bold for individuals who had either BECTS or ABPE and electrical status in sleep, which were subsumed under the phenotype of CSWS.
[a]Array-based CGH (aCGH) analysis identified a duplication at 8q11.23 and a duplication at 15q26.1q26.2. [b]aCGH analysis identified a *de novo* microduplication at 22q11.21 and a small maternally inherited intronic deletion of *NRXN1*.

of 83.9%) and 46 additional individuals with IFE. Out of 286 cases, 3 (1.0%) were identified with exon-disrupting microdeletions within *GRIN2A* (**Supplementary Fig. 4**). An additional intronic duplication was found to segregate with the phenotype but remains of unknown significance (**Supplementary Fig. 4**). CNVs of *GRIN2A* occurred significantly more often in affected individuals than in controls (3/286 versus 0/1,520; $P = 0.004$).

Our investigations have identified mutations as well as exon-disrupting CNVs within *GRIN2A* in a significant subset of individuals with IFE. We therefore postulate that genetic alterations in *GRIN2A* are not only a major genetic risk factor but are also compatible with IFE being a monogenetic trait in up to 7.5% of affected individuals. Additional modifying factors might explain phenotypic variability. As in other idiopathic epilepsies[12], mutations were occasionally identified in apparently unaffected relatives, suggesting mosaic status or incomplete penetrance (although EEG abnormalities and rare seizures might have been missed in these individuals). A significant trend toward higher mutation rates in more severe phenotypes was demonstrated, which is similar to what has been observed for other epilepsy-associated genes such as *SCN1A*[13,14].

NMDA receptors are tetrameric ligand-gated ion channels composed of two NR1 subunits and two of four possible NR2 subunits (NR2A–NR2D), which bind glutamate and determine the location of the NMDA receptor subtype as well as functional properties of synaptic transmission and plasticity[15]. Changes in NMDA receptor function have been demonstrated in animal models of temporal lobe epilepsy following induced status epilepticus[16–18] and in the Stargazer mouse model of idiopathic absence epilepsy[19]. Earlier studies also provided evidence for a role of *GRIN2A* alterations in individual subjects with epilepsy[10,20,21]. NMDA receptors are tonically inhibited by Zn^{2+}, a mechanism that has been shown *in vitro* to protect neurons against NMDA receptor–mediated overexcitation and glutamate toxicity.

Functional analysis of the missense alteration p.Ala243Val (index subject 1) demonstrated impaired reduction of receptor currents by low concentrations of Zn^{2+}, suggesting increased activation of the NR1-NR2A heteromer due to reduced high-affinity Zn^{2+}-mediated inhibition *in vivo*. Accordingly, relief of NMDA receptors from tonic Zn^{2+}-mediated inhibition results in higher susceptibility to repeated activation and enhanced Ca^{2+} influx. This mechanism should be of particular importance at synaptically localized NR1-NR2A receptors, owing to their high-affinity Zn^{2+}-binding site. This finding is in stark contrast to the loss of function predicted for truncating mutations, frameshift mutations and deletions of *GRIN2A*[10,20]. However, different molecular alterations of NMDA receptor subunit genes might lead to similar changes in subunit composition, resulting in comparable changes in the electrophysiological properties of the receptor[22]. Moreover, similar phenomena are known from other epilepsy-associated genes, such as activating and deactivating mutations and deletions of *SCN1A* that have been described in Dravet syndrome[23].

IFE is characterized by an age-dependent clinical phenotype. NMDA receptor subunit composition is also age dependent, with a switch from predominantly NR2B expression in early development to more prominent NR2A expression at later stages[15]. Therefore, alterations in NR2A may become relevant only in specific age groups[24].

In summary, we report genetic alterations in *GRIN2A* in 7.5% of individuals with IFE, rendering alterations of *GRIN2A* a major genetic risk factor. This finding is particularly noteworthy, as NMDA receptors are promising targets for epilepsy treatment[25].

URLs. dbSNP Build 135, http://www.ncbi.nlm.nih.gov/projects/SNP/; 1000 Genomes Project database, http://www.1000genomes.org/; Exome Variant Server (release ESP6500SI, accessed November 2012), http://evs.gs.washington.edu/EVS/; PolyPhen-2, http://genetics.bwh.harvard.edu/pph2/; MutationTaster, http://www.mutationtaster.org/;

SpliceView, http://zeus2.itb.cnr.it/~webgene/wwwspliceview_ex.html; HSF2.4, http://www.umd.be/HSF/; R statistical environment v2.15.1, http://www.R-project.org/; EuroEPINOMICS, http://www.euroepinomics.org/.

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

Study design: J.R.L., S.v.S., I.H., S. Biskup, E.M.R., F.Z., D.L., B.A.N. and H.L. Subject ascertainment and phenotyping: I.H., J.A.J., H.M., U.S., R.B., W.v.P., R.C., N.F., M.A., S.W., P.D.J., J.L., R.S.M., H.H., L.A., S.T., E.H., D.K.P., K.V., U.V., T.T., P.D., R.G.L., J.M.S., T.L., A.-E.L., S. Buerki, G.W., J.K., A.N.D., S.R., M.W., B.F., G. Kurlemann, G. Kluger, A.H., D.E.H., C.K., J.S., F.B., Y.G.W., H.L., M.F., H.S., B.N., G.M.R., U.G.-S., J.G., F.Z., B.A.N., J.R.L. and S.v.S. Mutation analysis of cohort I: S.v.S., I.H., K.F., M. Schilhabel and A.F. Next-generation sequencing panel analysis of index subjects: I.S. and S. Biskup Mutation analysis of cohort II: C.W., J.R.L. and S. Biskup Segregation analysis of cohort II: C.W. and S. Biskup Mutation analysis of cohort III: E.M.R., D.L., J.A., M.R.T., H.T. and P.N. Segregation analysis of cohort III: E.M.R. and D.L. CNV control cohort: P.H. and S.H. Statistical analysis: M.N. Functional analysis of *GRIN2A* missense mutation: M. Schwake, K.G. and B.L. Data interpretation: H.L., R.J.H., M. Schwake, B.L., J.R.L., I.H., S.v.S., S. Biskup, D.L., E.M.R., M.N., B.A.N. and F.Z. Manuscript writing: J.R.L., S.v.S., S. Biskup, B.L., M.N., E.M.R., F.Z., D.L. and B.A.N. All authors contributed to the final version of the manuscript.

COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

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ONLINE METHODS

Study design. The overall study design is shown in **Supplementary Figure 5**. Following reports on microdeletions including *GRIN2A* in individuals with complex neurodevelopmental phenotypes, epilepsy and CTS as a common feature of the EEG[10], we started sequencing a small cohort of 39 individuals with IFE (screening cohort) and identified a first index subject with BECTS, learning difficulties and a missense mutation (encoding p.Ala243Val) in *GRIN2A*. In addition, consecutive next-generation sequencing epilepsy panel analysis for diagnostic purposes identified two further index subjects with LKS and mutations in *GRIN2A* (c.1007+1G>A; IVS4, p.? and encoding p.Arg681*). In these subjects, molecular genetic analysis of 323 genes that are known to be involved in epilepsy was performed using a targeted next-generation sequencing approach (epilepsy panel version 2) as recently described[11]. No other subjects with mutations in *GRIN2A* have been identified using this analysis method so far.

Motivated by these findings, we analyzed a second cohort (validation cohort) of 119 individuals with IFE and confirmed our findings by analysis of a third cohort (replication cohort) of an additional 240 affected individuals. For statistical analysis, data from cohorts II and III were combined, giving a total cohort of 359 subjects with IFE of childhood, and compared to publically available control sequence data from EVS.

In addition to sequence analysis, we performed CNV analysis in a fourth cohort (CNV cohort) and compared these data to CNV data from 1,520 platform- and ancestry-matched in-house controls.

Subjects. Cohort I (screening cohort) comprised individuals with IFE with rolandic spikes recruited at the Department of Neuropediatrics at the University Hospital Schleswig-Holstein (Kiel, Germany) and the Northern German Epilepsy Center for Children and Adolescents (Schwentinental, Germany).

For follow-up studies, study cohort II (validation cohort) was recruited by partners at European and Argentinian epilepsy centers, children's hospitals and departments of neuropediatrics and neurology.

For replication, subjects for study cohort III (replication cohort) were recruited by collaborating centers of the EuroEPINOMICS-CoGIE (Complex Genetics of Idiopathic Epilepsies) initiative.

Cohort IV for CNV analysis (CNV cohort) included all subjects from cohort III as well as additional affected individuals recruited at the participating centers.

A summary of cohorts I–IV is given in **Supplementary Table 4**.

Phenotyping was performed according to the 2001 and 2010 International League Against Epilepsy (ILAE) classification schemes[1,26]. For analysis, the following epilepsy syndromes were used: benign childhood epilepsy with centrotemporal spikes (BECTS, also rolandic epilepsy), atypical benign partial epilepsy (ABPE), Landau-Kleffner syndrome (LKS) and epileptic encephalopathy with continuous spike and waves during sleep (CSWS). Rare forms of benign occipital epilepsy (Panayiotopoulos syndrome and Gastaut syndrome) were subsumed under BECTS, as these syndromes often show overlapping features. Individuals who had either BECTS or ABPE and electrical status in sleep were subsumed under the phenotype of CSWS, as this is a rather atypical feature in these epilepsy syndromes and will influence and probably change clinical outcome. In LKS, electrical status in sleep is a frequent symptom of the syndrome. CSWS was used synonymously with electrical-status epilepticus in slow-wave sleep (ESES)[27].

All affected individuals and/or their legal guardians gave written informed consent. The study protocol was approved at all sequencing centers (Kiel, Tübingen, Cologne, all in Germany). Approval for subject recruitment and inclusion in epilepsy genetics studies is available at all participating centers. The investigators were not blinded to allocation during experiments and outcome assessment.

Control cohorts. For statistical analysis of mutation frequencies, control data were derived from EVS from the National Heart, Lung, and Blood Institute (NHLBI) GO Exome Sequencing Project (ESP).

Data from CNV analysis were compared to those for 1,520 in-house controls matched for analysis platform and geographic origin. This cohort was drawn from the HNR (Heinz Nixdorf RECALL) population-based epidemiological study consisting of males and females aged 45 to 75 years from an unselected urban population from the Ruhr area in Germany[28].

DNA extraction from blood samples. DNA from individual blood samples was extracted locally at the recruitment centers using commercially available kits.

Mutation screening and CNV analysis. *Sequence analysis of cohorts I and II.* For cohorts I and II, mutation analysis of *GRIN2A*, including all coding exons and exon-intron boundaries, was performed using the primers whose sequences are given in **Supplementary Table 5a**,**b**. PCR amplification and bidirectional sequencing were carried out following standard protocols[29]. New and known polymorphisms as well as indels were identified by NovoSNP[30] and Sequence Pilot (JSI Medical Systems).

Sequence analysis of cohort III. Sequence analysis of cohort III was performed using next-generation sequencing techniques. In brief, DNA was fragmented using sonication technology (Covaris), and fragments were end repaired and adaptor ligated. SeqCap EZ Human Exome Library v2.0 (Roche NimbleGen) was used for enrichment, and samples were analyzed on the Illumina HiSeq 2000 sequencer. Only exome data with an average coverage of >30× for 85% of the target sequences were included in the analysis. Data were filtered using Illumina Real-Time Analysis (RTA) software v1.8 and mapped to human genome reference build hg19 via the ELANDv2 alignment algorithm on a multinode compute cluster. PCR duplicates were excluded using CASAVA v1.8. Variant calling was performed by SAMtools (version 0.1.7) for indel detection. Scripts developed in house at the Cologne Center for Genomics (Cologne, Germany) were applied to detect protein changes, affected splice sites and overlaps with known variants. In particular, variants were filtered for high-quality, previously unknown variants in *GRIN2A* compared to an in-house variant database, dbSNP Build 135, the 1000 Genomes Project database and EVS.

The pathogenic implications of identified coding variants were assessed by different *in silico* analysis programs (PolyPhen-2 and MutationTaster). For intronic SNPs, splice-site analysis was performed using SpliceView and HSF2.4.

CNV analysis. Whole-blood DNA was genotyped for 730,525 markers using the Illumina HumanOmniExpress BeadChip according to the manufacturer's protocol. Genotypes were analyzed with the Illumina GenomeStudio genotyping module (v.2011). CNV calls were generated using PennCNV software[31] by the use of the log R ratio (LRR) and B allele frequency (BAF) for all probes included on the genotyping chips. Analysis was restricted to CNVs larger than 30 kb and coverage of at least five consecutive probes. All potential microdeletions were manually inspected for the regional SNP heterozygosity state and $\log_2$ ratios of the signal intensities to exclude technical artifacts. Subsequently, *GRIN2A* CNV validation of the index subject and CNV segregation in the family was conducted by multiplex ligation-dependent probe amplification (MLPA; MRC-Holland) and CNV analysis of whole-exome sequencing data, respectively.

Statistical analysis. *Sequencing studies.* For statistical analysis of sequencing data, affected individuals from cohorts II and III (validation and replication cohorts) were combined. Owing to the small sample size of cohort I and isolated index subjects 2 and 3, affected individuals from this screening cohort were excluded from statistical analysis.

Fisher's exact test was used to compare mutation frequencies in cases and EVS controls. To exclude potential population stratification effects, an additional analysis was performed comparing only affected individuals with European ancestry (with geographic origin determined by surname and/or self-reported ancestry; excluding individuals from Turkey and Russia, as these countries cross borders between Europe and Asia) against the EVS European-American controls.

Cochran-Armitage test for trend was used to test the hypothesis of higher mutation frequencies in more severe phenotypes, and Pearson's corrected contingency coefficient was calculated to demonstrate an association between the severity of phenotypes and the type of mutation. All tests were carried out using the R statistical environment v2.15.1.

Two-point linkage analysis was performed using the LINKAGE package[32]. We assumed a dominant mode of inheritance with a reduced penetrance of 80% and complete linkage between *GRIN2A* and the disease locus.

CNV studies. A Fisher's exact test was used to compare the frequency of exon-disrupting CNVs in cases and in-house controls.

Functional studies. For *X. laevis* oocyte experiments, *GRIN2A* and *GRIN1* constructs and capped cRNAs were generated as described previously[21]. Individual oocytes between stages V and VI were obtained from anesthetized frogs and were isolated by collagenase treatment. Total *GRIN1* and *GRIN2A* cRNAs (10 ng) were injected into oocytes. After injection, oocytes were kept at 17 °C in ND96 solution (96 mM NaCl, 2 mM KCl, 1.8 mM $CaCl_2$, 1 mM $MgCl_2$ and 5 mM HEPES, pH 7.4). Glutamate, glycine and Zn^{2+} dose-response curves for wild-type NR1-NR2A and mutant NR1–NR2A Ala243Val NMDA receptors were analyzed by two-electrode voltage clamp recording. Molecular modeling of the NMDA receptor subunits was performed according to Endele *et al.*[21].

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