Rare genetic risk factors in common idiopathic epilepsy syndromes

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1 Introduction

1.1 Epilepsy

The term “epilepsy” encompasses a number of different syndromes whose key feature is defined by recurrent, unprovoked seizures (Berg et al. 2010). Epilepsy is one of the most common chronic neurologic diseases worldwide with a prevalence of 0.5-1% and a lifetime incidence of 3% (Hauser et al., 1993). Recurrent seizures critically change quality of life, particularly of those 30% of epilepsy patients who are drug-resistant (Berg et al., 2009). The economic burden of epilepsies is high, it has been estimated that the 6 million people with active epilepsy in Europe cost over €20 billion per year (Cross et al., 2011; www.who.int).

Epilepsy syndromes are highly heterogeneous in their clinical manifestation. In 1989, the International League Against Epilepsy (ILAE) proposed a classification scheme (Commission on Classification and Terminology of the International League against Epilepsy, 1989.) which has been revised in recently (Berg et al., 2010). Epilepsy syndromes are classified by their etiology and symptomatology. Symptomatic epilepsy is considered an acquired disorder, developing in response to congenital or acquired brain insults such as malformations, tumors or infections. Genetic (formerly: idiopathic) epilepsies represent about 40% of all epilepsies in childhood and approximately 20% of epilepsies in adulthood (Steinlein et al., 1999). Off note, due to our previous studies on the same patient cohort, before classification change, we keep in the presented follow up studies in this dissertation the term "idiopathic" (Commission et al.,1989) epilepsies instead of "genetic" (Berg et al., 2010) epilepsies. Idiopathic (genetic) epilepsies are characterized by recurrent seizures in otherwise healthy individuals. The absence of known or suspected cerebral lesions and their familial aggregation implicated a genetic basis (Ottman, 2005). Given the strong impact of genetic factors in the pathogenesis of idiopathic epilepsies, molecular genetic studies provide a promising approach to dissect the responsible susceptibility genes and to determine molecular pathways in epileptogenes. The most common forms of idiopathic epilepsies include the generalized i) idiopathic generalized epilepsy (IGE) and the focal ii) rolandic epilepsy (RE) (Figure 1).

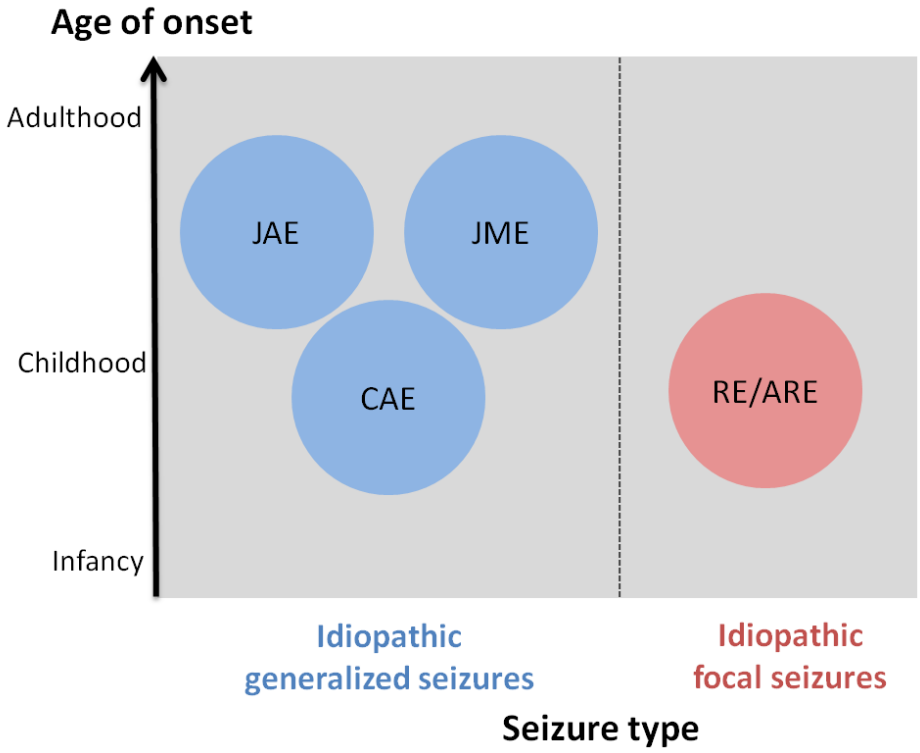

Figure 1 Overview of common idiopathic epilepsy types. The epilepsy types are distributed by age of onset as well as seizure type. Abbreviations: JAE= Juvenile absence epilepsy; JME= Juvenile myoclonic epilepsy; CAE= Childhood absence epilepsy; RE= Rolandic epilepsy; ARE= Atypical rolandic epilepsy.

1.2 Idiopathic epilepsies investigated

1.2.1 Idiopathic generalized epilepsies (IGE)

The group of idiopathic generalized epilepsies (IGEs) represent about one-third of all epilepsies (Jallon et al., 2011). These epilepsies are characterized by their age-related manifestation of generalized seizure types (Nordli et al., 2005). Generalized spike-wave discharges are the characteristic electroencephalographic signature, which reflects a synchronized hyperexcitable state of thalamocortical circuits (Blumenfeld et al., 2005). IGEs can be clinically subdivided by leading seizure types and age-of-onset into four major IGE syndromes: childhood absence epilepsy (CAE), juvenile absence epilepsy (JAE), juvenile myoclonic epilepsy (JME) and epilepsy with generalized tonic–clonic seizures alone (EGTCS). In addition, a large variety of other genetic epilepsies exist, however, quantitatively they represent a small minority of less than 10% (Nordli et al., 2005). The characteristic seizure types of the common IGE syndromes are absence seizures (CAE and JAE), bilateral myoclonic seizures on awakening (JME) and generalized tonic–clonic seizures (EGTCS). Family and

twin studies suggest a predominant genetic predisposition of common IGE syndromes with heritability estimates up to 80% (reviewed in Helbig et al., 2008).

1.2.1.1 Genetics

A significant complex genetic predisposition is indicated by concordance rates of 70–80% for IGE in monozygotic twin pairs (Berkovic et al., 1998) compared to rapidly declining recurrence risks of IGEs in siblings ranging from four to 10% depending on the IGE subtype (Beck-Mannagetta et al., 1991). IGEs follow a complex mode of inheritance, suggesting that several genetic factors contribute to generalized seizures (Greenberg et al., 1992; Sander et al., 1996; Berkovic et al., 1998). The recurrence risk for IGE ranges from 70% to 95% in monozygotic twins and is 10- to15-fold higher than for first-degree relatives (5–8%), and more than 100-fold greater than the prevalence of 0.6% in the general population (ratio of sibling risk to population prevalence: $\lambda_S \approx 8$) (Sander et al., 1996). However, CAE, JAE and JME cluster in families, and, frequently, absence seizures are followed by myoclonic seizures in the same patient in an age-dependent manner. These observations support the neurobiological concept that the common IGE subtypes share an overlapping genetic predisposition (Berkovic et al., 1987; Beck-Mannagetta et al., 1991; Reutens et al., 1991; Wirrell et al., 1991; Janz et al., 1997). Furthermore, supporting this concept, the age-related expression of various seizure types arises from a genetically determined impairment of brain maturation or is influenced by the stage of brain maturation (Shinnar et al., 1991). In rare monogenic types of idiopathic epilepsies, several genes have been identified of which the majority of genes encode voltage-gated or ligand-gated ion channels (e.g. *SCN1A, GABRA1, KCNQ2, KCNQ3, CHRNA4* and many others) (Reid et al., 2009). The molecular dissection of genes conferring susceptibility to the genetically complex idiopathic epilepsies remains a challenge. In contrast to gene mapping strategies applied in rare monogenic epilepsies, a large number of linkage and candidate gene association studies failed to identify susceptibility genes for common IGE syndromes with complex genetic predisposition (Gardiner, 2005; Hempelmann et al., 2006; Kasperaviciute et al., 2010; Greenberg and Subaran, 2011). The failure to identify replicable risk genes for common epilepsies most likely reflects the underestimated degree of genetic complexity and heterogeneity in human epilepsies.

1.2.2 Rolandic epilepsy (RE) and the RE spectrum

Benign epilepsy with centrotemporal spikes (BECTS) was first described by Martinus Rulandus in 1597 (Van Huffelen, 1989). The syndrome is now named rolandic epilepsy (RE) because of the characteristic features of partial seizures involving the brain region around the lower portion of the rolandic fissure. RE is one of the most common epilepsy syndromes in children, accounting for about 15 % of epilepsies beginning before the age of 16 years (Freitag et al., 2001; Shinnar et al., 2002). Onset typically occurs from 4–12 years in otherwise healthy children (Lerman et al., 1986). Seizures have a focal origin and frequently start with one-sided sensorimotor symptoms of lips tongue, and face, resulting in hypersalivation and speech arrest (summarized by Strug et al., 2009). The prognosis of RE is benign. It resolves spontaneously and by far of most affected children remain intellectually normal. There is, however, an increased comorbidity with attention deficit hyperactivity disorder (ADHD) and specific mild cognitive deficits (approx. 20%) (Smith et al., 2012).

RE is related to much rarer, and less benign epilepsy syndromes, including atypical benign partial epilepsy of childhood (ABPE), Landau-Kleffner syndrome (LKS) and epileptic encephalopathy with continuous spike-and-waves during sleep (CSWSS) (Doose et al., 2001; Gobbi et al., 2006; Guerrini and Pellacani, 2012; Hahn et al., 2001), referred to as RE related syndromes, or atypical rolandic epilepsy (ARE) by some authors (Fejerman, 2009). RE and the ARE share blunt, high-voltage, characteristically shaped centrotemporal spikes (CTS) of characteristic morphology in the EEG. However, ARE denote more severe forms of focal childhood epilepsies with various additional seizure types, developmental language delay or regression, speech dyspraxia and variable neuropsychiatric deficits in up to 50% of the affected (Gobbi et al., 2006; Hughes et al., 2011).

1.2.2.1 Genetics

The majority of RE patients have a complex genetic predisposition as implicated by the relative decline of the recurrence risks of seizures in 9.8% of first-degree relatives, 3% in second- and 1.5% in third-degree relatives as well as the intriguing phenotypic variability observed among family members (Vears et al., 2012). The age-dependent EEG endophenotype of centrotemporal sharp waves (CTS) represents the electroencephalographic hallmark of RE. The CTS-EEG may also be present in

clinically unaffected family members and was earlier reported to be inherited in an autosomal-dominant manner (Heijbel et al., 1975; Bali et al., 2007). However these findings were questioned for methodological reasons (Ottman et al., 1989) ARE belongs to an extended spectrum of Rolandic epilepsy, a rate of 40% EEG concordance for siblings investigated at the age of maximum expression (3 to 10 years) is observed (Doose et al., 2001). Despite the strong genetic predisposition of the CTS-EEG trait, the etiology of epilepsies with centrotemporal spikes is largely unknown. Various loci have been reported for classic or rare forms of RE (Scheffer et al., 1995; Neubauer et al., 1998; Guerrini et al., 1999; Roll et al., 2006; Strug et al., 2007; Strug et al., 2009).

Furthermore, rare variants in *KCNQ2*, *KCNQ3* and *SRPX2* have been associated with the RE or ARE in small subsets of patients (Neubauer et al., 2008; Roll et al., 2006). Recently, rare structural variations at 16p13 deleting the genomic region of *GRIN2A* have been observed for patients with ARE (Reutlinger et al., 2010). All these variants and loci have never been replicated and might account for only a small fraction of the genetic risk for the rolandic epilepsies, whereas the major cause(s) remain unknown.

2 Goals and objectives

The aim of the present studies was the molecular genetic dissection of risk factors with strong epileptogenic effects in idiopathic epilepsies (Figure 2) and the elucidation of their molecular pathways in epileptogenesis. Taking into account the low power of our case-control study for genome-wide scans, our current strategy focused on a candidate gene/locus approach of genes and CNVs that have either been implicated in the pathogenesis of epilepsy or represent plausible candidate genes with a presumed impact on neuronal excitability. This candidate gene/locus approach was restricted to the identification of genetic risk factors with a strong impact on epileptogenesis according to the common disease-rare variant (CD-RV) model (Pritchard, 2001; McClellan et al., 2007). The CD-RV model implies that multiple, heterogeneous rare variants with strong effects contribute to the genetic architecture underlying common idiopathic epilepsies. In line with the CD-RV model, we had previously demonstrated that large recurrent microdeletions in the chromosomal regions 15q11.2, 15q13.3 and 16p13.11 constitute individually rare but collectively substantial genetic risk factors of IGE (Helbig et al., 2009; De Kovel et al., 2010).

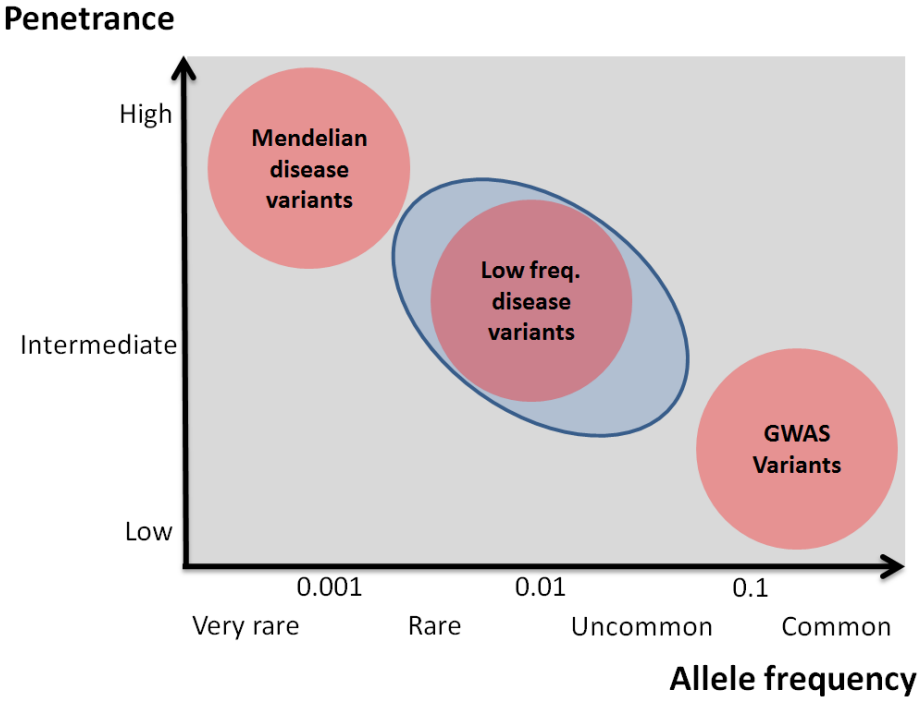

Figure 2: Expected distribution of disease variants. Theory on common disease variation predicts a continuum of frequency and effect size of disease variants present in the population. Rare high−risk variations are mainly expected for Mendelian diseases. Less frequent disease variants with moderate to large effects (highlighted in blue) will be detected in our CNV and mutation studies. The figure is modified from McCarthy et al., 2008.

Two strategies were applied to identify genetic risk factors for idiopathic epilepsies: 1) copy number variation (CNV) analysis using high-resolution SNP arrays and 2) whole exome sequencing (WES) to detect mutations in the coding regions. Taking into account our cohort sizes and the amount of expected variants, the study power was small. Therefore, our analyses focused on genetic variants affecting genes involved in neuronal excitability.

2.1 Discovery cohorts

The discovery cohorts of idiopathic epilepsies included two case-control cohorts: i) 1582 unrelated IGE and 2795 populations controls of North-Western European descent, and ii) 308 patients with RE-spectrum epilepsies and 1512 controls of European descent. In addition, we performed candidate gene sequence analysis in 242 patients with RE-spectrum epilepsies. The IGE study cohort was collected by a European concerted action organized by the EPICURE Consortium (EPICURE GWAS discovery cohorts; EPICURE et al., 2012). The standardized ascertainment scheme, clinical protocols and diagnostic criteria are available at http://portal.ccg.uni-koeln.de/ccg/research/epilepsy-genetics/sampling-procedure/. The cohort of 281 patients with RE–spectrum epilepsies were recruited in the framework of the European collaborative research project EuroEPINOMICS. In a multi-centre effort, RE/ARE patients were recruited from Germany, Austria, Canada and Australia. Ninety-eight of the patients were ascertained through multiplex-families with at least two affected siblings. Diagnosis of RE was performed according to the International Classification of Seizures and Epilepsies (Commission on Classification and Terminology of the International League against Epilepsy 1989; Berg et al., 2010). The various syndromes classified as RE-spectrum epilepsies fulfilled the diagnostic criteria as specified previously (Aicardi and Chevrie, 1982; Doose et al., 2001; Hahn et al., 2001). In total, the discovery cohort comprised 230 patients affected by typical RE and 51 patients with ARE (165 males and 116 females). Both screening cohorts represent the largest assemblies of IGE and RE/ARE patients reported so far.

2.2 Molecular genetic screening methods

The present studies applied two molecular genetic screening techniques to identify genetic risk factors with strong effects: i) copy number variation (CNV) analyses using high-resolution SNP arrays (IGE and RE cohorts), and ii) candidate gene

sequence analysis based on an ongoing whole exome sequencing project (RE/ARE, n =242).

2.3 Selection of candidate genes

We investigated large recurrent CNVs at 1q21, 15q11.2, 15q13.3, 16p11.2, 16p13.11 and 22q11.2 (Helbig et al., 2009; Coe et al., 2012; De Kovel et al., 2013); as well as the high-ranking candidate genes *GRIN2A* (Reutlinger et al., 2010; Lesca et al., 2012), *GPHN* (Förstera et al., 2010; Lionel et al., 2013), *RBFOX1* and *RBFOX3* (Bhalla et al., 2004; Martin et al., 2007; Gallant et al., 2011; Gehman et al., 2011) as well as *DEPDC5* (Dibbens et al., 2013; Ishida et al., 2013). The investigated CNV loci have been implicated in the pathogenesis of epilepsies or other neurodevelopmental disorders, whereas the candidate genes are presumed to have a strong functional impact in pathways controlling neuronal excitability.

2.4 Scope of the ongoing research activities

In the presented cumulative thesis I report the results of our current CNV and candidate gene sequence analyses resulting in three articles already published in peer review journals (Lal et al., 2013a; Lal et al., 2013b, Lemke et al., 2013). Another three papers are currently in revision (Lal et al., Dejanovic et al., Reinthaler et al.,). Our ongoing studies identified CNVs at 16p11.2, *RBFOX1*, *RBFOX3* and *GRIN2A* and putative deleterious sequence mutations in *GRIN2A* and *DEPDC5* in patients with idiopathic epilepsies. These results offer new insight into the genetic architecture of common epilepsy syndromes by: i) the delineation of the phenotypic spectrum associated with these epilepsy genes, ii) the characterization of their allelic spectra and modes of inheritance (de novo, inherited, estimates of the effect size, penetrance, expressivity), and iii) a first step in the elucidation of the underlying molecular pathomechanisms in epileptogenesis. In the long term, our study results might accelerate the prospects to translate the rapidly increasing knowledge into clinically relevant actions.

3 Publications

3.1 Publications on IGE

3.1.1 *RBFOX1* in IGE (published)

Lal D, Trucks H, Møller RS, Hjalgrim H, Koeleman BPC, et al. (2013) Rare exonic deletions of the RBFOX1 gene increase risk of idiopathic generalized epilepsy. **Epilepsia** 54: 265–271. doi:10.1111/epi.12084

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FULL-LENGTH ORIGINAL RESEARCH

Rare exonic deletions of the *RBFOX1* gene increase risk of idiopathic generalized epilepsy

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Summary

Purpose: Structural variations disrupting the gene encoding the neuron-specific splicing regulator RBFOX1 have been reported in three patients exhibiting epilepsy in comorbidity with other neuropsychiatric disorders. Consistently, the *Rbfox1* knockout mouse model showed an increased susceptibility of seizures. The present candidate gene study tested whether exon-disrupting deletions of *RBFOX1* increase the risk of idiopathic generalized epilepsies (IGEs), representing the largest group of genetically determined epilepsies.

Methods: Screening of microdeletions (size: >40 kb, coverage >20 markers) affecting the genomic sequence of the *RBFOX1* gene was carried out by high-resolution single-nucleotide polymorphism (SNP) arrays in 1,408 European patients with idiopathic generalized epilepsy (IGE) and 2,256 population controls. Validation of *RBFOX1* deletions and familial segregation analysis were performed by quantitative polymerase chain reaction (qPCR).

Key Findings: We detected five exon-disrupting *RBFOX1* deletions in the IGE patients, whereas none was observed in the controls ($p = 0.008$, Fisher's exact test). The size of the exonic deletions ranged from 68 to 896 kb and affected the untranslated 5′-terminal *RBFOX1* exons. Segregation analysis in four families indicated that the deletions were inherited, display incomplete penetrance, and heterogeneous cosegregation patterns with IGE.

Significance: Rare deletions affecting the untranslated 5′-terminal *RBFOX1* exons increase risk of common IGE syndromes. Variable expressivity, incomplete penetrance, and heterogeneous cosegregation patterns suggest that *RBFOX1* deletions act as susceptibility factor in a genetically complex etiology, where heterogeneous combinations of genetic factors determine the disease phenotype.

KEY WORDS: Idiopathic generalized epilepsy, Microdeletion, *RBFOX1*, Genetics.

The idiopathic generalized epilepsies (IGEs) affect up to 0.3% of the general population and account for 30% of all epilepsies (Jallon et al., 2001). Genetic factors play a predominant role in the etiology of common IGE syndromes. Heritability estimates are >80% and recurrence risk for first-degree relatives varies between 4% and 9% depending on the IGE subtype (Helbig et al., 2008). The genetic architecture is likely to display a biologic continuum, in which a small fraction follows monogenic inheritance, whereas the majority of IGE patients presumably display an oligogenic/polygenic predisposition. Molecular genetic studies have identified causative gene mutations in mainly rare monogenic forms of genetic epilepsies. Most of the

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[2]Members of the EPICURE Consortium are Appendix 2.

currently known genes for human genetic epilepsies encode voltage-gated or ligand-gated ion channels (Reid et al., 2009; Pandolfo, 2011). Despite extensive research, the majority of genetic variants predisposing to common IGE syndromes remain elusive.

Large-scale analysis of structural genomic variations using high-resolution whole-genome oligonucleotide arrays suggests that copy number variations (CNVs) play a substantial role in about 3% of patients with idiopathic epilepsies (de Kovel et al., 2010; Heinzen et al., 2010; Mefford et al., 2011). Recurrent microdeletions on 15q11.2, 15q13.3, and 16p13.11 increase risk of IGE and a wide range of neurodevelopmental disorders (Helbig et al., 2009; de Kovel et al., 2010; Heinzen et al., 2010; for review see Hochstenbach et al., 2011). The genes deleted by these microdeletions are thought to play a key role in the regulation of neuronal excitation and cortical synchronization.

Structural variations disrupting the gene encoding the neuronal splicing regulator *RBFOX1* (also assigned as *A2BP1, HRNBP1*, or *FOX1*) have been reported in three patients exhibiting epilepsy in comorbidity with autism, intellectual disability, or pontocerebellar hypoplasia (Bhalla et al., 2004; Martin et al., 2007; Gallant et al., 2011). The *RBFOX1* gene is located in the chromosomal region 16p13.3 to which a linkage locus for photoparoxysmal response in families of IGE subjects has been mapped (Pinto et al., 2005). The *RBFOX1* gene plays a key role in the regulation of neuronal excitation and influences susceptibility of epilepsy (Gehman et al., 2011; Voineagu et al., 2011). The *RBFOX1* protein regulates splicing of many neuronal transcripts by binding the sequence (U)GCAUG in introns flanking alternative exons (Jin et al., 2003; Auweter et al., 2006; Voineagu et al., 2011; Fogel et al., 2012). A number of *RBFOX1* target transcripts (e.g., *SNAP25*, *SCN8A*, *GRIN1*, *GABRG2*, *DCX*, *GAD2*, *KCNQ2*, *SLC12A5*, *SV2B*, *SYN1*) have been implicated to play a role in epileptogenesis (Barnby et al., 2005; Corradini et al., 2009; Papale et al., 2009; Pandolfo, 2011; Fogel et al., 2012; Veeramah et al., 2012) and show differentially spliced RNA transcripts in *Rbfox1* knockout mice (Gehman et al., 2011) Notably, brain-specific homozygous and heterozygous *Rbfox1* knockouts in mice do not alter brain morphology but display spontaneous seizures and a dramatic epileptogenic response to kainic acid resulting in status epilepticus (Gehman et al., 2011). Consistent with the splicing alterations in mice, a RNA interference–mediated 50% knockdown of *RBFOX1* transcripts in human neurons changes the alternative splicing pattern and expression of primarily neuronal genes involved in synapse formation and function (Voineagu et al., 2011; Fogel et al., 2012).

The present candidate gene association study tested whether exon-disrupting deletions of *RBFOX1* increase risk of common IGE syndromes. We found a significant excess of exon-disrupting deletions of the *RBFOX1* gene in IGE patients compared to population controls. Familial cosegregation analysis implicates that exon-disrupting *RBFOX1* deletions represent susceptibility factors that increase risk of IGE but are not sufficient for the expression of IGE in most of the families.

Subjects and Methods

Study participants

The study protocol was approved by the local institutional review boards of the participating centers, and all study participants gave informed consent. The patients with common IGE syndromes were recruited as a concerted effort of epilepsy genetics programs integrated in the European EPICURE Project (http://www.epicureproject.eu; EPICURE Consortium et al., 2012). Phenotyping and diagnostic classification of IGE syndromes were carried out according to standardized protocols (available at: http://portal.ccg.uni-koeln.de/ccg/research/epilepsy-genetics/sampling-procedure). Patients with IGE exhibit unprovoked generalized seizures but are typically otherwise normal and have no anatomic brain abnormalities (Commission on Classification & Terminology of the International League Against Epilepsy, 1989; Nordli, 2005). Accordingly, the ascertainment scheme applied in this multicenter study did not include IGE patients with severe intellectual disability (no basic education, permanently requiring professional support in their daily life). All subjects of the case–control association cohorts were typed by the Affymetrix Genome-Wide Human SNP Array 6.0 (Affymetrix, Santa Clara, CA, U.S.A.). To ensure highly confident CNV calls, we excluded all individuals (351 of 4,015) carrying a genome-wide excess of more than 50 microdeletions (size >40 kb, coverage >20 markers) prior to the assessment of *RBFOX1* microdeletions (Elia et al., 2012).

The case–control sample included in this candidate gene CNV study comprised 1,408 unrelated patients with IGE of self-identified Northwestern European ancestry (869 females/539 males; childhood absence epilepsy [CAE] n = 413, juvenile absence epilepsy [JAE] n = 207, unspecified idiopathic absence epilepsy [IAE] n = 7, juvenile myoclonic epilepsy [JME] n = 557, epilepsies with generalized tonic–clonic seizures alone [EGTCS] n = 224) and 2,256 German population controls (1,077 females/1,179 males). Array data of 2,256 German control subjects were obtained from the PopGen biobank (University Hospital Schleswig-Holstein, Campus Kiel, Kiel, Germany) and the KORA (Cooperative Health Research in the Region of Augsburg) research platform representing epidemiologically recruited cohorts from the Northern (Schleswig-Holstein, PopGen) and Southern (Augsburg, KORA) regions of Germany. The control subjects have not been screened for epilepsy or other neurodevelopmental disorders. EIGENSTRAT principal component analysis (Price et al., 2006) was applied to remove ancestry outliers and to match the European ancestry of the case–control cohorts (EPICURE Consortium et al., 2012).

RBFOX1 microdeletion screening

For all DNA samples of the case–control cohorts, we assessed the signal intensities of 1.8 million probe sets on the Affymetrix Genome-Wide Human SNP Array 6.0. CNV analysis of all Affymetrix SNP 6.0 arrays was performed at the Cologne Center for Genomics, using the algorithm implemented in the Affymetrix Genotyping Console version 4.1.1. To achieve high accuracy in CNV calling across Affymetrix SNP 6.0 arrays processed at different laboratories, microdeletion screening was restricted to deletions covered by at least 20 probes and spanning >40 kb in size (Pinto et al., 2011).

Array-based screening of *RBFOX1* deletions captured all deletions affecting the genomic sequence of the *RBFOX1* gene (chr16:6,069,131-7,763,339, human genome build 37/hg19). The *RBFOX1* gene is located in the chromosomal region 16p13.3 and consists of 21 exons (NM_018723.3), which form six mRNA transcripts encoding five known protein isoforms (Fig. 1; *RBFOX1* exon annotation adapted from Fogel et al., 2012). Notably the 5′-terminal exons 1, 2, 3, 1B and part of exon 4 are untranslated (Fig. 1). All potential *RBFOX1* microdeletions were manually inspected for the regional SNP heterozygosity state and log2 ratios of the signal intensities to exclude technical artifacts. Subsequently, the copy number state of all *RBFOX1* microdeletions identified by the array-based CNV analysis was examined by real-time quantitative PCR (qPCR), using seven TaqMan CNV assays covering the 5′-terminal *RBFOX1* exons 1-4 (Life Technologies, Carlsbad, CA, U.S.A.; Fig. S1).

Statistical analysis

Case–control association analysis was carried out using a two-sided Fisher's exact test.

Results

Detection of *RBFOX1* deletions in patients with IGE and controls

Microdeletions (size >40 kb, coverage >20 markers) affecting the genomic sequence of the *RBFOX1* gene were found in 8 (0.6%) of 1,408 individuals with IGE, whereas two deletions were observed in 2,256 controls ($p = 0.017$, Fisher's exact test; odds ratio [OR] 6.4, 95% confidence interval [CI] 1.2–62.35; Figs 1 and S1). The size of the deletions ranged from 41 to 896 kb. All 10 *RBFOX1* deletions were located in the 5′-terminal *RBFOX1* region encompassing the untranslated exons 1–4. Remarkably, the two *RBFOX1* deletions observed in the controls were both located in intronic sequences and were smaller (41 and 56 kb) than the deletions observed in the IGE patients (68–896 kb; Fig. S1). Specifically exon-disrupting *RBFOX1* deletions were present in 5 (0.35%) of 1,408 individuals with IGE (Table 1) and none was detected in 2,256 controls ($p = 0.008$, Fisher's exact test; Figs 1 and S2). The hemizygous copy number state of all *RBFOX1* deletions detected by the array-based CNV scan could be confirmed in the IGE patients by TaqMan qPCR assays. DNA samples of the control subjects were not available for qPCR validation.

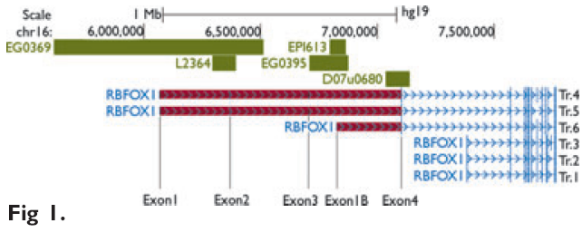

Fig 1.
Exon-disrupting *RBFOX1* deletions. Overview of exon-disrupting *RBFOX1* deletions, genomic localization, and overview of the transcript variants (hg19). The largest transcript variants 4–5 (NM_018723.3, NM_001142333.1) cover almost the entire 1.7 Mb of the gene and encode isoforms 4 and 5. The third largest transcript variant 6 also encodes isoform 4 (NM_001142334.1), whereas transcript variants 1, 2, and 3 cover only approximately 380 kb of the 3′-region of the gene. Green bars represent microdeletion size and location for each individual IGE patient. The red bars indicate the untranslated 5′-terminal *RBFOX1* exons. Tr., Transcript variant ID. The exon annotation refers to the genomic organization of *RBFOX1* as shown in Fogel et al. (2012).
Epilepsia © ILAE

Table 1. *RBFOX1* exon-disrupting deletions in IGE index patients

Family	Index patient	Deletion size (kb)	Breakpoints at chr16 (Mb)	Diagnosis/age-at-onset/seizure types	Familial comorbidity
1	EG0369	896	5.616–6.512	CAE:3/abs,7/GTCS	Developmental delay, LD, sudden death
2	EPI613	68	6.797–6.865	JAE:11/FS,15/abs,15/GTCS	No neuropsychiatric disorders
3	D07u0680	103	7.035–7.138	JME:16/myo,16/GTCS	ASD, LD, myopia
4	EG0395	165	6.709–6.874	CAE:3/abs, 3/GTCS	No neuropsychiatric disorders
–	L2364	100	6.294–6.394	JME:14/myo,14/GTCS	No neuropsychiatric disorders

Diagnosis: ASD, autism spectrum disorder; CAE, childhood absence epilepsy; EGTCS, epilepsy with generalized tonic–clonic seizures alone; FS, febrile seizure; JME, juvenile myoclonic epilepsy; IGE, idiopathic generalized epilepsy; LD, learning disability; Seizure types: myo, myoclonic seizure; abs, absence seizure.
Survey on *RBFOX1* exon-disrupting deletions in index patients with idiopathic generalized epilepsies.
Values indicate the age-of-onset in years.

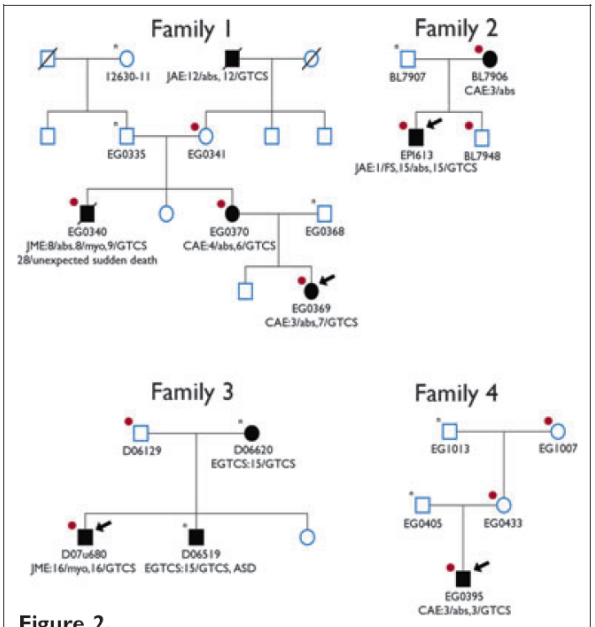

Figure 2.
Familial segregation of the exonic *RBFOX1* deletions. Familial segregation of exon-disrupting *RBFOX1* microdeletions. Deletion carriers of *RBFOX1* are marked with a red dot, "n" indicates an analyzed sample without deletion. Crossed individuals, deceased; black symbols, individuals affected by IGE; CAE, childhood absence epilepsy; JME, juvenile myoclonic epilepsy; EGTCS, epilepsy with generalized tonic–clonic seizures alone; FS, febrile seizures; myo, myoclonic seizures; abs, absence seizures; ASD, autism spectrum disorder.
Epilepsia © ILAE

Familial segregation and comorbidity analysis of the exonic *RBFOX1* deletions

The segregation of exonic *RBFOX1* deletions identified in the IGE index cases was tracked in four families (Fig. 2). The copy number status of the *RBFOX1* was assessed in 20 available family members using TaqMan qPCR assays (Table S1). In total, 12 family members carried an exon-disrupting *RBFOX1* deletion (6 females, 6 males). All *RBFOX1* deletions identified in the IGE index patients were inherited. Overall, the deletions were transmitted five times maternally and one time paternally. Seven of 12 deletion carriers were affected by IGE, and five carriers were clinically unaffected. Seven of nine IGE patients investigated carried an exonic *RBFOX1* deletion. In families 1 and 2, exonic *RBFOX1* deletions were detected in all investigated family members with IGE. In contrast, the *RBFOX1* deletion identified in the IGE index patient did not cosegregate in family 3. The phenotypic features of the IGE syndromes of the seven affected exonic deletion carriers did not differ from those IGE patients lacking a *RBFOX1* deletion. Notably, six of seven IGE patients with *RBFOX1* exon-disrupting deletions exhibited typical absence seizures. Comorbidity with other neuropsychiatric disorders was observed in families 1 and 3. In family 1, the index patient EG0369 was affected by a classical CAE but also exhibited neurodevelopmental problems with delayed speech and attention and memory problems resulting in a learning disability that required special education. Learning disability also occurred in the IGE-affected siblings of family 3 (D07u680 and D06519). However, only D07u680 carried a *RBFOX1* deletion, whereas the *RBFOX1* deletion was missing in sibling D06519, who was affected by IGE since the age of 15 years but also had pervasive developmental disorder, which is part of the diagnostic group of autism spectrum disorders. Moreover, vision impairment due to a strong myopia was present in the IGE-affected mother and all three siblings in family 3. Comorbidity with neuropsychiatric disorders was not reported in families 2 and 4. Magnetic resonance imaging scans of three IGE patients carrying an exonic *RBFOX1* deletion (family 1, EG0340; family 2, EPI613; family 4, EG0395) did not reveal any structural abnormalities of the brain, other than for bifrontal lesions in patient EG0340 due to a traumatic brain contusion occurring 16 years after the onset of the IGE.

Discussion

The present candidate gene CNV study revealed a significant excess of intronic and exonic deletions affecting the neuron-specific *RBFOX1* gene in patients with IGE compared with population controls. Specifically, we found *RBFOX1* exon-disrupting deletions in 5 (0.35%) of 1,408 IGE patients, whereas none was detected in 2,256 controls. Considering the key role of the splicing regulator *RBFOX1* in the control of neuronal excitation and seizure susceptibility (Gehman et al., 2011), the present findings suggest that rare microdeletions affecting the *RBFOX1* gene increase the risk of common IGE syndromes.

The four exonic *RBFOX1* deletions tested for familial segregation were all inherited. They differed considerably in size, ranging from 68 to 896 kb, and were all located in the 5′-terminal *RBFOX1* region encompassing the untranslated exons 1–4 (Fig. S1). The *RBFOX1* 5′-terminal exons represent highly conserved genomic sequences (Fig. S3) and are predominantly expressed in brain, suggesting that the 5′-terminal *RBFOX1* region contains important regulatory elements (Damianov & Black, 2010). Consistent with our findings, the structural genomic variations of the *RBFOX1* gene reported previously in three single patients with neurodevelopmental disorders and epilepsy also disrupted the 5′-terminal *RBFOX1* exons (Bhalla et al., 2004; Martin et al., 2007; Gallant et al., 2011). Moreover, a female with autism carrying a deletion of *RBFOX1* exon 1 due to a de novo translocation t(15p;16p) displayed a significantly reduced *RBFOX1* mRNA expression in lymphocytes (Martin et al., 2007). Accordingly, a similar reduction in *RBFOX1* mRNA expression can be expected in the

members of IGE-multiplex family 1 carrying the large 896 kb microdeletion that deletes the *RBFOX1* exons 1–2. IGE-multiplex family 1 is of particular interest because of the consistent cosegregation of the IGE trait with the *RBFOX1* deletion (Fig. 2). Notably, none of the previously identified IGE-associated microdeletions at 15q11.2, 15q13.3, and 16p13.11 (Helbig et al., 2009; de Kovel et al., 2010; Heinzen et al., 2010; for review see: Hochstenbach et al., 2011) was found in the IGE index patients carrying a *RBFOX1* deletion.

The potential functional alterations of the four smaller deletions involving the *RBFOX1* 5′-terminal exons 2–4 and exons 1B and 4 remain elusive (Figs 1 and S1). In particular, IGE-multiplex family 3 does not show a co-segregation of the IGE-trait with the 163 kb spanning deletion affecting exon 4. Similar heterogeneous cosegregation patterns, incomplete penetrance, and variable phenotypic expressivity have been observed for the recurrent 15q13.3 microdeletion, representing the strongest genetic risk factor for IGE (OR 68; 95% CI 29–181) identified so far (Dibbens et al., 2009; Helbig et al., 2009; de Kovel et al., 2010; Mefford et al., 2011; Mulley et al., 2011). Together these lines of evidence support an oligogenic/polygenic heterogeneity model for the genetic architecture of the majority of common IGE syndromes and other common neurodevelopmental disorders. Accordingly, the effect of each genetic risk factor alone is not sufficient to express IGE phenotypes, but the interactive effects of heterogeneous sets of rare and low frequency susceptibility factors together promote ictogenesis and epileptogenesis (Dibbens et al., 2007). Depending on the heterogeneous composition of genetic risk factors, the phenotypic expression of exonic *RBFOX1* microdeletions is likely to exhibit extensive phenotypic variability as observed for the large recurrent microdeletions at 1q21.1, 15q11.2, 15q13.3, 16p11.2, 16p13.11, and 22q11.2 (Coe et al., 2012). These pathogenic microdeletions seem to affect normal neurodevelopment, resulting in an impaired homeostatic regulation of neuronal networks. In combination with other genetic susceptibility factors, a set of genomic structural variations may contribute to the genetic variability and phenotypic overlap of a wide spectrum of common neuropsychiatric diseases sharing a neurodevelopmental pathogenesis (Coe et al., 2012). With regard to the pivotal role of RBFOX1 in regulating both splicing and transcriptional networks in human neurodevelopmental processes (Fogel et al., 2012), the highly variable spatiotemporal expression of the *RBFOX1* gene in differentiating human neurons (Gehman et al., 2011; Fogel et al., 2012; Lin et al., 2012) and the variable expressivity of the large number of downstream gene transcripts may also contribute to the pleiotropic effects of exon-disrupting *RBFOX1* deletions. In line with the oligogenic/polygenic heterogeneity model, we observed a familial comorbidity with other neurodevelopmental disorders, such as learning disability and autism spectrum disorder, in two families (1 and 3) with *RBFOX1* exon-disrupting deletions (Table 1, Fig. 2). Taking into account that the ascertainment scheme for subjects with IGE applied in this study leads to an exclusion of individuals with severe intellectual disability or predominant neuropsychiatric disorders, comorbidity of generalized seizures with other neurodevelopmental disorders should be more common.

In summary, the present candidate gene CNV study of the neuron-specific splicing regulator gene *RBFOX1* suggests that microdeletions affecting the untranslated 5′-terminal *RBFOX1* exons increase risk of common IGE syndromes. The present findings warrant further studies to replicate an involvement of *RBFOX1* in the genetic predisposition of IGE syndromes and other common neurodevelopmental disorders and to elucidate the pathogenic mechanisms of epileptogenesis resulting from RBFOX1-mediated alterations of the splicing process of neuronal genes.

Acknowledgments

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Disclosure

None of the authors has any conflict of interest to disclose. We confirm that we have read the Journal's position on issues involved in ethical publication and affirm that this report is consistent with those guidelines.

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Bhalla K, Phillips HA, Crawford J, McKenzie OLD, Mulley JC, Eyre H, Gardner AE, Kremmidiotis G, Callen DF. (2004) The de novo chromosome 16 translocations of two patients with abnormal

SUPPLEMENTARY INFORMATION

Rare exonic deletions of the *RBFOX1* gene increase risk of idiopathic generalized epilepsy

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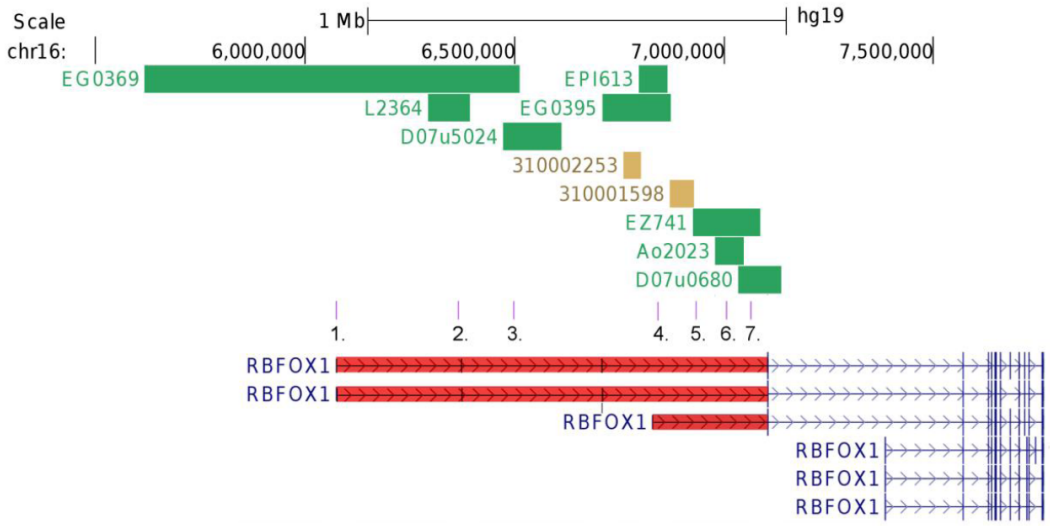
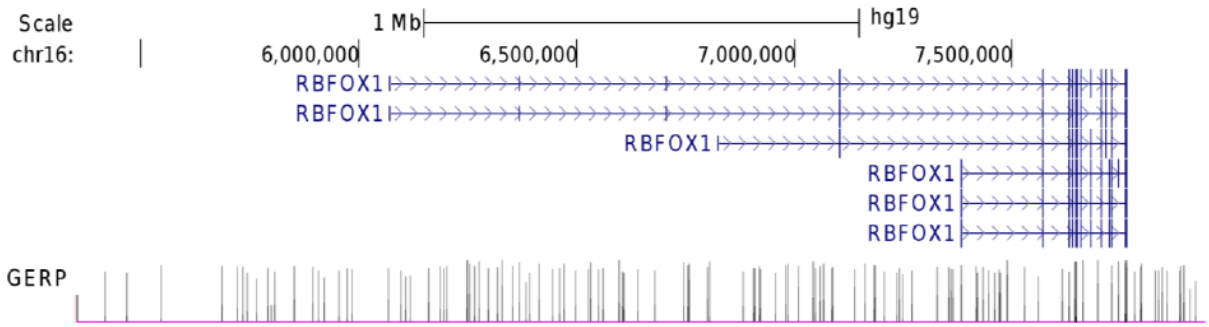
Figure S1



Overview of all deletions affecting the *RBFOX1* gene including locations of the TaqMan qPCR assays. Green bars define microdeletion size and location in IGE patients, brown bars in population controls; purple vertical lines indicate the genomic location of each individual TaqMan Copy Number Assay (1.-7.); the red bars indicate the untranslated 5'-terminal *RBFOX1* exons 1-4. All deletions were hemizygous and (if possible) have been confirmed by qPCR using copy number assays located in the deletions. *RBFOX1* genomic organization and exon annotation adapted from Fogel et al., 2012.

Figure S3



UCSC Genome Browser *RBFOX1* regulatory annotation tracks. *RBFOX1* Genomic Evolutionary Rate Profiling (GERP) scores. The rejected substitutions score (RS) is based on an alignment of 35 mammal scores, only peaks above a RS score of 2 are shown. A RS score threshold of 2 provides high sensitivity while still strongly enriching sequence conservation sites (http://www.genome.ucsc.edu). For *RBFOX1,* high RS scores were observed across the entire gene including the introns.

Table S1

			RBFOX1 qPCR CN states						
Family	Sample_ID	Diagnosis/age-at-onset /seizure types	Assay1	Assay2	Assay3	Assay4	Assay5	Assay6	Assay7
1	**EG0369**	CAE:3/abs,7/GTCS	**1**	**1**	**1**	2			
1	EG0370	CAE:4/abs,6/GTCS	**1**	**1**	**1**	2			
1	EG0340	JME:8/abs,8/myo,9GTCS, 28/unexpected sudden death	**1**	**1**	**1**	2			
1	EG0341		**1**	**1**	**1**	2			
1	EG0368		2	2	2	2			
1	EG0335		2	2	2	2			
1	12630-11		2	2	2	2			
2	**EPI613**	JAE:1/FS,15/abs,15/GTCS, CAE:3/abs			2	**1**	2	2	
2	BL7907					2	2	2	
2	BL7906	CAE:3/abs			2	**1**	2	2	
2	BL7948				2	1	2		
4	**EG0395**	CAE:3/abs,3/GTCS			2	1	2		
4	EG0433				2	**1**	2		
4	EG0405				2	2	2		
4	EG1007				2	**1**	2		
4	EG1013				2	2	2		
3	**D07u680**	JME:16/myo,16/GTCS					2	2	**1**
3	D06519	EGTCS:15/GTCS,ASD					2	2	2
3	D06129						2	2	**1**
3	D06620	EGTCS:15/GTCS					2	2	2
	Ao2023	JME:19JME,19/EGMA,gsw					2	**1**	2
	EZ741	JME:5/myo,15/GTCS,gsw,PPR					2	**1**	2
	D07u5024	JME:16/myo,16/GTCS		2	**1**	2			
	L2364	JME:14/myo,14/GTCS	2	**1**	2				

Epilepsy phenotype of individuals examined by qPCR and overview of all *RBFOX1* deletions validated by TaqMan qPCR assays. Bold sample IDs indicate index-cases for which the *RBFOX1* deletion was initially detected by the Affymetrix SNP 6.0 array. The copy number state of 1 (CN = 1) indicates a hemizygous deletion, whereas a CN = 2 represents the normal diploid copy number. All estimates of the CN states examined by qPCR achieved the

highest confidence level according to the quality scores assessed by the CopyCaller Software (Life Technologies, Carlsbad, CA, USA). The genomic position of the TaqMan CNV assays refers to the physical nucleotide position on chromosome 16 according to the human genome build 37/hg19: Assay 1: 6070126bp, Assay 2: 6360716 bp, Assay 3: 6494059 bp, Assay 4: 6837750 bp, Assay 5: 6927498 bp, Assay 6: 7000712 bp, Assay 7: 7058025 bp. Abbreviations: Diagnosis: ASD = autism spectrum disorder; CAE = childhood absence epilepsy; EGTCS = epilepsy with generalized tonic-clonic seizures alone; FS = febrile seizure; JME = juvenile myoclonic epilepsy; IGE = idiopathic generalized epilepsy; LD = learning disability; Seizure types: myo = myoclonic seizure; abs = absence seizure. Values indicate the age-of-onset in years.

3.1.2 *GPHN* in IGE (in revision)

Dejanovic B*, **Lal D***, Catarino BC*, Arjune S et al. Exonic microdeletions of the gephyrin gene exert a dominant-negative effect on GABAergic synaptic inhibition in two patients with idiopathic generalized epilepsy. **Human Molecular Genetics** (in revision)

**These authors contributed equally to this work*

Exonic microdeletions of the gephyrin gene impair GABAergic synaptic inhibition in patients with idiopathic generalized epilepsy

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ABSTRACT

Gephyrin is a postsynaptic scaffolding protein, essential for the clustering of glycine and γ-aminobutyric acid type-A receptors ($GABA_ARs$) at inhibitory synapses. An impairment of GABAergic synaptic inhibition represents a key pathway of epileptogenesis. Recently, exonic microdeletions in the gephyrin gene (*GPHN*) have been associated with neurodevelopmental disorders including autism spectrum disorder, schizophrenia and epileptic seizures. Here we report the identification of novel exonic *GPHN* microdeletions in two patients with idiopathic generalized epilepsy (IGE), representing the most common group of genetically determined epilepsies. The identified *GPHN* microdeletions involve exons 5-9 (Δ5-9) and 2-3 (Δ2-3), both affecting the gephyrin G-domain. Molecular characterization of the *GPHN* Δ5-9 variant demonstrated that it perturbs the clustering of regular gephyrin at inhibitory synapses in cultured mouse hippocampal neurons in a dominant-negative manner, resulting in a significant loss of γ_2-subunit containing $GABA_ARs$. *GPHN* Δ2-3 causes a frameshift resulting in a premature stop codon (p.V22Gfs*7) that most likely leads to haplo-insufficiency of the gene. Our results demonstrate that structural exonic microdeletions affecting the *GPHN* gene constitute a rare genetic risk factor for IGE and other neuropsychiatric disorders by an impairment of the GABAergic inhibitory synaptic transmission.

KEYWORDS

Idiopathic generalized epilepsy; Microdeletion; *GPHN*; gephyrin;

ABBREVIATIONS

$GABA_AR$, γ-aminobutyric acid type-A receptors; CNV, copy number variations; IGE, idiopathic generalized epilepsie; TMS, transcranial magnetic stimulation; DTI,

diffusion tensor imaging; HEK293, Human Embryonic Kidney 293; TLE, temporal lobe epilepsy; qPCR, quantitative polymerase chain reaction;

INTRODUCTION

Epilepsy is characterized by recurrent spontaneous seizures due to a neuronal hyperexcitability and an abnormal cortical synchronization. Approximately 3% of the general population are affected by epilepsy until the age of 40 years [1]. The idiopathic generalized epilepsies (IGEs) represent the most common group of genetically determined epilepsies, accounting for 20-30% of all epilepsies [2]. Their clinical features are characterized by age-related recurrent unprovoked generalized seizures, in the absence of detectable brain lesions or metabolic abnormalities [3,4]. Genetic factors play a predominant role in the etiology of IGE with heritability estimates of 80%. However, the vast majority of IGE syndromes have an oligo-/polygenic predisposition and their genetic basis remains elusive [5].

Structural genomic copy number variations (CNVs) account for a substantial fraction of the genetic variance in about 3% of patients with idiopathic epilepsies [6-9]. Recurrent microdeletions at 15q11.2, 15q13.3, 16p13.11 [6,7] and exonic deletions in *NRXN1* [10] and *RBFOX1* [11] increase the risk of IGE and a wide range of neurodevelopmental disorders [12]. Recently, exonic microdeletions in the gene encoding the synaptic scaffolding protein gephyrin (*GPHN*) have been found to represent a rare cause of neurodevelopmental disorders, including autism spectrum disorder (ASD), schizophrenia and epileptic seizures [13]. Two out of six patients carrying exonic *GPHN* deletions exhibited seizures [13].

Gephyrin is a postsynaptic scaffolding protein, essential for the clustering and localization of glycine and a subset of $GABA_A$ receptors at inhibitory synapses [14-19].

Gephyrin is composed of three domains - the C-terminal E-domain binds directly to the glycine and $GABA_A$ receptor subunits, whereas the N-terminal G-domain is crucial for gephyrin's oligomerization. Both domains are connected by the central (C) domain [18]. Aberrant function of gephyrin impairs GABAergic synaptic inhibition, which thereby may promote neuronal hyperexcitability and seizure susceptibility. Previously, we have reported a gephyrin-specific effect in temporal lobe epilepsy (TLE), where stress-induced irregular splicing of *GPHN* resulted in the expression of truncated gephyrin variants that impaired the function of regular gephyrin by dominant-negative interaction [20]. Additionally, reduced gephyrin expression has been detected in temporal lobe surgical specimens of patients with medically refractory TLE, as well as in rat models [21]. These accumulating lines of evidence support the hypothesis that gephyrin dysfunction impairs GABAergic synaptic inhibition and thereby contributes to epileptogenesis [21,22]. In the present study, we screened the *GPHN* gene for microdeletions in 1469 European patients with common IGE syndromes and 2256 population controls [11]. We identified exonic *GPHN* deletions affecting the gephyrin N-terminal G-domain in two IGE patients and characterized the underlying molecular mechanism causing functional alterations in gephyrin clustering thus promoting epileptogenesis.

MATERIALS AND METHODS

Study cohort and CNV screening

This study has been approved by the local Research Ethics Committees. All study participants including the family members of the *GPHN* deletion-carriers provided an informed written consent. CNV screening of the genomic *GPHN* sequence (chr14:66,974,125-67,648,525; human genome build 37/hg19) was carried out in 1,469 unrelated patients with IGE of self-identified North-Western European ancestry

and 2,256 German population controls [11]. DNA samples were investigated by the Affymetrix Genome-Wide Human SNP Array 6.0 (Affymetrix, Santa Clara, CA, USA). CNV analysis was performed, using the Birdsuit algorithm implemented in the Affymetrix Genotyping Console version 4.1.1. Regional log2 ratios of the signal intensities and the SNP heterozygosity state were visualized in the Chromosome Analysis Suite v1.2.2 (Affymetrix, Santa Clara, CA, USA). The copy number state of the *GPHN* microdeletions identified by the array-based CNV analysis were examined by real-time quantitative PCR (qPCR), using a TaqMan® CNV assays located in *GPHN* exon 7 (Assay ID: Hs01711518_cn) and intron 2 (Assay ID: Hs07084813_cn; Life Technologies, Carlsbad, CA, USA). Genome-wide CNV screening beyond the *GPHN* locus was restricted to CNVs with a segment size > 500kb [23] and a minimum of 50 markers to achieve a high accuracy and reliability. Again, regional log2 ratios of the signal intensities and the SNP heterozygosity state were visualized for all CNVs in the Chromosome Analysis Suite v1.2.2 (Affymetrix, Santa Clara, CA, USA) and were manually inspected.

Transcript analysis

RNA was isolated from whole blood samples using the PAXgene Blood RNA Kit (Qiagen, Hilden, Germany). For amplification of the Δ5-9 deletion flanking primers were designed: Forward (within exon 4) AGGAACAGGATTTGCACCAC and reverse (within exon 13) GCGATGTCTTCTAGCCACCT. Amplification of the Δ2-3 deletion was performed with primers: Forward (within exon 1) CCGAGGGAATGATCCTTACTAA and reverse (within exon 7) TCAAGTTCATCATGCACCTCC. The sizes of the amplified DNA fragments were assessed using a 1.5% agarose gel electrophoresis and the Agilent 2200 TapeStation Nucleic Acid System (Agilent Technologies, Santa Clara, California,

USA). Bidirectional Sanger sequencing of the identified *GPHN* cDNA amplicons was done following standard protocols.

Paired-pulse transcranial magnetic stimulation

For Family 1, the patient, his father and six healthy controls with no personal history of seizures or other neurological disorders and no medication (three females, median age 38 years, range 24-44 years) had transcranial magnetic stimulation (TMS). After evaluating the resting motor threshold (MT), paired-pulse TMS was used to study intra-cortical inhibition (ICI; interstimulus interval [ISI] of 3 ms) and intra-cortical facilitation (ICF; ISI of 13 ms). The protocol used was adapted from Werhahn and colleagues [24]. IBM SPSS Statistics 21.0 software was used for statistical analysis.

Expression construct

Regular enhanced green fluorescent protein (EGFP)-tagged gephyrin [25] served as the basis for Δ5-9geph expression constructs. Fragments encoding gephyrin exons 1-4 and 10-30 were amplified from regular gephyrin construct and connected by fusion PCR. The two fragments were connected by fusion PCR and introduced into the pEGFP-C2 vector (Clontech) using XhoI and HindIII restriction sites. To create myc-tagged gephyrin, EGFP-tagged gephyrin was amplified with primers that introduce the sequence for the myc-tag (EQKLISEEDL) at the N-terminus of gephyrin and a stop-codon after the last amino acid of gephyrin. Fragment was introduced into pEGFP-N2 the vector (Clontech, Mountain View, CA, USA) using XhoI and HindIII restriction sites.

Hippocampal neuron cultures and transfection

Primary neuron cultures were prepared from hippocampi of C57BL/6 mice and plated on poly-L-lysine coated coverslips at a density of 75,000/24-well dish. Neurons were cultured in Neurobasal medium, supplemented with B-27, N-2 and glutamine.

Neurons were transfected after 11 days in vitro (DIV) unless otherwise stated. Transfection was carried out with Lipofectamin 2000 (Invitrogen) according to manufactures manual. Constructs were expressed for 48 hours unless otherwise stated.

Human embryonic kidney cell culture and transfection

Human embryonic kidney (HEK293) cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum and 2 mM L-glutamine at 37 °C and 5% CO_2. For confocal laser-scanning microscopy, 1 × 10^5 HEK-293 cells were seeded on collagenized coverslips in 12-well plates and immediately transfected with polyethylenimine (1 mg/ml, diluted in H_2O, pH 7.0) using standard protocols. Cells were grown for 24-48 h depending on the assay.

Immunostaining of cultured cells

Cells were fixed with 4% PFA for 10 min at room temperature. Unspecific binding sites were blocked with blocking solution (2% BSA, 10% goat serum, 0.2% Triton X-100) for 1h and primary antibodies were applied for 1 h in 10% goat serum. After three washing steps in PBS, cells were incubated with secondary antibodies in 10% goat serum for 1 h at room temperature. After the final three washing steps in PBS, slides were mounted on cover slips with Fluoro gel II containing DAPI (Science Service, Munich, Germany) to stain the nuclei. Antibodies used for the staining: mouse anti-gephyrin (1:50 cell culture supernatant, clone 3B11); rabbit anti-gephyrin (1:1,000, rGeph-C, epitope EDLPSPPPPLSPPP, Eurogentec, Belgium); guinea-pig anti-$GABA_A$-gamma2 receptor (Jean-Marc Fritschy, ETH, Zurich); rabbit anti-VGAT (1:500, Synaptic Systems, Göttingen, Germany); mouse anti-myc-tag (1:10 cell culture supernatant, clone 9E10). Secondary antibodies were all goat raised Alexa Fluor antibodies (1:500, Invitrogen, Carlsbad, CA, USA).

Image analysis and quantification

Images were acquired with a confocal laser scanning microscope (Nikon A1) at 60x using a resolution of 1024x1024. Images were processed using the software NIS Elements (NIKON). For analysis of neuronal cultures at least two independent preparations per condition were used. Images were acquired as a z-stack with three optical sections with 0.5 µm steps. Maximum intensity projections were created and analyzed using NIS Elements software. Usually two 20 x 5 µm region of interests (ROI) were placed on dendrites and clusters were counted using the analyze particles option in NIS Elements. For statistical analysis, gephyrin cluster were compared pairwise between EGFP and Δ5-9geph expressing neurons. Mean values were compared for significance using Student's *t*-test with the software SigmaPlot (Systat Software, Chicago, IL, USA). Errors are presented as standard error of the mean (SEM). Significance levels are indicated as $*P < 0.01$, $**P < 0.05$, $***P < 0.001$. Images were processed with the software ImageJ (NIH).

Co-immunoprecipitation

Protein extracts were prepared in an IP-buffer (25 mM Tris, pH 7.4, 150 mM NaCl, 1% Triton X-100, protease and phosphatase inhibitor cocktail (Roche, Mannheim, Germany)). The post-nuclear fraction was incubated with primary antibodies for 1 h at room-temperature or overnight at 4 °C. 20 µl Protein-G Sepharose beads were incubated with the extracts for 2 hours at room temperature. After a brief centrifugation (500 *g* for 3 min), the immune-beads were washed three times with IP buffer. The adsorbed proteins were eluted from the immune-beads by boiling in 50 µl SDS-loading buffer. Immunoprecipitated samples were subjected to SDS-PAGE followed by immunoblotting.

Western Blot analysis and antibodies

For immunoblotting, a standard protocol was followed and detection was carried out using chemiluminescence and an ECL system with a cooled CCD camera (Decon Science Tec, Germany). The following primary antibodies were used and diluted in TBS-Tween containing 1% dry-milk: anti-Gephyrin (1:50, clone 3B11 cell culture supernatant), rabbit anti-ß-tubulin (1:100, Santa Cruz, Santa Cruz, CA, USA), rabbit anti-GFP (1:5,000, Abcam, Cambridge, UK), mouse anti-myc-tag (1:10 cell culture supernatant, clone 9E10). As secondary antibodies anti-mouse or anti-rabbit conjugates were used in a 1:10,000 dilutions in TBS-Tween containing 1% dry milk.

RESULTS

Detection of exonic *GPHN* microdeletions and mRNA transcription analysis

We screened the genomic *GPHN* sequence (14q23.3:66,974,124-67,648,524, reference coding sequence: NM_020806, CCDS9777.1; hg19) for microdeletions (size > 40 kb, number of probe sets > 20) using high coverage microarrays from 1,469 unrelated European patients with common IGE syndromes and 2,256 German population controls. We identified two hemizygous exonic *GPHN* microdeletions in the IGE cohort: i) a 129 kb microdeletion (chr14:67,314,917-67,443,729) affecting *GPHN* exons 5-9 (Δ5-9) in a German male patient with juvenile myoclonic epilepsy (JME, Family 1), and ii) a 158 kb microdeletion (chr14:67,136,658-67,295,196) encompassing *GPHN* exons 2-3 (Δ2-3) in a German male patient with myoclonic astatic epilepsy (Doose syndrome, Family 2) (Fig. 1A). None of the 2,256 German controls carried a microdeletion at the *GPHN* locus. The hemizygous copy number state of the *GPHN* deletions was validated by TaqMan quantitative PCR (qPCR).

Exome sequencing of the *GPHN* coding regions and exon/intron boundaries did not reveal any additional indels and rare missense mutations in the parent-offspring trio of Family 1. Direct blood cDNA amplification of *GPHN* exons 4-13 for Family 1 and exons 1-7 for Family 2 followed by Sanger sequencing of the major amplicons confirmed the transcription of the truncated *GPHN* variants and revealed a paternal transmission of both microdeletions (Fig. 1B-D and Supplementary Material S1). Genome-wide screening for CNVs previously associated with epilepsy [6,8,9,26,27], revealed no additional known pathogenic CNVs in both index patients.

On protein level, the *GPHN* Δ5-9 microdeletion leads to the truncation of the gephyrin G- and C-domain (hereafter termed "Δ5-9geph" - Fig. 1E). Given that residue 22 is encoded by a splitted codon composed by exons 1 and 2, the *GPHN* Δ2-3 variant results in a frameshift and a premature stop codon (p.V22Gfs*7, Fig. 1F). We assume that, although through first strand synthesis we were able to amplify the *GPHN* Δ2-3 variant, most of the transcript will be degraded by nonsense-mediated mRNA decay, a translation-dependent posttranscriptional process that selectively recognizes and degrades mRNAs whose open reading frame is truncated by a premature translation termination codon [28], resulting in negligible amounts of the protein.

Clinical features of the *GPHN* deletion carriers and segregation analysis

Family 1 (GPHN Δ5-9 deletion)

The pedigree of Family 1 is presented in Fig. 1A. The non-consanguineous German parents (57-years-old father, 55-years-old mother) and the patient's 30-year-old sister had no unequivocal history of seizures. The *GPHN* Δ5-9 deletion was paternally inherited. The mother showed a diploid copy number status at the *GPHN* locus. DNA

from the clinically unaffected sister was not available. The 33-year-old male index IGE patient was born at term after an uneventful pregnancy and delivery. He had recurrent simple febrile seizures from the age of six months to six years, unprovoked generalized tonic-clonic seizures since the age of five years and myoclonic seizures recorded by video-EEG starting at the age of 16 years. Complete remission of epileptic seizures by monotherapy with levetiracetam was reached since the age of 24 years. Neurological examinations and brain MRIs (3-T) were normal. The epilepsy diagnosis was classified as JME. Neuropsychiatric and developmental comorbidities were mild deficits in motor coordination, hyperactivity, and learning difficulties during childhood. Neuropsychological testing at the age of 19 years showed normal cognitive abilities with average performance scores. Recurrent episodes of major depression with suicide attempts started at the age of 16 years, accompanied by generalized anxiety, panic attacks and phobic vertigo. Non-epileptic psychogenic attacks became evident since the age of 32 years. The patient's father experienced three unprovoked events of loss of consciousness between 4 and 6 years of age. There is a strong family history of migraine affecting the maternal branch of the family. Any history of psychiatric disorders, dysmorphism, developmental delay, intellectual disability or miscarriages was absent.

Neurophysiological examinations

To evaluate cortical excitability, we performed paired-pulse transcranial magnetic stimulation (TMS) in Family 1 [29]. For both, the JME index-patient and his father, intra-cortical inhibition was significantly reduced as compared to six healthy controls ($P < 0.001$), while intra-cortical facilitation was more pronounced than in controls ($P < 0.001$- Fig. 2). Likewise, diffusion tensor imaging (DTI) showed an enhanced structural connectivity between the mesial frontal region and the descending motor

pathways in the index patient and his father that was beyond the range seen in healthy controls (Supplementary Material Fig. S3). For the patient the peak voxels reached statistical significance for the connectivity to the motor pathways ($P < 0.001$), whereas the father's values showed only a trend but did not reach statistical significance.

Family 2 (GPHN Δ2-3 deletion)

The pedigree of Family 2 is presented in Fig. 1A. The non-consanguineous parents (50-year-old father, 48-year-old mother) are of German ancestry. The *GPHN* Δ2-3 deletion was paternally inherited. The mother and the brother had diploid copy numbers at the *GPHN* locus. The 16-year-old male index IGE patient was affected by febrile seizures at the age of 15 months and by myoclonic astatic seizures starting at the age of 1.5 years. Since antiepileptic treatment with valproate, which was started shortly after epilepsy-onset, he is seizure-free. The EEG exhibited irregular sharp-slow wave discharges at 3-Hz. The patient showed a normal physical but delayed cognitive development with persistent learning disability. His epilepsy was classified as idiopathic myoclonic-astatic epilepsy (Doose syndrome). The father had no history of seizures and a normal psychomotor development. The mother experienced three seizures during infancy with spontaneous remission and normal psychomotor development. Her EEGs displayed interictal epileptiform discharges. The 18-year-old brother of the index patient had two febrile seizures during childhood while no subsequent history of afebrile seizures, normal psychomotor development and EEG were reported. None of the family members had a history of psychiatric disorders.

Molecular and biochemical characterization of the *GPHN* Δ5-9 variant

Gephyrin forms postsynaptic clusters along the neurites as well as soma, while following its overexpression in non-neuronal cells, it forms cytosolic protein-

aggregates (“blobs”, Fig. 3A,B). To examine the effects of the *GPHN* Δ5-9 deletion, we expressed GFP-tagged Δ5-9geph in cultured hippocampal neurons and HEK293 cells. Δ5-9geph-GFP was homogenously distributed in the cytosol of HEK293 cells and neurons including all morphological structures, i.e. spines (Fig. 3A,B), suggesting its inability of form postsynaptic clusters. To see how clustering deficient Δ5-9geph influences regular gephyrin, we first co-expressed myc-tagged gephyrin and Δ5-9geph-GFP in HEK293 cells. We found that gephyrin blobs were dissolved resulting in either diffuse regular gephyrin or the formation of microclusters co-localizing with Δ5-9geph-GFP (Fig. 3C,D). This demonstrates a dominant-negative effect of Δ5-9geph on the oligomerization of regular gephyrin. Notably, Δ5-9geph-GFP was stably expressed at levels equivalent to regular gephyrin-GFP (Fig. 3E). The observed dominant-negative effect on regular gephyrin clustering is mediated by direct interaction with Δ5-9geph, as shown by co-immunoprecipitation (Fig. 3F).

To evaluate the consequences of dominant-negative Δ5-9geph action on neuronal gephyrin clustering, we expressed Δ5-9geph-GFP or GFP alone in cultured hippocampal neurons. To distinguish between endogenous gephyrin and Δ5-9geph-GFP, we used a monoclonal antibody against a peptide in the C-domain that is deleted in Δ5-9geph (rGeph-C, Fig. 4A). Expression of Δ5-9geph-GFP significantly reduced the number of endogenous gephyrin clusters confirming the previously stated dominant-negative effect (Fig. 4B,C). Moreover, remaining gephyrin clusters were significantly reduced in size indicating that Δ5-9geph disturbs the clustering of regular gephyrin (Fig. 4D). To determine how Δ5-9geph-GFP expression influences the clustering of $GABA_ARs$, we visualized the γ_2-subunit (Fig. 4E), which plays a central role in gephyrin-dependent postsynaptic clustering of $GABA_ARs$ [30,31]. Number and cluster size of γ_2-containing $GABA_ARs$ were significantly decreased in the

presence of Δ5-9geph-GFP (Fig. 4F,G). Together with the observed reduced quantity of gephyrin clusters, our data imply that receptor homeostasis responds to decreased amounts of clustered gephyrin.

To investigate whether Δ5-9geph-mediated reduction in GABAergic innervation influences synaptic spines, typical morphological structures of the excitatory synapse [32], we visualized spines in control and Δ5-9geph-GFP-expressing neurons by thresholding the EGFP-signal to an identical value. Spine head morphology and amount was similar in EGFP and Δ5-9geph-expressing neurons, suggesting that diminished GABAergic synapses did not interfere with synaptic spines (Fig. 4H-J).

Besides its synaptic function, gephyrin catalyzes the last step of the molybdenum cofactor (Moco) biosynthesis being essential for the activity of four molybdenum-dependent enzymes [33-35]. We measured typical urinary biomarkers of two Moco-dependent enzymes, xanthine oxidase (uric acid, xanthine) and sulfite oxidase (S-sulfocysteine) as well as the Moco degradation product urothion [36,37] in the patient and his parents of Family 1. Concentrations of all analyzed metabolites were inconspicuous, suggesting that the hemizygous *GPHN* Δ5-9 variant did not cause any subclinical Moco deficiency phenotype in the affected patient (Supplementary Material Fig. S3).

DISCUSSION

We present a comprehensive clinical, genetic, functional and neurophysiological characterization of two IGE patients with novel hemizygous microdeletions encompassing *GPHN* exons that code for the N-terminal gephyrin G-domain. Our finding strengthens a previous report of six unrelated subjects with a wide range of neurodevelopmental disorders (ASD, schizophrenia, seizures) who carried hemizygous *GPHN* microdeletions affecting the gephyrin G-domain [13], and extends the phenotypic spectrum related to N-terminal *GPHN* microdeletions by IGE syndromes (Supplementary Material Fig. S4). The study reported by Lionel *et al*. deduced evidence of pathogenicity of *GPHN* microdeletions from the significant association with neurodevelopmental disorders (P = 0.009; 6/8,775 cases versus 3/27,019 controls) and found that three out of five *GPHN* microdeletions tested for inheritance arose as *de novo* events [13]. In this study, we present additional functional data elucidating the molecular mechanisms by which the N-terminal *GPHN* deletions cause dysfunctional GABAergic synaptic inhibition and thereby increase susceptibility of IGE.

Molecular characterization demonstrates that the truncated *GPHN* Δ5-9 variant (Family 1) exerts a dominant-negative effect on the structural synaptic organization of gephyrin and $GABA_AR$ clusters. Gephyrin oligomerization is thought to be a concerted interaction mediated by G-domain trimerization [38], G- and E-domain interaction [18], E-domain dimerization [25] and C-domain binding to other proteins controlled by posttranslational modifications [39]. The truncated Δ5-9geph protein itself is not able to form clusters and impairs the oligomerization process of regular gephyrin (Fig. 4), which can be explained by the loss of the G-domain trimerization interface encoded mainly by exon 6 [38]. Instead, Δ5-9geph probably forms irregular dimers by interaction of the intact E-domain with regular gephyrin E-domains [25].

Thereby, it stochastically affects oligmerization of regular gephyrin in a dominant-negative manner. Importantly, the size of remaining gephyrin and $GABA_AR$ clusters was significantly reduced, which will probably impair the recently described homeostatic regulation of gephyrin scaffolds and normal development of the neuronal inhibitory GABAergic circuits [40]. Consistently with our findings, it was speculated that cellular stressors such as elevated temperature or alkalosis, which can arise from seizure activity, might induce irregular exon skipping in *GPHN* mRNA leading to deletions within the G- and C-domain thus causing a dominant-negative effect on the oligomerization of synaptic gephyrin clusters [20]. Moreover, given the constitutional expression of the truncated Δ5-9geph protein, it is also likely that during development Δ5-9geph expression negatively affects the establishment of certain networks which cumulatively contribute to the genetic variance of the IGE phenotype.

In Family 2, the *GPHN* Δ2-3 variant causes a frameshift with premature stop codon, resulting in a truncated *GPHN* protein consists of the first 21 amino acids encoded by exon 1, followed by seven residues encoded by out of frame codons before the premature stop codon (p.V22Gfs*7 – Fig. 1F). Notably, five out of six *GPHN* multi-exon deletions (Δ2-5 (n = 2), Δ3-8, Δ3-11 and Δ3-12) reported by Lionel *et al*. [13] in patients with neurodevelopmental disorders result in frameshifts and premature stop codons, causing truncated *GPHN* proteins including the first 21 or 47 amino acids encoded by exon 1 or exons 1-2, respectively. Although truncated gephyrins comprising the first 47 amino acids have the capacity to impair the oligomerization of regular gephyrin [20], the expression of these frameshift-causing *GPHN* multi-exon deletions will probably be negligible due to nonsense-mediated mRNA decay [28]. Considering the fact that we were able to amplify the *GPHN* Δ2-3 transcript in cDNA derived from blood cells (Supplementary Material Fig. S2), at least a partial

expression of the *GPHN* Δ2-3 transcript is possible. However, we assume that in these patients the pathogenic effect of the truncated *GPHN* results predominantly from haplo-insufficiency rather than from effects on the oligomerization of regular gephyrin.

Given the intriguing impact of an impaired GABAergic synaptic transmission in epileptogenesis [41], the reduced amount of synaptic $GABA_AR$s in the presence of truncated gephyrins exerts a functionally convergent effect similar to that observed by mutations of genes encoding $GABA_AR$ subunits (*GABRA1, GABRB3, GABRG2* and *GABRD*) in rare families with dominantly inherited IGE syndromes (for reviews see [42,43]). On molecular level, *GABR* gene mutations generally lead to a reduced surface expression of $GABA_AR$s, which results in the reduction of the amplitude of GABA-evoked currents. Although *GPHN* deletions also should affect glycine receptor clustering, this seems to have no significant pathogenic consequences considering that typical symptoms of glycinergic deficits, such as hyperekplexia, are missing.

Interestingly, in Family 1, the clinically unaffected father as well as the JME-affected index patient carrying the *GPHN* Δ5-9 deletion exhibited an impaired cortical inhibition leading to hyperexcitability, as demonstrated by TMS, which mainly reflects the functional state of GABAergic interneuronal circuits [44]. This imbalance of the inhibitory/excitatory ratio corresponds well with the experimentally validated dominant-negative effect of the *GPHN* Δ5-9 deletion on the reduced size and number of $GABA_AR$ clusters at inhibitory synapses. Moreover, diffusion tensor imaging showed a statistically significant increase in the structural connectivity of the mesial frontal region and the descending motor circuits in the JME-affected index patient and a slightly enhanced connectivity in the father of Family 1. An altered structural connectivity of the mesial frontal region has recently been found in JME [45] and may

explain the predominant manifestation of myoclonic seizures in the JME-index patient of Family 1. Altogether, our present findings add to convergent lines of evidence that dysfunctional GABAergic synaptic inhibition represent a key pathogenic process shared by a wide spectrum of neurodevelopmental disorders, such as epilepsy, ASD, schizophrenia, and intellectual disability [46]. Consistently, a high prevalence of epilepsy has been found in autism spectrum disorder (ASD), ranging from 21% in ASD subjects with an intellectual disability to 8% in those without intellectual disability [47]. Notably, the index patient of Family 1 had a typical JME but also exhibited a broad range of psychiatric and behavioral disorders.

Carriers of *GPHN* microdeletions encompassing the N-terminal exons display a high phenotypic variability and incomplete penetrance, strongly depending on the genetic background. This pleiotropic expression has been observed for all microdeletions (at 1q21.1, 15q11.2, 15q13.3, 16p11.2, 16p13.11, 22q11.2, *A2PB1*, *NRXN1*) that have been associated with IGE or other neurodevelopmental disorders [48]. Most likely, these microdeletions impair neurodevelopmental processes in a rather unspecific manner and contribute to the genetic variance of a wide spectrum of neurodevelopmental disorders. The individual disease phenotype is probably further specified by the interplay with genetic background effects and environmental influences following an oligo-/polygenic inheritance model with substantial genetic heterogeneity [5]. In particular, bi-parental genetic risk factors may contribute to the epilepsy phenotype of the index subject. Accordingly, segregation analysis in Family 2 suggests a complex bi-parental inheritance pattern composed by a paternal inheritance of the *GPHN* Δ2-3 deletion and a predominant epileptogenic susceptibility factor(s) transmitted by the mother, who had afebrile seizures during infancy. Likewise, the brother showing a diploid *GPHN* copy status had a history of febrile

seizures. Similar inheritance patterns were also reported earlier for exonic *GPHN* microdeletions [13] and other recurrent microdeletions increasing risk for IGE [42,43]. As we did not observe metabolic Moco deficiency symptoms, the hemizygous microdeletions of N-terminal *GPHN* exons presumably affect only the synaptic functions of gephyrin. However, it cannot be excluded that minor alterations in the Moco-enzymes activity during brain development might contribute to disease manifestation.

In summary, our study strengthens a previous statistical association of *GPHN* microdeletions affecting the gephyrin G-domain with a wide range of neuropsychiatric disorders and expands this spectrum to IGE syndromes [13]. *GPHN* microdeletions affecting the N-terminal gephyrin domain confer a susceptibility effect with high phenotypic variability and incomplete penetrance depending on the genetic background. We provide genetic, functional and neurophysiological evidence that structural exonic microdeletions affecting the gephyrin G-domain can increase neuronal excitability by an impairment of GABAergic synaptic inhibition, and thereby confer susceptibility for IGE.

SUPPLEMENTARY DATA

Further data are provided as Supplementary Material.

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FIGURE LEGENDS

Figure 1. Genetic analyses of *GPHN* microdeletions

(A) Genomic localization (hg19) and *GPHN* deletions identified in the IGE index patients. The deletion segregating in Family 1 is spanning approximately 129 kb involving exons 5-9. The microdeletion of Family 2 is approximately 158 kb in size and deletes exons 2-3. (B) Familial segregation of exonic *GPHN* microdeletions. Deletion carriers are indicated by copy number state (CN) = 1, whereas CN = 2 represents an analyzed individual without deletion; black symbols = affected by IGE; TMS path.= increased cortical excitability without seizures; arrows highlight index cases. (C) Electropherogram of the cDNA Δ5-9 *GPHN* transcript sequence of the index patient F1II.1 and his father F1I.1, confirming the transcription of a truncated Δ5-9 *GPHN* transcript. Arrow separates the sequence of exon 4 from the fused adjacent sequence of exon 10. The exon annotation refers to the genomic organization of *GPHN* (NM_020806.4). (D) Electropherogram of the cDNA Δ2-3 *GPHN* transcript sequence of the index patient F2II.2, confirming the transcription of a truncated Δ2-3 *GPHN* transcript (NM_020806.4). Arrow separates the sequence of exon 1 from the fused adjacent sequence of exon 4. (E) Schematic structure of regular gephyrin and Δ5-9geph with numbering of deleted residues in Δ5-9geph. (F) Sequence of the truncated Δ2-3 *GPHN* transcript expressed by father and son F2II.2. Residue Val22 is encoded by a splitted codon composed by exons 1 and 2; the *GPHN* Δ2-3 variant results in a frameshift and a premature stop codon (p.V22Gfs*7, NP_001019389.1).

Figure 2. Paired-pulse transcranial magnetic stimulation

The relative variation of the average peak-to-peak amplitude of the motor evoked potential in relation to the baseline (set at 100%), is plotted as a percentage, for the conditions “intracortical inhibition” (ISI 3 ms), white bars; and “intracortical facilitation” (ISI 13 ms), black bars. Both the patient and his father exhibited an increased cortical excitability, with decreased ICI and increased ICF, statistically significant compared to healthy controls (***$P < 0.001$, ANOVA).

Figure 3. Δ5-9geph interacts with regular gephyrin and affects oligomerization in a dominant-negative manner.

(A) Morphology and localization of GFP-tagged regular gephyrin and Δ5-9geph in cultured hippocampal neurons. Insets show high-magnification of the dendrites. Arrowheads point to spine heads, morphological structures of excitatory synapses. Scale bar = 20 µm. (B) Myc-tagged gephyrin forms large “blobs” upon expression in HEK293 cells, while Δ5-9geph-GFP is diffusively distributed. Upon co-expression, Δ5-9geph-GFP dominant negatively affected gephyrin oligomerization leading to either completely diffuse regular gephyrin (C1) or the formation of microclusters harboring regular and truncated gephyrin-variants (C2). (D) Quantification of gephyrin blobs and diffuse gephyrin in 100 cells of each condition derived from three independent transfections. (E) Immunoblots of GFP-tagged gephyrin and Δ5-9geph expressed in HEK293 cells indicate that the truncated gephyrin is stable and expressed at equivalent levels to regular gephyrin. ß-tubulin served as loading control. (F) Δ5-9geph-GFP immunoprecipitated with myc-tagged gephyrin in HEK293 cell-lysates following co-expression of both proteins.

Figure 4. GABAergic innervation is decreased upon expression of Δ5-9geph-GFP

(A) Schematic structure of gephyrin and the epitope of the rGeph-C antibody, which was used to distinguish between endogenous gephyrin and Δ5-9geph. Representative dendrites (20 µm) of EGFP- or Δ5-9geph-expressing cultured hippocampal neurons (11+2 DIV) immunostained for endogenous gephyrin (B), $GABA_AR$ γ_2 subunit (E) or VGAT (H). (B-G) Δ5-9geph-GFP significantly reduced number and size of endogenous gephyrin clusters and $GABA_A$ γ2-containing receptors. (H-J) Morphology and numbers of spine heads were not affected by Δ5-9geph-GFP. Arrowheads point to exemplary spines that have been quantified. Scale bar = 10 µm. All data are expressed as mean ± SEM (***P* < 0.05; ****P* < 0.001, Student's *t*-test; n = 14-18 neurons per condition from two independent preparations).

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APPENDIX

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Figures

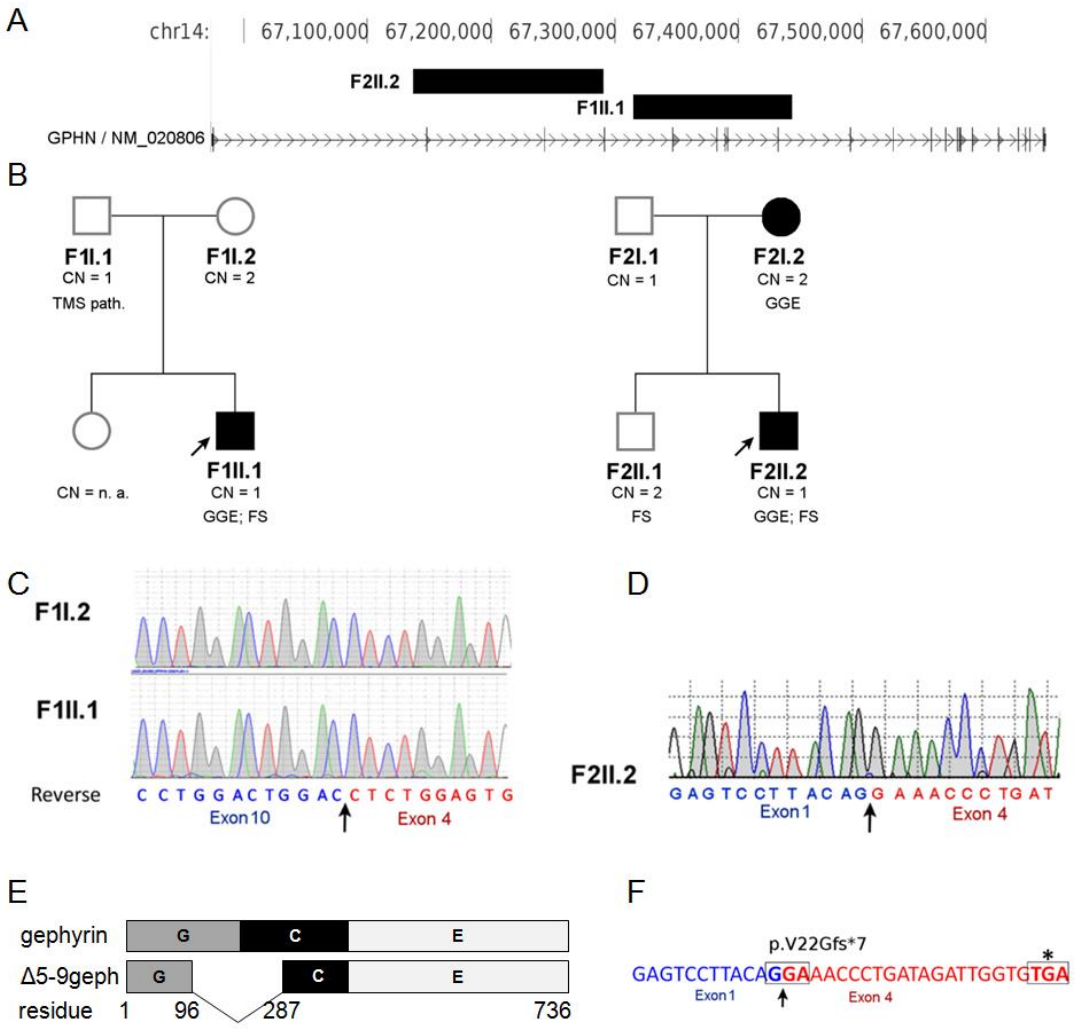

Figure 1
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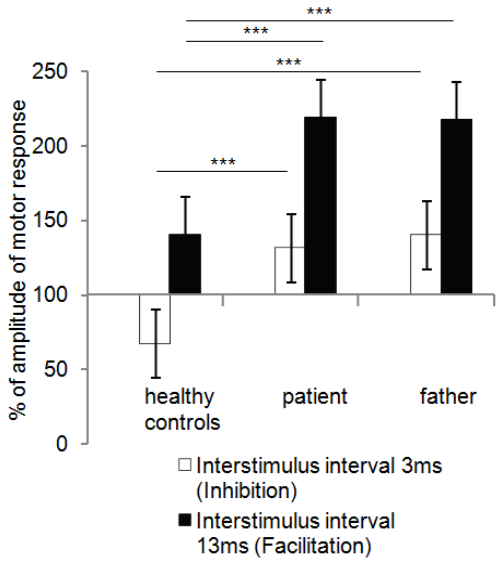

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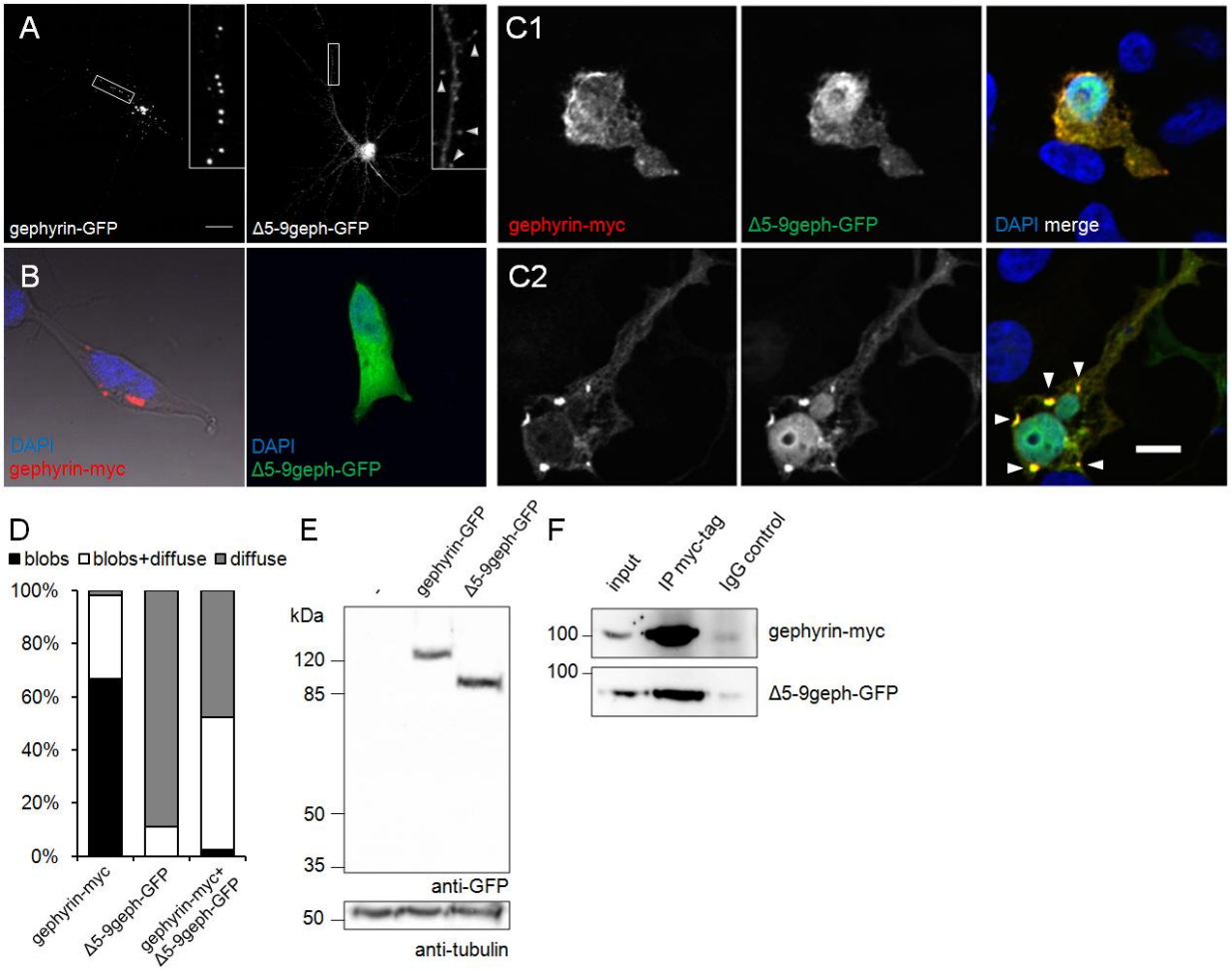

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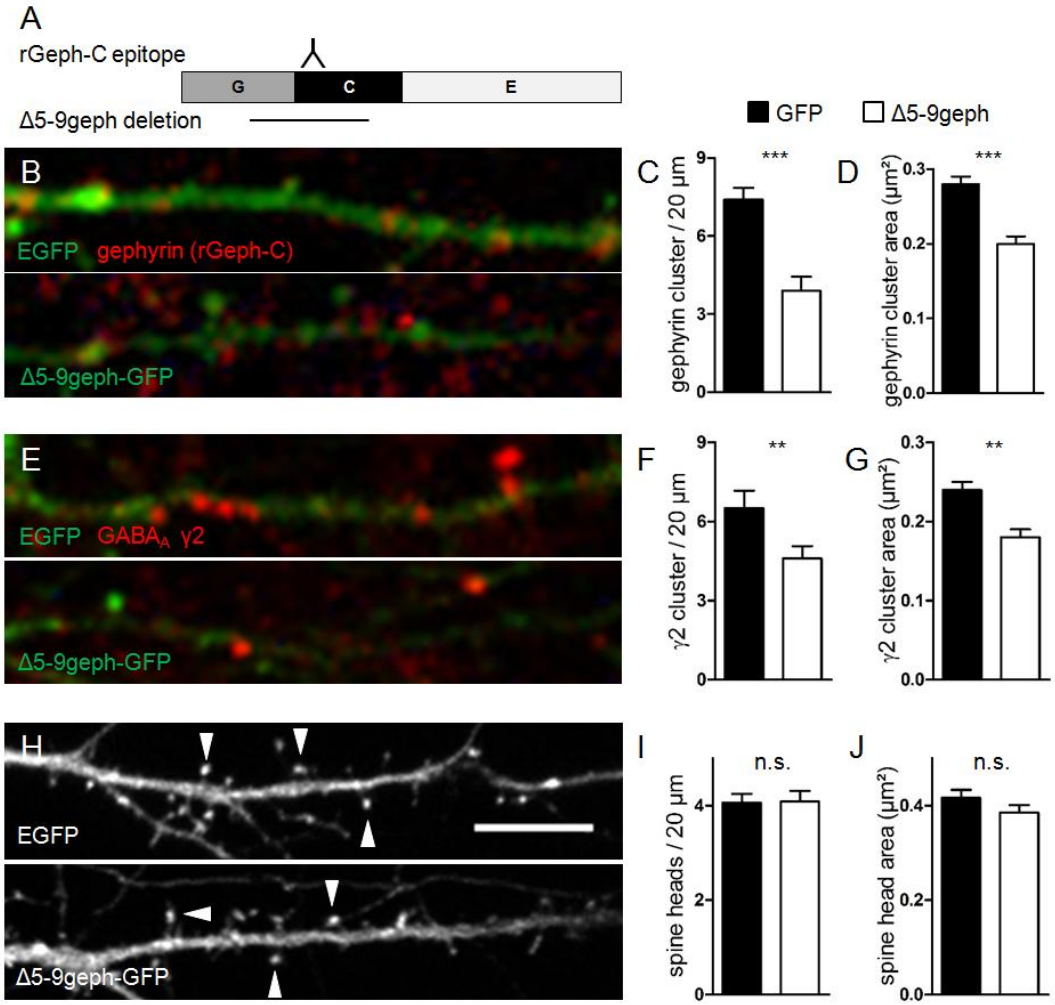

Figure 4
Dejanovic et al.

SUPPLEMENTARY INFORMATION

Exonic microdeletions of the gephyrin gene impair GABAergic synaptic inhibition in patients with idiopathic generalized epilepsy

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EPICURE Consortium

* Authors contributed equally

Equal senior authorship

FIGURES

Figure S1. Genetic analyses (A) Familial segregation of exonic *GPHN* microdeletions. Deletion carriers are indicated by copy number state (cn) = 1, whereas cn = 2 represents an analyzed individual without deletion; black symbols = affected by IGE; TMS path.= increased cortical excitability without seizures; arrows highlight index cases. (B) Genomic localization (hg19) and *GPHN* deletions identified in the IGE index patients. The deletion segregating in Family 1 is spanning approximately 129 kb involving exons 5-9. The microdeletion of Family 2 is approximately 158 kb in size and deletes exons 2-3. (C) Electropherogram of the cDNA Δ5-9 *GPHN* transcript sequence of the index patient F1II.1 and his father F1I.1, confirming the transcription of a truncated Δ5-9 *GPHN* transcript. Red coloring indicates the sequence of exon 4 whereas the sequence of exon 10 is highlighted in blue. The exon annotation refers to the genomic organization of *GPHN* (NM_020806.4). (D) Schematic structure of regular gephyrin and Δ5-9geph with numbering of deleted residues in Δ5-9geph. (E) Electropherogram of the cDNA Δ2-3 *GPHN* transcript sequence of the index patient F2II.2, confirming the transcription of a truncated Δ2-3 *GPHN* transcript (NM_020806.4). Red coloring indicates the sequence of exon 4 whereas the sequence of exon 1 is highlighted in blue. (F) Sequence of the truncated transcript (see panel E) expressed by father and son F2II.2. The arrow highlights the fusion of exon 1 and 4. Residue Val22 is encoded by a splitted codon composed by exons 1 and 2; the *GPHN* Δ2-3 variant results in a frameshift and a premature stop codon (p.V22Gfs*7, NP_001019389.1).

Figure S2. Family 1 (*GPHN* Δ5-9 deletion). Assessment of the copy number state by TaqMan qPCR assay located in *GPHN* exon 7 (Assay ID: Hs01711518_cn; Life Technologies, Carlsbad, CA, USA). The copy number state of 1 (CN = 1) indicates a hemizygous deletion, whereas a CN = 2 represents the normal diploid CN state.

Figure S3. Blood cell cDNA amplification with primers flanking the *GPHN* Δ5-9 deletion in Family 1. Left: Agarose gel electrophoresis illustrates the expression of the truncated Δ5-9 *GPHN* transcript in F1II.1 (index-patient) and F1I.1 (father). Right: Analysis of the same cDNA by more sensitive Agilent 2200 TapeStation Nucleic Acid

System indicates also the presence of the 793 bps wt transcript in father and patient. Targeted band sizes are highlighted by asterisks; green = 793 bps wt transcript; red = 125 bps truncated Δ5-9 *GPHN* transcript.

Figure S4. Blood cell cDNA amplification with primers flanking the *GPHN* Δ2-3 deletion in Family 2. Using the sensitive Agilent 2200 Tape Station Nucleic Acid System, the 544 bps wt transcript of the G-domain is present in all cDNAs analyzed. The truncated *GPHN* Δ2-3 transcript of 407 bps was observed in the father (F2I.1) and the index patient (F2II.2).

Figure S5. Diffusion tensor imaging of *GPHN* Δ5-9 deletion carriers of Family 1. Results of the probabilistic tractography from the mesial frontal region in healthy controls (blue) compared to the index patient and his father (yellow/orange), overlaid on the MNI 152 T1 template image in three orthogonal slices (MNI coordinates given in figure). The connectivity reaches beyond the range seen in healthy controls, both in the index-patient and his father.

Figure S6. Validation of the rGeph-C antibody. Δ5-9geph was transiently expressed in HEK293 cells and immunostained either with the commercial mouse monoclonal 3B11 antibody (top panels), that binds to the E-domain or rabbit monoclonal antibody here termed rGeph-C (lower panels), whose epitope lies within the C-domain that is deleted in Δ5-9geph. The antibody only recognizes endogenous gephyrin but not Δ5-9geph. Scale bar = 25 µm.

Figure S7. Representative images of cultured hippocampal neurons expressing either EGFP or Δ5-9geph. Framed dendritic segments are shown at higher magnification in Figure 3. Scale bar = 20 µm.

Figure S8. Urinary metabolite analysis of samples derived from the patient (P), his mother (M) and father (F), control and Moco deficient individuals (MoCD), who were

previously diagnosed (1). All concentrations of metabolites were normalized to creatinine concentration in urine samples.

Figure S9. Genomic localization (hg19) of the *GPHN* microdeletions identified in the IGE index-patients in this study (dark grey) and six patients with autism, schizophrenia and seizures recently reported by Lionel et al. (light gray) (2).

MATERIALS AND METHODS

Exome sequencing

Sequence analysis of the whole exome was performed in the IGE proband and both parents using next generation sequencing techniques as described previously (3). The exome data had an average coverage >30x for 78-79% of the exome target sequences. The *GPHN* gene an average coverage of 49-55x with a minimal coverage for an exon of 16x (ENST00000478722, NM_020806.4). Variants were filtered for high-quality novel *GPHN* variants compared to an in-house variation database (>400 exomes, dbSNP build 135 (www.ncbi.nlm.nih.gov/projects/SNP/), 1000Genomes database (www.1000genomes.org/) and the Exome Variant Server (http://evs.gs.washington.edu/EVS/).

Diffusion tensor imaging

Data was acquired in Family 1 for the index-patient, his father and 15 healthy controls with no personal history of seizures or other neurological disorders and no medication (7 female, mean age 34 years, range 22-54 years). The mother could not tolerate any MRI studies due to claustrophobia. Images were recorded on a 3T GE Excite HDx scanner using an acquisition scheme with 64 diffusion weighted directions (b-value 1,000 s/mm^2) and one B0 image with a slice thickness of 2.4 mm and 96 x 96 voxels in plane resolution, a TE of 87 ms and TR of 16,000 ms. Images were processed using FSL 4.1.9 software (www.fmrib.ox.uk), estimating a two fibre per voxel model. Probabilistic tractography was performed as recently described (4) seeding from a region of interest (ROI) in the left mesial frontal lobe and normalizing the resulting tracts to a common acquisition specific template in MNI space.

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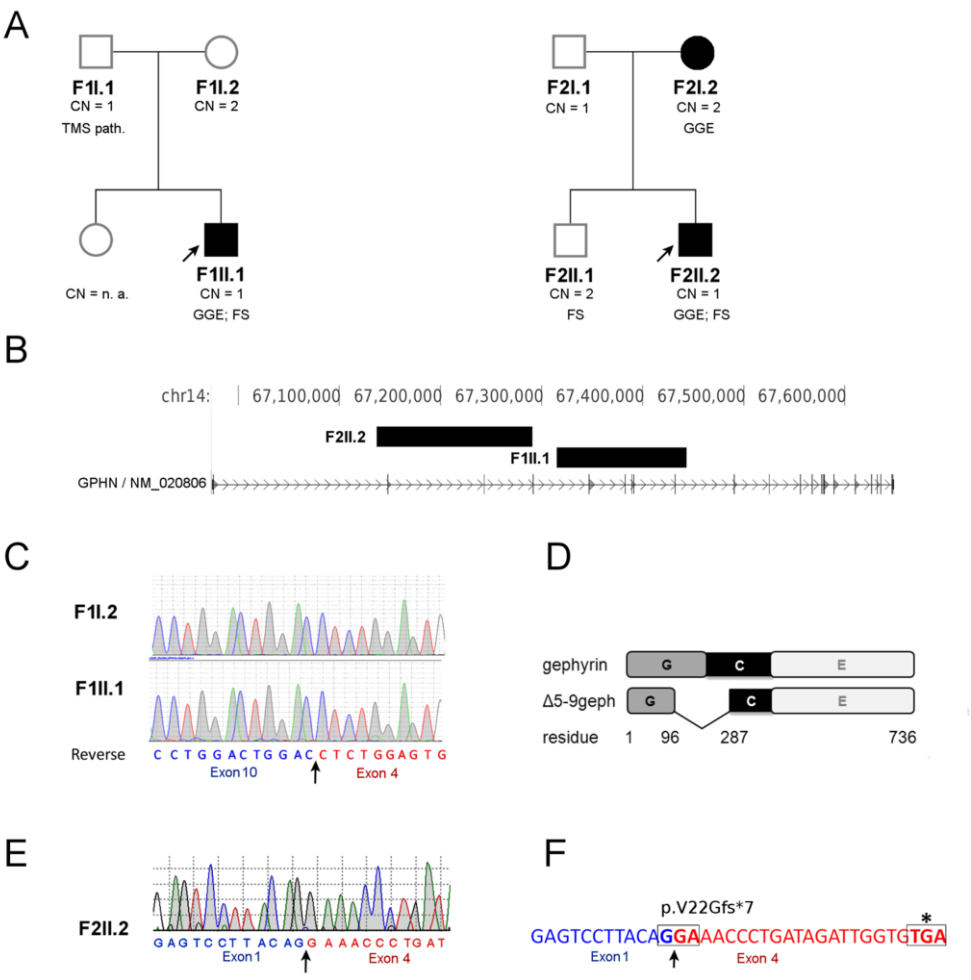
Figure 1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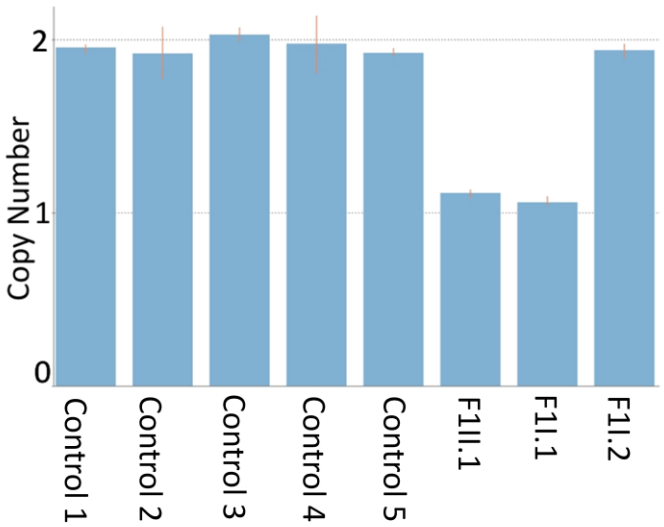

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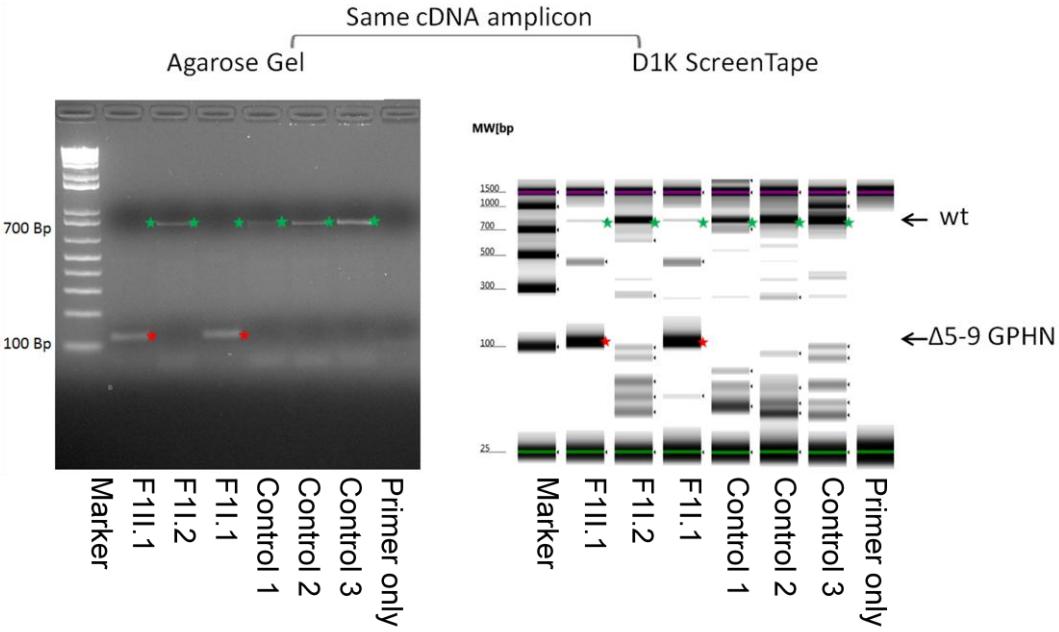

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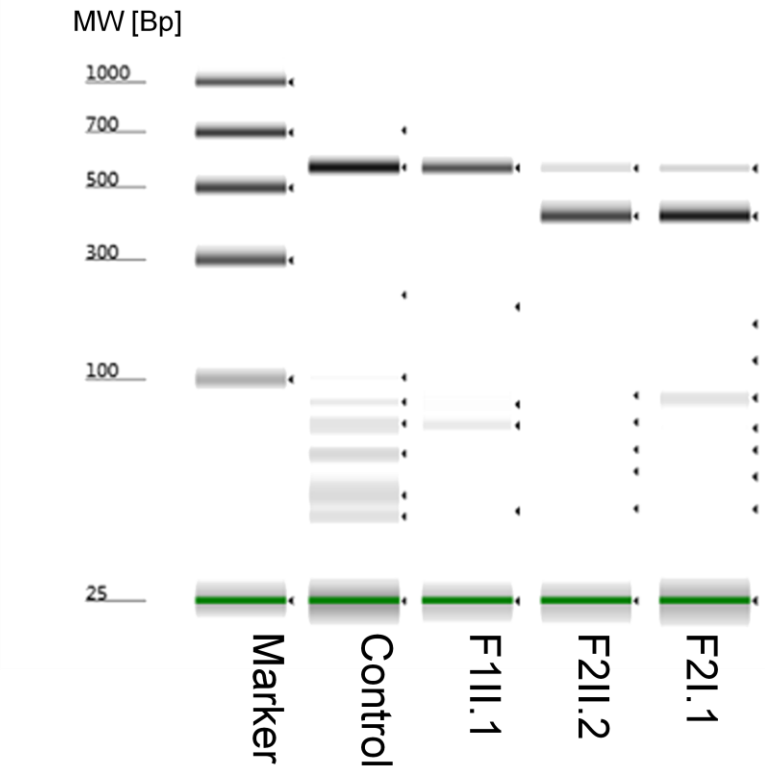

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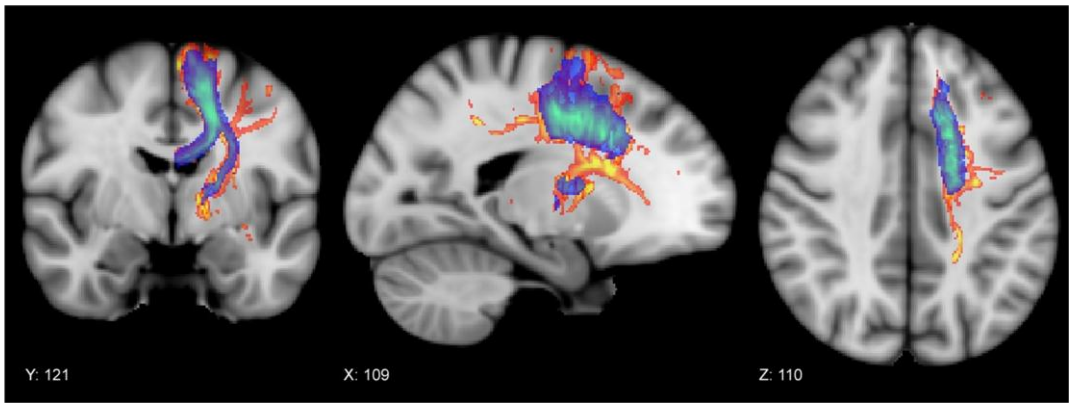

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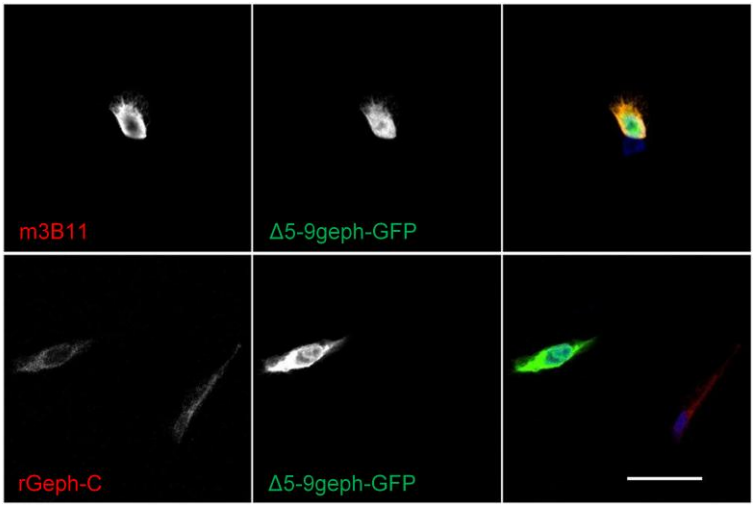

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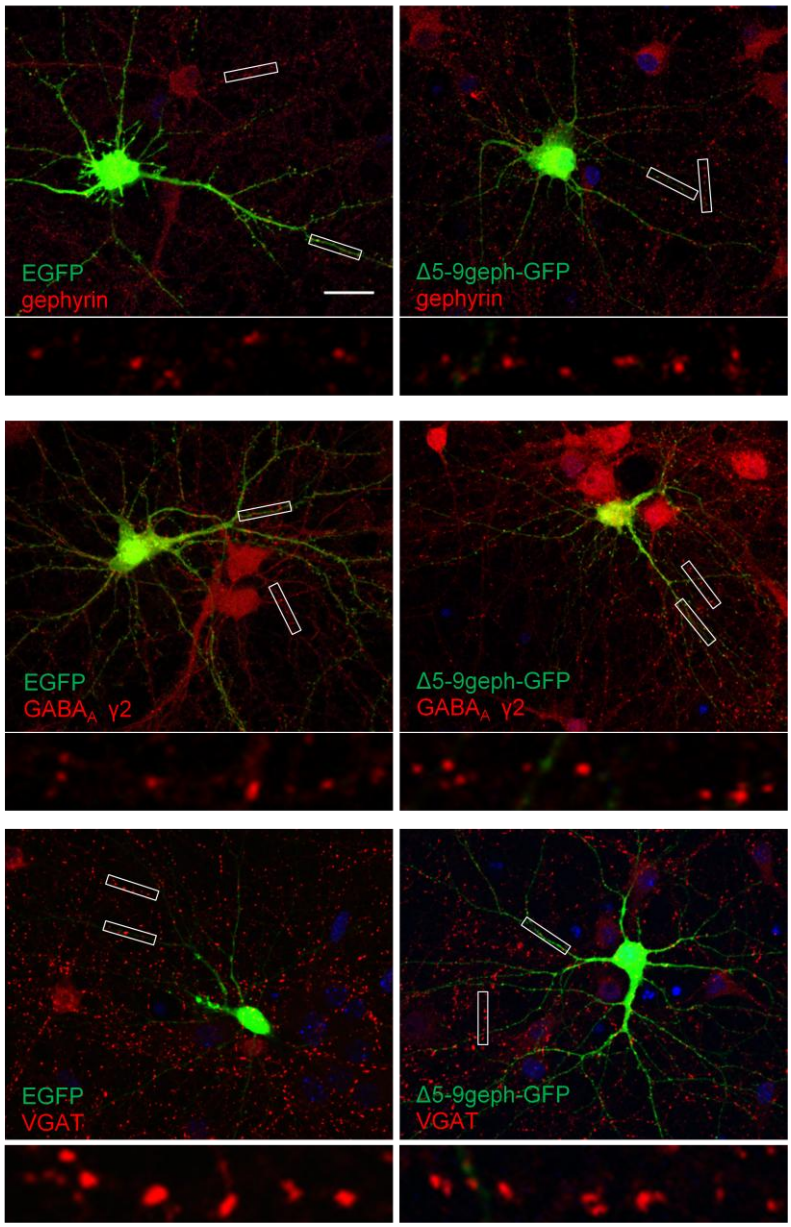
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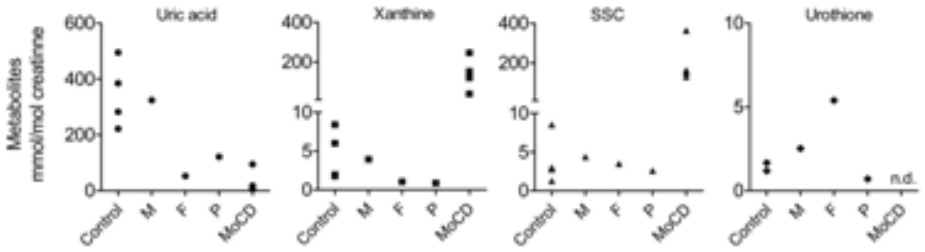
Figure S8



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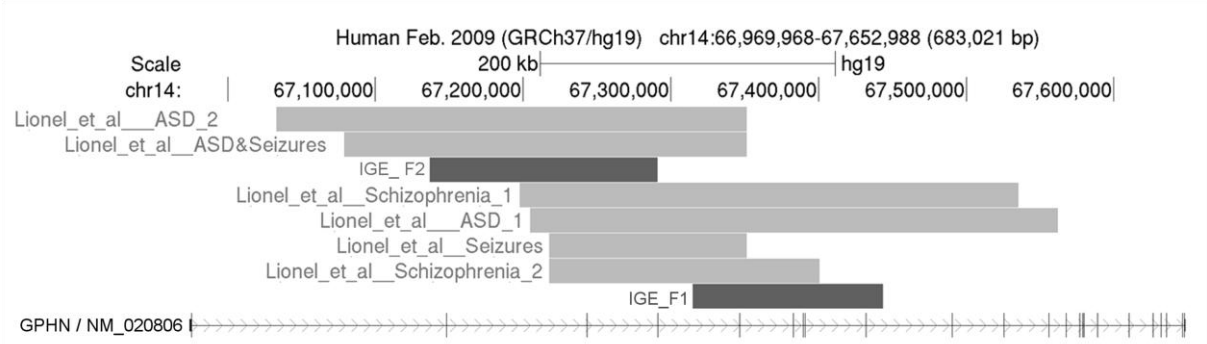

3.2 Publications on RE

3.2.1 *RBFOX* genes in RE (published)

Lal D, Reinthaler EM, Altmüller J, Toliat MR, Thiele H, et al. (2013) RBFOX1 and RBFOX3 Mutations in Rolandic Epilepsy. **PLoS ONE** 8: e73323. doi:10.1371/journal.pone.0073323.

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PLOS ONE

RBFOX1 and *RBFOX3* Mutations in Rolandic Epilepsy

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Abstract

Partial deletions of the gene encoding the neuronal splicing regulator *RBFOX1* have been reported in a range of neurodevelopmental diseases, including idiopathic generalized epilepsy. The RBFOX1 protein and its homologues (RBFOX2 and RBFOX3) regulate alternative splicing of many neuronal transcripts involved in the homeostatic control of neuronal excitability. In this study, we explored if structural microdeletions and exonic sequence variations in *RBFOX1*, *RBFOX2*, *RBFOX3* confer susceptibility to rolandic epilepsy (RE), a common idiopathic focal childhood epilepsy. By high-density SNP array screening of 289 unrelated RE patients, we identified two hemizygous deletions, a 365 kb deletion affecting two untranslated 5′-terminal exons of *RBFOX1* and a 43 kb deletion spanning exon 3 of *RBFOX3*. Exome sequencing of 242 RE patients revealed two novel probably deleterious variants in *RBFOX1*, a frameshift mutation (p.A233Vfs*74) and a hexanucleotide deletion (p.A299_A300del), and a novel nonsense mutation in *RBFOX3* (p.Y287*). Although the three variants were inherited from unaffected parents, they were present in all family members exhibiting the RE trait clinically or electroencephalographically with only one exception. In contrast, no deleterious mutations of *RBFOX1* and *RBFOX3* were found in the exomes of 6503 non-RE subjects deposited in the Exome Variant Server database. The observed *RBFOX3* exon 3 deletion and nonsense mutation suggest that *RBFOX3* represents a novel risk factor for RE, indicating that exon deletions and truncating mutations of *RBFOX1* and *RBFOX3* contribute to the genetic variance of partial and generalized idiopathic epilepsy syndromes.

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Introduction

Rolandic epilepsy (RE), or benign epilepsy with centrotemporal spikes (BECTS), is one of the most common epilepsy syndromes of childhood, comprising about 15% of epilepsies in children under the age of 16 years [1]. Age of onset ranges from years 3 to 13 and peaks between 8–9. Characteristic features are 1.) a somatosensory onset with unilateral paresthesias involving the tongue, lips, gums, and inner cheeks 2.) unilateral, tonic, clonic or tonic-clonic convulsions involving the face, lips, tongue as well as the pharyngeal and laryngeal muscles, causing 3.) speech arrest and drooling due to sialorrhea and saliva pooling. At this stage the seizure may end, or it may develop into a generalized tonic clonic seizure. Nocturnal seizures, the most frequent variant of this syndrome, frequently become generalized. The electroencephalographic hallmark, a prerequisite of diagnosis, are blunt high-voltage characteristically shaped centrotemporal spikes (CTS), often followed by slow waves [2,3]. Based on a number of family studies it is generally assumed that, like in all other common idiopathic epilepsies a multifactorial mode of inheritance appears most likely [2,4,5]. The most recent family study on RE reported an increased rate of RE, febrile seizures, and an "epilepsy aphasia spectrum disorder" in relatives of children with RE [6]. To date, a number of loci or genes have been linked to various forms of idiopathic focal epilepsies or the EEG endophenotype of centrotemporal spikes (CTS). Linkage was reported to markers on 15q13.2 and to 16p12-11.2 [7,8]. But no causative gene has been reported at either loci yet. In a small number of cases variants and mutations in *KCNQ2* and *KCNQ3* were found to be associated with RE and the respective EEG trait [9]. In addition, mutations in *SRPX2* in two families with mental retardation, severe language dysfunction and rolandic seizures have been reported [10]. By genome-wide linkage analysis RE and the CTS trait have been associated with the elongator protein complex 4 [11]. Furthermore, in a girl with early-onset epileptic encephalopathy and CTS a *de novo GRIN2A* mutation was identified [12]. However, all these findings in single or few patients or small families still lack replication.

Rare copy number abnormalities of *RBFOX1* have also been associated with mental retardation in comorbidity with and without seizures, attention deficit disorder and autism [13,14,15,16]. Recently, we have shown in 1408 idiopathic

generalized epilepsy (IGE) patients and 2256 population controls that deletions affecting 5′ located exons of *RBFOX1* are significantly enriched in IGE cases compared to population matched controls [17]. The *RBFOX* genes (*RBFOX1*: chr16:6069132-7763340, NM_001142333; *RBFOX2*:, chr22:36134783-36424585, NM_001082578, and *RBFOX3*: chr17:77085427-77512230; NM_001082575) encode neuron-specific splicing factors predicted to regulate neuronal splicing networks. Several epilepsy candidate genes are downstream targets of Rbfox proteins (*FLNA, SLC1A3, DCX, GABRB3, GAD2, KCNQ2, SCN8A, SLC12A5, SV2B, SYN1*) and their regulation of expression and splicing has been demonstrated [18,19]. FOX family members regulate splicing of the other FOX members and autoregulate themselves [20,21].

The present candidate gene analysis tested whether (i) deletions in *RBFOX1, RBFOX2* and *RBFOX3* might increase risk of RE and (ii) exonic mutations affecting the protein structure occur more frequently in RE compared to control subjects.

Methods

The institutional review board of the University Giessen, Germany specifically approved this study. Registration number: No 03/11. Written informed consent was obtained from all subjects or their legal guardians according to study protocols approved by the institutional ethical review board of the University Giessen, Germany under the title "genomic variation in patients with idiopathic epilepsy".

Diagnostic Criteria

Diagnosis of RE was performed according to the International Classification of Seizures and Epilepsies as described [3]. Sleep activation, characteristic shape, and classification by two independent individuals were required for classification of the EEG trait. Electrical Status Epilepticus in slow sleep (ESES) was diagnosed if prolonged generalized discharges of CTS dominated sleep EEG recordings [22,23,24]. Atypical benign partial epilepsy of childhood (ARE) was diagnosed employing the following criteria: Characteristic EEG trait of CTS, however, with trains of continuous generalized nocturnal discharges as a prerequisite of diagnosis in all ABPE cases. In addition at least one of the following two features needed to be present: (1) seizures compatible with BECTS plus one or more additive seizure types like astatic seizures, atypical absences ("dreamy states") or myoclonic seizures as reported. (2) seizures compatible with BECTS plus a significant mental handicap, and/or severe developmental speech disorder [25,26,27]. If a child had a seizure symptomatology compatible with BECTS but had prolonged generalized discharges of CTS during sleep EEG, without any additional seizure types and a normal global and speech development it was diagnosed as BECTS.

Patient Cohort

Families. The investigated cohort consisted of 98 index cases selected from 98 multiplex families with at least two affected siblings. In 96 families at least one of the affected probands suffered from RE or ARE, the second affected sibling presented either with RE, ARE or the EEG trait only. In two families all affected children did not suffer from seizures, but displayed the EEG trait only. Of all 98 index patients tested, 78 presented with RE, 15 with ARE, 3 with ESES, and 2 with CTS only.

Sporadic cases. 191 non-familial cases were included into the study cohort. Of these patients 153 suffered from classic RE, 11 from ESES, 26 from ARE and 1 with CTS only.

Genotyping and Copy Number Variation Detection

Whole blood DNA from the patients was genotyped for using the Infinium OmniExpressExome BeadChip (Illumina Inc., San Diego, CA) according to the manufacture's protocol. Briefly, 200 ng of DNA were amplified, biotin labeled, and hybridized to the microarray. CNV calls were generated with the PennCNV software [28], using the log R ratio (LRR) and B allele frequency (BAF) for 730.525 probes designed for the genotyping array. CNV analysis was restricted to microdeletions covered by at least 20 probes and spanning 40 kb or more in size. To exclude technical artifacts, all potential microdeletions were manually inspected for regional SNP heterozygosity state and log2 ratios of the signal intensities using the Illumina Genome Viewer (Illumina Inc., San Diego, CA). Only one *RBFOX1* deletion and one *RBFOX3* deletion could be confirmed by manual variant evaluation. Segregation in the families of the microdeletions was examined by real-time quantitative PCR using TaqMan CNV probes (*RBFOX1:* Hs04461212_cn; *RBFOX3:* Hs03975574_cn) (Life Technologies, Darmstadt, Germany).

Exonic Sequence Analysis

Sequence analysis was performed using next generation sequencing techniques. In brief, DNA was fragmented using sonification technology (Covaris, Woburn, MA, USA) and fragments were end repaired and adaptor ligated. SeqCap EZ Human Exome Library® v2.0 (Roche NimbleGen, Madison, WI, USA) was used for enrichment and samples were analyzed on the Illumina HiSeq 2000® sequencer. For 242 patients exome data were generated which featured an average coverage >30x for 77% of the target sequences. Data were filtered using Illumina Realtime Analysis® (RTA) software v1.8 and mapped to the 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 unknown variants in *RBFOX1, RBFOX2,* and *RBFOX3* by comparison to an in-house variation database, dbSNP build 137 (www.ncbi.nlm.nih.gov/projects/SNP/), 1000 Genomes database (www.1000genomes.org/), and the Exome Variant Server (http://evs.gs.washington.edu/EVS/). Variant validation and segregation analyses were performed by Sanger sequencing following standard protocols.

Results

Detection of *RBFOX1* and *RBFOX3* Microdeletions in RE Patients

In total, 289 RE patients were screened for copy number variations in *RBFOX1, RBFOX2,* and *RBFOX3* using the Infinium OmniExpressExome BeadChip® (Illumina Inc., San Diego, CA). We identified one RE patient among 289 (0.34%) with a hemizygous deletion in both *RBFOX1* and *RBFOX3*. A deletion of 365 kb was found to be located in the genomic region of *RBFOX1* affecting the untranslated 5′-terminal exons 3 and 1B (Fig. 1A, S1, exon annotation according to [17]) whereas a smaller deletion of 43 kb affected *RBFOX3* by removing exon 3 of the known isoform NM_001082575 along with flanking intronic sequences (Fig.1B, S1). The *RBFOX1* gene deletion affects the largest transcript variants 4–5 and 6 (NM_018723, NM_001142333, NM_001142334). No additional mutations were

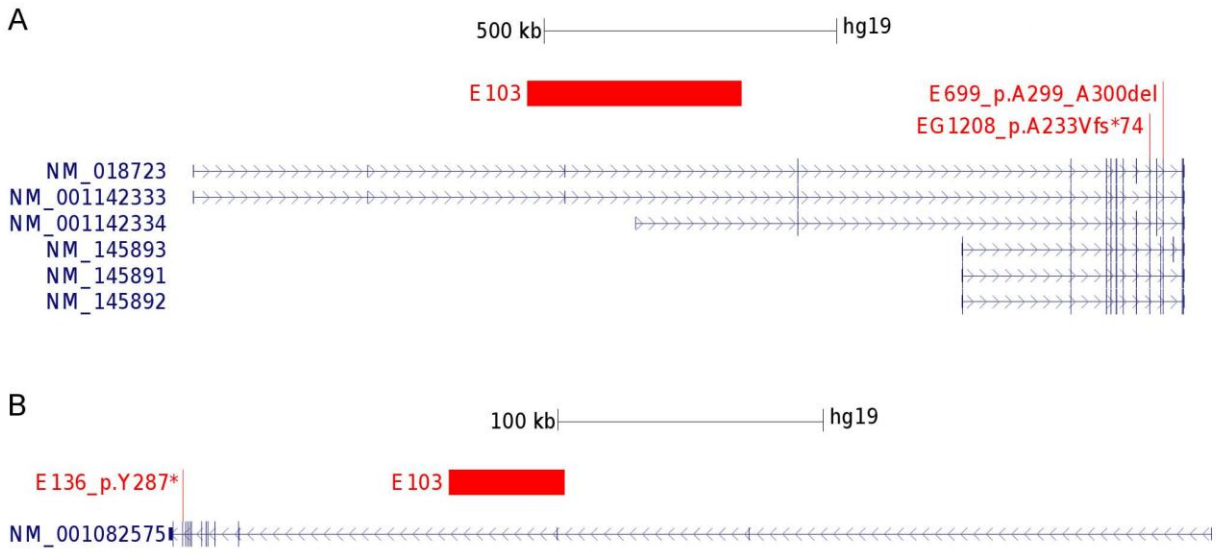

Figure 1. Overview of *RBFOX1* and *RBFOX3* affecting variants. Hg19 genomic localization and overview of known transcript variants. Red bars represent microdeletion size and location for the patient. Red dashes indicate the genomic location of single nucleotide variants. (A) Overview *RBFOX1* (B) *RBFOX3*.
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identified in the remaining undeleted *RBFOX1* and *RBFOX3* exonic sequences.

Detection of Rare Exonic Variants in *RBFOX1*and *RBFOX*3

Mutational screening of *RBFOX1*, *RBFOX2*, *RBFOX3* in 242 RE patients did not reveal any mutation in *RBFOX2*, while a total of three rare mutations (1.2%) were identified in *RBFOX1* and *RBFOX3* (Fig. 1A,1B; Table 1). In contrast to the identified *RBFOX1* and *RBFOX3* microdeletions, all exonic variants were located near the 3′-terminal region of both genes. The identified exonic variants included two *RBFOX1* variants in exon 11 (c.690_696delGTATCCAins(GTATCCA)2; p.A233Vfs*74, NM_001142333) and exon 13 (c.893_898delCTGCCG, p.A299_A300del, NM_001142333), as well as a nonsense mutation in exon 13 of the *RBFOX3* gene (c.861C>A, p.Y287*, NM_001082575). The p.A299_A300del variant of patient E699 (Table 1, S1) deletes two out of three consecutive alanine residues which are conserved among mammals but not vertebrates (Fig. S2, S3).

To our knowledge nonsense mutations in *FOX* genes have not been described in the literature and are not found in any of the available databanks. They are absent from 6503 individuals whose exomes were deposited in the EVS database. Furthermore, no deleterious mutations have been identified in >450 exomes of our in-house database (various non epilepsy projects; about 80% Caucasian ancestry). In the truncated proteins the C-terminal fragments of RBFOX1 and RBFOX3 are affected which are critical for cassette-exon activation and repression [29] and the nuclear localization of the FOX proteins [21,30,31].

Familial Segregation and Comorbidity Analysis

The segregation of *RBFOX1* and *RBFOX3* variants identified in the RE index-patients were tracked in four families (Fig. 2). Where testing was possible (n = 3), all variants identified were inherited, one maternally and two paternally. Five out of ten variant carriers were affected by RE, one by an encephalopathy with status epilepticus during sleep (ESES) and one by the RE-characteristic CTS EEG-trait only. The transmitting parents were all unaffected at the date of evaluation. Notably, we cannot rule out that the parents might have expressed the RE-characteristic CTS EEG-trait at younger age, considering its age-related expression with maximum manifestation during childhood. Six out of eight family members affected by either RE, CTS or ESES carried one exonic variant of *RBFOX1* or *RBFOX3* and one RE patient had a deletion in both genes (Fig. 2). The index patient in Family 1 had RE and exon-removing microdeletions in both *RBFOX1* and *RBFOX3* whereas her sister with the CTS EEG-trait alone carried only the *RBFOX1* microdeletion. In Families 1, 2 and 4, all affected family members carried a *RBFOX1* deletion or one of the truncating mutations. In family 3, the RBFOX1 p.A299_A300del deletion identified in the RE-index patient was not present in his RE-affected sister. The phenotypic RE features of the four variant carrying index cases did not differ from those RE patients lacking *RBFOX*1 and *RBFOX3* variants (Table 1). The ESES phenotype variant is generally assumed to represent the most severe expression of the RE CTS EEG-trait. A mild to moderate developmental speech delay, that frequently resolves later, is a known feature in many RE patients [32].

Discussion

A previous study of 1408 unrelated individuals with idiopathic generalized epilepsy revealed exon-removing *RBFOX1* microdeletions in five patients, whereas none was found in 2256 ethnically matched controls [17]. Furthermore, rare copy number abnormalities of *RBFOX1* have been reported for patients with neurological diseases like epilepsy, mental retardation and autism [13,14,15,16] strongly indicating that partial *RBFOX1* deletions are a recurrent risk factor of neurodevelopmental defects in human. The heterozygous *Rbfox1* knockout mouse model shows deregulated splicing which impacts genes involved in synaptic transmission and membrane excitability, leading to an increased susceptibility for seizure events. Notably, homo- and heterozygous *Rbfox1* knockouts display normal brain morphology [33]. Consistent with the splicing alterations in mice, RNA interference-

Table 1. *RBFOX1and RBFOX3* variants and phenotype of index-patients.

Family	Index-patient	Patient	Variant	Epilepsy Syndrome	Diagnosis/seizure types	Comorbidity
1	x	E103	RBFOX1:365kb Deletion, RBFOX3:43 kb Deletion	RE	Nocturnal generalized tonic clonic seizures, Postictal speech arrest	None (normal global development, normal speech acquisition)
1		E103b	RBFOX1:365 kb Deletion	CTS only	No seizures, EEG trait only	None (normal global development, normal speech acquisition)
2	x	EG1208	RBFOX1: p.A233Vfs*74	RE	Nocturnal rolandic seizures with postictal speech arrest	Initially delayed language development, later normal
2		EG1209	RBFOX1: p.A233Vfs*74	RE	Nocturnal and diurnal rolandic seizures with postictal speech arrest	Initially delayed language development, later normal
3	x	E699	RBFOX1: p.A299_A300del	RE	Nocturnal rolandic seizures with postictal speech arrest	None (normal global development, normal speech acquisition)
3		E699b	-	RE	Nocturnal rolandic seizures with postictal speech arrest	None (normal global development, normal speech acquisition)
4	x	E136	RBFOX3: p.Y287*	RE	Nocturnal generalized tonic clonic seizures with postictal speech arrest	Initially delayed language development, later normal
4		E679c	RBFOX3: p.Y287*	ESES	ESES without seizures	Moderate developmetal delay, delayed speech development, mild oral dyspraxia

Survey on *RBFOX1* and *RBFOX3* variants in patients. Seizure type and comorbidity overview of variant carrier. Abbreviations: RE = rolandic epilepsy; CTS = centrotemporal spikes; ESES = epileptic encephalopathy with status epilepticus during sleep.
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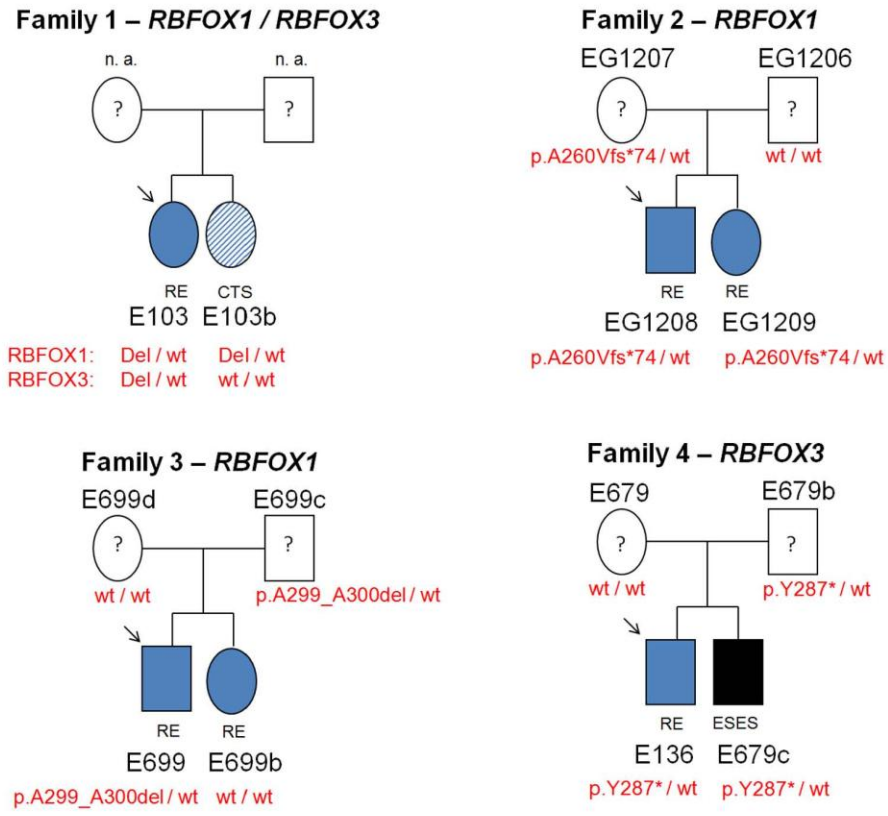

Figure 2. Segregation of *RBFOX1* and *RBFOX3* affecting variants. For three mutations for which DNA samples of family members were available, segregation analyses could be performed. The respective *RBFOX1* and *RBFOX3* truncating mutations co-segregated with a variable phenotype of either seizures or pathologic EEG patterns in most family members. Only a few individuals carried the respective familial mutation but did not present any clinical features, indicating incomplete penetrance of the mutations. However, subclinical phenotypes (e.g. EEG patterns) have not been investigated in these individuals (indicated by question mark). In family 3 the variant (deletion of two consecutive alanine residues at position 299–300 of RBFOX1) did not segregate with the epilepsy phenotype. Abbreviations: n.a = DNA was not available for testing; RE = rolandic epilepsy; CTS = centrotemporal spikes; ESES = encephalopathy with status epilepticus during sleep.
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mediated 50% knockdown of *RBFOX1* transcripts in human neurons changes the alternative splicing pattern and expression of primarily neuronal genes involved in synapse formation and function [19].

Our identification of a RE patient carrying a rare microdeletion affecting the 5′ part of *RBFOX1* replicates the association found in idiopathic epilepsy, but now in an entirely different, i.e. focal, epilepsy syndrome. Her sister, who also carried the deletion, exhibited the RE-specific EEG trait but did not suffer from overt seizures. In agreement with our previous IGE study, this indicates that *RBFOX1* microdeletions act as a susceptibility factor but not as a highly penetrant variant. The frequency of the *RBFOX1* deletion in the RE patient cohort (0.34%) is in the range of that observed for IGE (0.35%; [17]). The *RBFOX1* microdeletion presented here is located in the untranslated 5′-terminal region of the gene, like the previously reported microdeletions and translocations [13–17]. A female with autism carrying a 5′-terminal microdeletion of *RBFOX1* due to a *de novo* translocation t(15p;16p) displayed a significantly reduced *RBFOX1* mRNA expression in lymphocytes [14]. A second *FOX* microdeletion also detected in patient E103 is affecting the paralogous *RBFOX3* gene which also encodes the highly conserved RNA recognition motif (RRM) [20]. Interestingly, the sister of E103, who also carries the *RBFOX1* deletion but lacks the *RBFOX3* deletion, only expressed CTS but without having epileptic seizures. Due to the lack of further statistical and functional evidence, we can only hypothesize that both deletions affect neuronal splicing in Family 1 synergistically. Microdeletions in *RBFOX3* are extremely rare. Only two other exon-removing microdeletions have been noted in the Database of Genomic Variants (DGV, accessed 2/2013) in the general population. Being present in control individuals might indicate a variable expressivity of *RBFOX3* microdeletions or a lack of careful assessment of neuropsychiatric phenotypes in these probands. Both microdeletions do not delete exon 3 (NM_001082575) of *RBFOX3* as in our RE patient. It has been demonstrated that Rbfox3 regulates alternative splicing and nonsense mediated decay of *Rbfox2* mRNA [21]. This complex interplay of Fox family members has been further reported in the *Rbfox1* knockout mouse model where the loss of *Rbfox1* inhibits an upregulation of *Rbfox2* [33]. Mutational screening did not reveal any exonic mutation in *RBFOX2*, while three rare mutations have been identified (1.2%) in *RBFOX1* and *RBFOX3* together. The C-terminal fragment is critical for cassette-exon activation and repression [29] in *RBFOX1* as well as for nuclear localization for RBFOX1 [30,31] and RBFOX3 [21]. These mechanisms are likely to be affected by the observed mutations. Furthermore, no frameshift nor nonsense mutations have been found in the exomes of 6503 control subjects reported in the ESV database. All individuals with an epileptic phenotype were carriers of the truncating mutations in family 1, 2 and 4. Only in family 3 the variant (deletion of two consecutive alanine residues at position 299–300 of *RBFOX1*) did not segregate with the epilepsy phenotype. This deleted sequence is conserved among mammals only, suggesting that most likely only truncating variants may be risk-conferring for RE. *In vitro* studies demonstrated that the C-terminal domain is critical for cellular localization in both *FOX* genes and also for targeted splicing in *RBFOX1* [21,30,31]. As a model for haploinsufficiency *in vitro* knock down of *RBFOX1* in primary human neural stem cells resulted in an altered expression and splicing of several epilepsy candidate genes (*FLNA, SLC1A3, DCX, GABRB3, GAD2, KCNQ2, SLC12A5, SV2B, SYN1*) [19]. Interestingly, variants and mutations in *KCNQ2* were recently found associated with RE [9].

In summary, our results strengthen the association of partial *RBFOX1* deletions in neurodevelopmental diseases and extend the *RBFOX1*-related phenotypic spectrum by RE. The present *RBFOX3* mutations highlight this neuronal gene as a plausible novel genetic risk factor of RE, and suggest that besides genomic microdeletions affecting the 5′-terminal exons, truncating mutations of *RBFOX1* and *RBFOX3* increase the risk of RE.

Supporting Information

Figure S1 Raw SNP intensity data of all samples carrying exon-disrupting microdeletions affecting the *RBFOX1* and *RBFOX3* genes. Red frames represent the area of the observed microdeletions. Signal intensities of a SNP probe are represented by dots, one dot per each probe (Log R ratio track). A decline of neighboring probe signal intensities and B allele frequencies (B Allele Freq track) near 1 and 0 indicate a genomic deletion. The deletions have been visualized using the Illumina Genome Studio Software.
(DOC)

Figure S2 UCSC Genome Browser *RBFOX1* transcript, common SNP and GERP conservation annotation tracks. Top track: Highlighted in red, deleted nucleotides of patient E699. Middle track: The deleted sequence is abundant all six known *RBFOX1* transcripts. Lower track: No common SNP (≤1%) is annotated in dbSNP137 for the shown sequence interval. Bottom track: *RBFOX1* Genomic Evolutionary Rate Profiling (GERP) scores. The rejected substitutions score (RS) is based on an alignment of 35 mammal scores. A RS score threshold of 2 provides high sensitivity while still strongly enriching sequence conservation sites (http://www.genome.ucsc.edu). For the deleted sequence of *RBFOX1,* high and low RS scores are shown.
(DOC)

Figure S3 Multiple Sequence Alignment *RBFOX1* variant A299_A300del (c.893_898delCTGCCG, p.A299_A300del, NM_001142333). Multiple sequence alignments: The top line indicates the human amino acid sequence according to genome build hg19. Amino acids highlighted in red are hemizygously deleted in patient E699. Three alanine residues are conserved among mammalians but only one alanine residue in none mammalian vertebrates. Sequence annotations were taken from the UCSC Genome Browser (http://www.genome.ucsc.edu) and for multiple sequence alignments we used ClustalW (http://www.ebi.ac.uk/Tools/services/web_clustalw2/).
(DOC)

Table S1. *RBFOX1* and *RBFOX3* exonic sequence variant.
(DOC)

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Author Contributions

Conceived and designed the experiments: DL BN. Performed the experiments: DL JA MT EMR. Analyzed the data: DL HT. Contributed reagents/materials/analysis tools: RSM AH TS PN FZ BN HM. Wrote the paper: DL EMR HL FZ TS PN BN.

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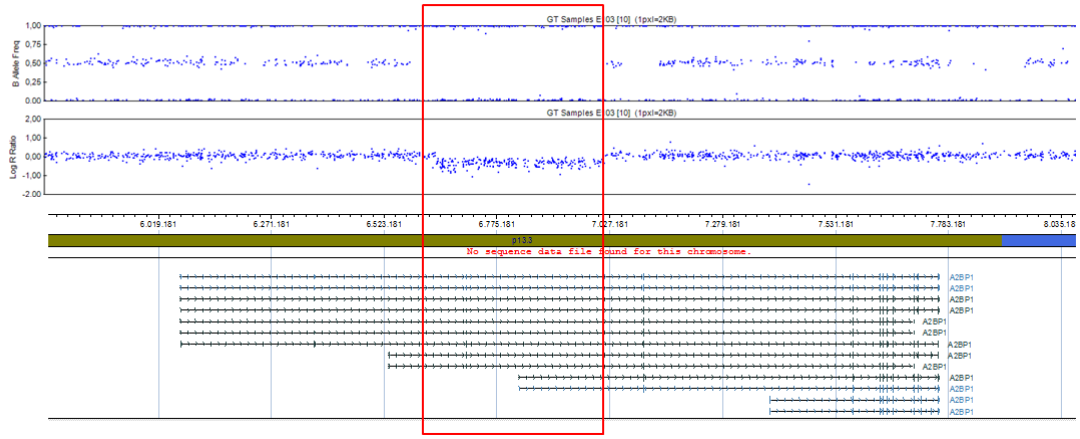
Figure S1 Raw SNP intensity data of all samples carrying exon-disrupting microdeletions affecting the *RBFOX1* and *RBFOX3* genes

RBFOX1 365 kb deletion



RBFOX3 43 kb deletion

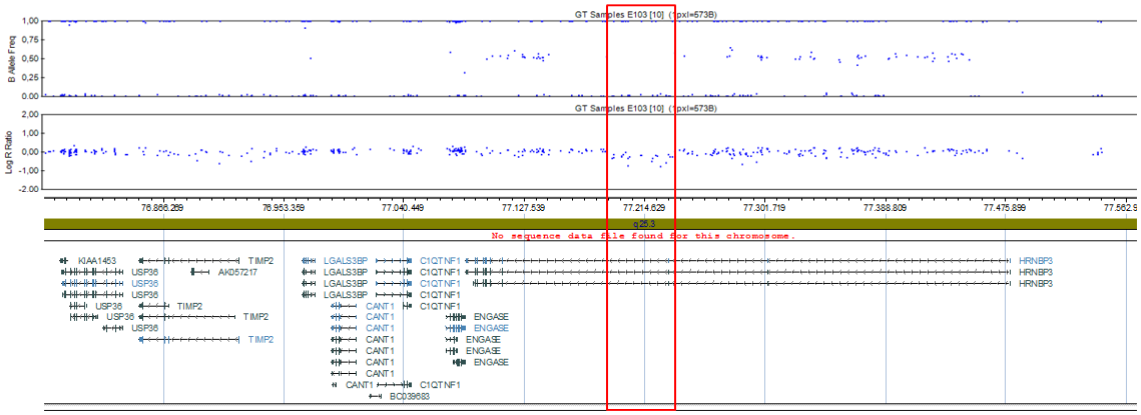


Figure S2 Flanking sequence information for *RBFOX1* variant p.A299_A300del (c.893_898delCTGCCG, p.A299_A300del, NM_001142333)

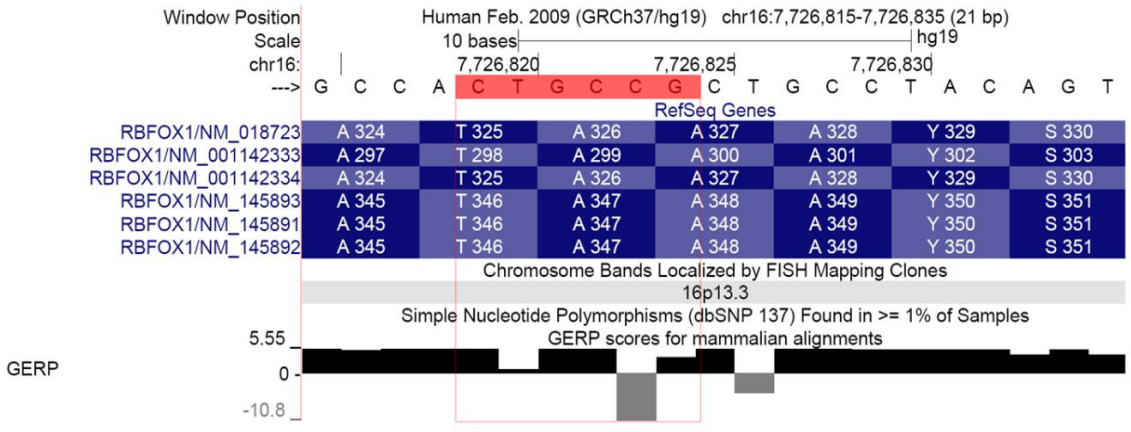


Figure S3 Multiple Sequence Alignment*RBFOX1* variant A299_A300del (c.893_898delCTGCCG; NM_001142333)

```
hg19                        ATAAAYS
Pan_troglodytes__Chimp_     ATAAAYS
Macaca_mulatta__Rhesus_     ATAAAYS
Bos_taurus__Cow_            ATAAAYS
Mus_musculus__Mouse_        ATAAAYS
Canis_familiaris__Dog_      ATAAAYS
Gallus_gallus__Chicken_     G-ADIY-
Danio_rerio__Zebrafish_     G-ADIY-
                            .  *  *
```

Table S1. *RBFOX1*and *RBFOX3* exonic sequence variants

	Start	End	Type	HGNC	CCDS	Consequence	MutCDNA	MutProt	Patient
16	7703830	7703836	INDEL	RBFOX1	CCDS55984.1	FRAMESHIFT	c.690_696delGTATCCAins(GTATCCA)2	p.A233Vfs*74	EG1208
16	7703830	7703836	INDEL	RBFOX1	CCDS55983.1	FRAMESHIFT	c.771_777delGTATCCAins(GTATCCA)2	p.A260Vfs*74	EG1208
16	7703830	7703836	INDEL	RBFOX1	CCDS55983.1	FRAMESHIFT	c.771_777delGTATCCAins(GTATCCA)2	p.A260Vfs*74	EG1208
16	7703830	7703836	INDEL	RBFOX1	CCDS10531.1	FRAMESHIFT	c.831_837delGTATCCAins(GTATCCA)2	p.A280Vfs*51	EG1208
16	7703830	7703836	INDEL	RBFOX1	CCDS45405.1	FRAMESHIFT	c.831_837delGTATCCAins(GTATCCA)2	p.A280Vfs*51	EG1208
16	7703830	7703836	INDEL	RBFOX1	CCDS10532.1	FRAMESHIFT	c.831_837delGTATCCAins(GTATCCA)2	p.A280Vfs*51	EG1208
16	7726819	7726824	DEL	RBFOX1	CCDS10531.1	DELETION	c.1037_1042delCTGCCG	p.A347_A348del	E699
16	7726819	7726824	DEL	RBFOX1	CCDS55984.1	DELETION	c.893_898delCTGCCG	p.A299_A300del	E699
16	7726819	7726824	DEL	RBFOX1	CCDS55983.1	DELETION	c.974_979delCTGCCG	p.A326_A327del	E699
16	7726819	7726824	DEL	RBFOX1	CCDS45405.1	DELETION	c.1037_1042delCTGCCG	p.A347_A348del	E699
16	7726819	7726824	DEL	RBFOX1	CCDS10532.1	DELETION	c.1037_1042delCTGCCG	p.A347_A348del	E699
16	7726819	7726824	DEL	RBFOX1	CCDS55983.1	DELETION	c.974_979delCTGCCG	p.A326_A327del	E699
17	77090608	77090608	SNP	RBFOX3	CCDS45805.1	STOP_GAINED	c.861C>A	p.Y287*	E136

Hg19, detailed description of variants identified in *RBFOX1* and *RBFOX3*

3.2.2 *GRIN2A* in RE (published)

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**These authors contributed equally to this work*

LETTERS

nature genetics

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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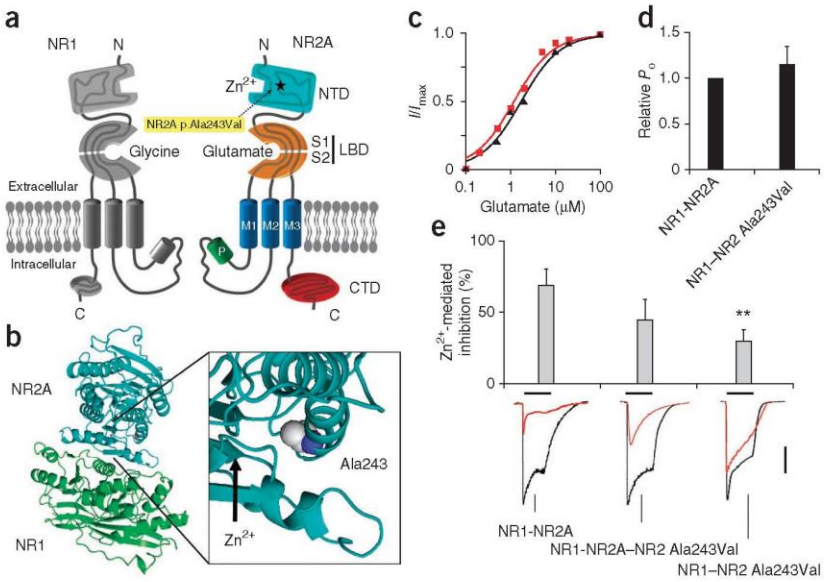

Figure 1 Structural and functional consequences of the missense mutation in *GRIN2A* encoding p.Ala243Val. Functional analysis of the missense alteration p.Ala243Val (index subject 1) showed a significant reduction in high-affinity Zn^{2+} inhibition, whereas current amplitude, glutamate and glycine 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

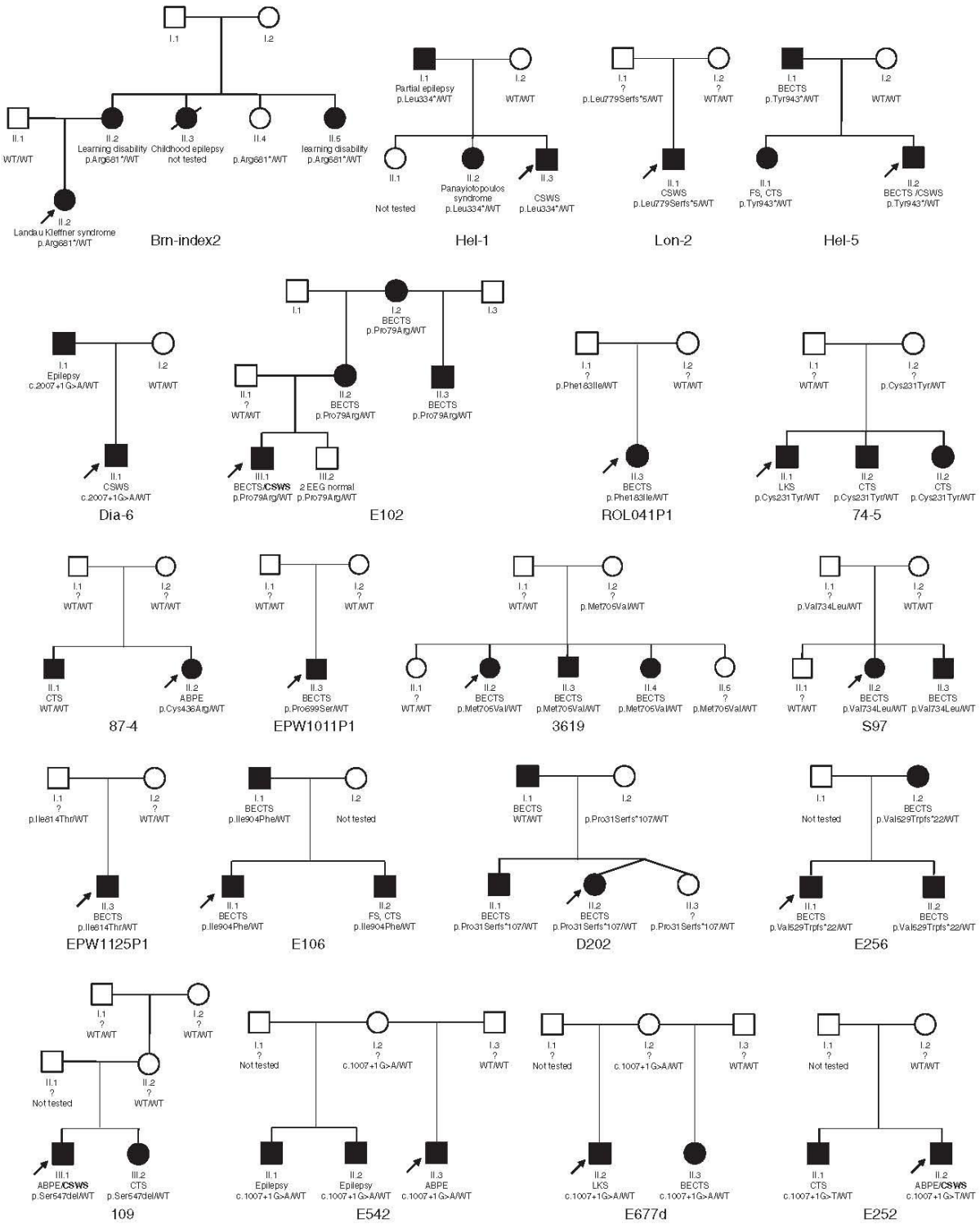

Figure 2 Pedigrees of affected individuals with available family information. Analysis of possible segregation of the respective mutation in family members could be performed for index subject 2 and for 19 of 27 mutation carriers in cohorts II and III where DNA samples were available from family members. 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_f-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_f-11	CSWS	c.2927A>G	p.Asn976Ser
4	K_f-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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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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Mutations in *GRIN2A* cause idiopathic focal epilepsy with rolandic spikes

Supplementary Material

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Supplementary Tables and Figures

References

Supplementary Table 1a Characteristics of novel *GRIN2A* mutations in patients with idiopathic focal epilepsy

#	Case	Phenotype	Nucleotide Mutation	Protein Alteration	PolyPhen2[1]		MutationTaster[2]		Inheritance/ Segregation (y/n)	ID dbSNP, MAF
					Score	Rating	Proba-bility	Rating		
	Ki-index1	BECTS	c.728C>T	p.Ala243Val	1.0	Probably damaging	0.917	Poly-morphism	Unknown	Novel
	Brn-index2	LKS	c.2041C>T	p.Arg681*	-	-	0.999	Disease causing	Maternal, y	Novel
1	Ant-18	BECTS	c.1108C>T	p.Arg370Trp	1.0	Probably damaging	0.999	Disease causing	Unknown	Novel
2	Ant-11	CSWS	c.2140G>A	p.Glu714Lys	0.19	Benign	0.729	Poly-morphism	Unknown	Novel
3	Ki-11	CSWS	c.2927A>G	p.Asn976Ser	0.186	Benign	0.571	Disease causing	Unknown	Novel
4	Ki-40	ABPE	c.594G>A	p.Trp198*	-	-	1.0	Disease causing	Unknown	Novel
5	Hel-1	CSWS	c.1001T>A	p.Leu334*	-	-	0.999	Disease causing	Paternal, y	Novel
6	Lon-2	CSWS	c.2334_2338delCTTGC	p.Leu779Ser *fs**5	-	-	1.0	Disease causing	Paternal, n	Novel
7	Hel-5	CSWS	c.2829C>G	p.Tyr943*	-	-	0.999	Disease causing	Paternal, y	Novel
9	E102	BECTS/ CSWS	c.236C>G	p.Pro79Arg	1.0	Probably damaging	0.833	Disease causing	Maternal, y	Novel
10	ROL 041P1	BECTS	c.547T>A	p.Phe183Ile	1.0	Probably damaging	0.723	Poly-morphism	Paternal, n	Novel
11	74-5	LKS	c.692G>A	p.Cys231Tyr	1.0	Probably damaging	0.996	Disease causing	Maternal, y	Novel
12	EPW 1109P1	BECTS	c.869C>T	p.Ala290Val	0.593	Possibly damaging	0.968	Poly-morphism	Unknown	Novel
13	87-4	ABPE	c.1306T>C	p.Cys436Arg	1.0	Probably damaging	0.999	Disease causing	*De novo*, n	Novel
14	EPW 1011P1	BECTS	c.2095C>T	p.Pro699Ser	1.0	Probably damaging	0.998	Disease causing	*De novo*, -	Novel
15	3619	BECTS	c.2113A>G	p.Met705Val	1.0	Probably damaging	0.999	Disease causing	Maternal, y	Novel
16	EPW 1083P1	BECTS	c.2179G>A	p.Ala727Thr	1.0	Probably damaging	0.998	Disease causing	Unknown	Novel
17	S97	BECTS	c.2200G>C	p.Val734Leu	1.0	Probably damaging	0.999	Disease causing	Paternal, y	Novel
18	EPW 1128P1	ABPE	c.2314A>G	p.Lys772Glu	1.0	Probably damaging	0.889	Disease causing	Unknown	Novel
19	EPW 1125P1	BECTS	c.2441T>C	p.Ile814Thr	0.157	Benign	0.983	Disease causing	Paternal, n	Novel
20	E106	BECTS	c.2710A>T	p.Ile904Phe	0.411	Benign	0.909	Disease causing	Paternal, y	Novel
21	ROL 057P1	ABPE	c.2927A>G	p.Asn976Ser	0.186	Benign	0.571	Disease causing	Unknown	Novel
22	D202	BECTS	c.90delTins(T)2	p.Pro31Ser *fs**107	-	-	1.0	Disease causing	Maternal, n	Novel
23	E256	BECTS	c.1585delG	p.Val529Trp *fs**22	-	-	1.0	Disease causing	Maternal, y	Novel
24	109-4	ABPE/ CSWS	c.1637_1639delCTT	p.Ser547del	-	-	-	-	Unknown, y	Novel

y = yes, n = no; MAF = Minor Allele Frequency; phenotype underlined: patients who had either BECTS or ABPE and electrical status in sleep were subsumed under the phenotype of CSWS.

Supplementary Table 1b Characteristics of novel *GRIN2A* splice site mutations in patients with idiopathic focal epilepsy

#	Case	Phenotype	Nucleotide Mutation	Protein Alteration	HSF[3]		SpliceView[4]		Inheritance/ Segregation (y/n)	ID dbSNP, MAF
					Varia-tion	Predict-ion	wt	mut		
	Brn-index3	LKS	c.1007+1G>A	IVS4, p.?	-32,77%	donor splice site disrupted	DSS 80%	No DSS	Unknown	Novel
8	Dia-6	CSWS	c.2007+1G>A	IVS7, p.?	-27,63%	donor splice site disrupted	DSS 89%	No DSS	Paternal, y	Novel
25	E542	ABPE	c.1007+1G>A	IVS4, p.?	-32,77%	donor splice site disrupted	DSS 80%	No DSS	Maternal, y	Novel
26	E677d	RE	c.1007+1G>A	IVS4, p.?	-32,77%	donor splice site disrupted	DSS 80%	No DSS	Maternal, y	Novel
27	E252	ABPE/ CSWS	c.1007+1G>T	IVS4, p.?	-32.77%	donor splice site disrupted	DSS 80%	No DSS	Unknown, y	Novel

wt = wildtype allele, mut = mutated allele, y = yes, n = no; MAF = Minor Allele Frequency; DSS = Donor Splice Site; phenotype underlined: patients who had either BECTS or ABPE and electrical status in sleep were subsumed under the phenotype of CSWS.

Discussion of Supplementary Tables 1a and 1b:

For nonsense and missense mutations as well as for analysis of splice site mutation, two *in-silico* prediction programs were used to assess the potential pathogenic impact of the mutations. We used two complementary programs to increase the validity of the prediction. Most variants are supposed to have a disease causing effect on protein function and both programs give consistent predictions in most cases. One mutation (c.2140G>A, p.E714K) was rated to be a polymorphism rather than a pathogenic variant. However, only subsequent functional studies will be able to fully assess the role of these mutations in the pathophysiology of idiopathic focal epilepsies.

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Supplementary Table 2 Characteristics of known *GRIN2A* non-synonymous, coding sequence alterations in patients with idiopathic focal epilepsy (cohorts I – III)

Case	Phenotype	Nucleotide Mutation	Protein Alteration	PolyPhen2[1]		MutationTaster[2]		Inheritance/ Segregation (y/n)	ID dbSNP, MAF
				Score	Rating	Proba-bility	Rating		
Ki-28	BECTS	c.91C>A	p.Pro31Thr	0.0	Benign	0.837	Disease causing	Unknown	(known in EVS®)
112-3	ABPE	c.422C>T	p.Thr141Met	0.016	Benign	0.626	Disease causing	Unknown	rs78631453, 0.0009
Cohort 1-40	ABPE	c.2899G>C	p.Val967Leu	0	Benign	0.984	Poly-morphism	Unknown	rs61731465, 0.004
Cohort 1-12	ABPE/ unclassified epilepsy	c.2899G>C	p.Val967Leu	0	Benign	0.984	Poly-morphism	Unknown	rs61731465, 0.004
Cohort 1-27	BECTS	c.2899G>C	p.Val967Leu	0	Benign	0.984	Poly-morphism	Unknown	rs61731465, 0.004
Ki-5	ABPE	c.2899G>C	p.Val967Leu	0	Benign	0.984	Poly-morphism	Unknown	rs61731465, 0.004
EPW 1023P1	BECTS	c.2899G>C	p.Val967Leu	0	Benign	0.984	Poly-morphism	Unknown	rs61731465, 0.004
EPW 1056P1	BECTS	c.2899G>C	p.Val967Leu	0	Benign	0.984	Poly-morphism	Unknown	rs61731465, 0.004
EPW 1085P1	BECTS	c.2899G>C	p.Val967Leu	0	Benign	0.984	Poly-morphism	Unknown	rs61731465, 0.004
KK 1065	BECTS	c.2899G>C	p.Val967Leu	0	Benign	0.984	Poly-morphism	Unknown	rs61731465, 0.004
EPW 1109P1	BECTS	c.2899G>C	p.Val967Leu	0	Benign	0.984	Poly-morphism	Unknown	rs61731465, 0.004
ROL 006P1	BECTS	c.2899G>C	p.Val967Leu	0	Benign	0.984	Poly-morphism	Unknown	rs61731465, 0.004
88-4	BECTS	c.2899G>C	p.Val967Leu	0	Benign	0.984	Poly-morphism	Unknown	rs61731465, 0.004
E130f	BECTS	c.3190A>G	p.Thr1064Ala	0	Benign	0.994	Poly-morphism	Unknown	rs138809301, N/A
Cohort 1-16	BECTS	c.3228G>C	p.Asn1076Lys	0.997	Probably damaging	0.995	Disease causing	Unknown	rs61758995, 0.002
Tar-13	BECTS	c.3228G>C	p.Asn1076Lys	0.997	Probably damaging	0.995	Disease causing	Paternal, ?	rs61758995, 0.002
Brn-7	LKS	c.3228G>C	p.Asn1076Lys	0.997	Probably damaging	0.995	Disease causing	Maternal, n	rs61758995, 0.002
Cohort 1-28	BECTS	c.3827A>G	p.Ala1276Gly	0.517	Possibly damaging	0.874	Disease causing	Unknown	rs145063086, 0.001
Ki-20	CSWS	c.3827A>G	p.Ala1276Gly	0.517	Possibly damaging	0.874	Disease causing	Unknown	rs145063086, 0.001

y = yes, n = no; MAF = Minor Allele Frequency; N/A = not known

Supplementary Table 3 Phenotypic characteristics of patients with novel mutations in *GRIN2A*

#	Case	Sex	Protein Alteration	Age-at-Onset (years)	Epilepsy Syndrome	EEG	Seizure Types	Motor Development	Cognitive Development	Speech Development	FH
	Ki-index1	m	p.Ala243Val	5.5	BECTS	CTS	Rolandic sz.	Normal	Learning problems	Normal	na
	Brn-index2	m	p.Arg681*	3.5	LKS	Multifocal sw/ ssw, CSWS	No seizures	Normal	Learning disability	Auditory agnosia, receptive+ expressive language disorder	+
	Brn-index3	m	c.1007+1G>A IVS4, p.?	4	LKS	Multifocal + bitemporal sw, CSWS	Rare nocturnal sec. GTCS	Normal	Mental retardation	Auditory agnosia, severe language disorder	+
1	Ant-18	f	p.Arg370Trp	7	BECTS	CTS	Nocturnal focal sz., postictal hemiparesis	Normal	Normal	Normal	+
2	Ant-11	m	p.Glu714Lys	4.5	CSWS	CTS, CSWS	Two episodes of non-convulsive status epilepticus	Normal	Mild attention deficit	Normal	na
3	Ki-11	m	p.Asn976Ser	na	CSWS	CSWS	No seizures	Normal	Dyslexia	Normal	
4	Ki-40[1]	m	p.Trp198*	3.5	ABPE	Multifocal sw, CTS	Atonic sz.	Milestones delayed, mild muscular hypotonia	Delayed	Delayed, slurred speech	na
5	Hel-1	f	p.Leu334*	4	Panayiot./ CSWS	Multifocal sw, activation in sleep, CSWS	Rolandic sz., ictal vomiting	Mild fine motor clumsiness	Lower normal range	Delayed	+
6	Lon-2[2]	m	p.Leu779Ser *fs**5	4	ABPE/ CSWS	CTS, extending to frontal and parietal, CSWS	Focal atonic sz., staring episodes	Motor coordination difficulties, right hemineglect	Difficulties in social communication and attention	Normal	
7	Hel-5	m	p.Tyr943*	2	CSWS	CTS, CSWS	Rolandic sz.	Fine/gross motor clumsiness	Mild-moderate retardation	Delayed	+
8	Dia-6	f	c.2007+1G>A IVS7, p.?	3.5	CSWS	CSWS	Absences	Fine/gross motor clumsiness	Normal	Problems in expressive language	+
9	E102	m	p.Pro79Arg	6	BECTS/ CSWS	CSWS	Nocturnal GTCS	Normal	Severe attention deficit S.	Delayed	+
10	ROL 041P1	m	p.Phe183Ile	na	BECTS	CTS	Rolandic sz.	na	Mildly delayed	na	na
11	74-5	m	p.Cys231Tyr	3	LKS	CSWS	FS, GTCS	Delayed	Mild intellectual impairment	Delayed	+
12	EPW 1109P1	f	p.Ala290Val	8.3	BECTS	CTS	Rolandic sz.	Normal	Normal	Normal	+
13	87-4	f	p.Cys436Arg	4	ABPE	CTS	Nocturnal GTCS, atonic seizures	Normal	Normal	Mild delay	+
14	EPW 1011P1	m	p.Pro699Ser	8.0	BECTS	CTS	Rolandic sz.	Normal	Normal	Normal	-
15	3619	f	p.Met705Val	8	BECTS	CTS	GTCS	Normal	Mild delay	Delayed	+
16	EPW 1083P1	f	p.Ala727Thr	6.0	BECTS	CTS	Rolandic sz.	Normal	Normal	Normal	+
17	S97	f	p.Val734Leu	6	BECTS	CTS	Rolandic sz.	Normal	na	na	+

18	EPW 1128P1	m	p.Lys772Glu	2.6	ABPE	CTS, multifocal shw	FS, rolandic sz.	Normal	Learning and reading problems	Normal	na
19	EPW 1125P1	m	p.Ile814Thr	4.8	BECTS	CTS/occipital spikes	CPS, GTCS	Normal	Normal	Normal	-
20	E106	m	p.Ile904Phe	7	BECTS	CTS	Rolandic sz., astatic sz.	Mild delayed fine motor skills	Lower normal range	Delayed	+
21	ROL 057P1	f	p.Asn976Ser	3.7	ABPE	CTS	Absences, myoclonic sz.	Normal	na	Normal	na
22	D202	m	p.Pro31Ser *fs**107	4	BECTS	CTS	Rolandic sz.	Normal	na	na	+
23	E256	m	p.Val529Trp *fs**22	2	BECTS	CTS	Rolandic sz.	Clumsy	Mild intellectual impairment with regression	Delayed	+
24	109-4	m	p.Ser547del	4	ABPE/ CSWS	CSWS	Nocturnal GTCS	Delayed	Mild intellectual impairment	Delayed	+
25	E542	m	c.1007+1G>A IVS4, p.?	4	ABPE	CTS	Serial astatic sz.	Normal	Delayed	Delayed	+
26	E677d	f	c.1007+1G>A IVS4, p.?	4	BECTS	CTS	Nocturnal GTCS	Normal	Delayed	Delayed	+
27	E252	m	c.1007+1G>T IVS4, p.?	4	ABPE/ CSWS	CSWS	Rolandic sz.	Normal	Mild delay	Delayed	+
28	NB3	?	CNVdel	na	ABPE	na	na	na	na	na	na
29	72-3	m	CNVdel	na	BECTS	CTS	Nocturnal GTCS, Rolandic sz.	Normal	Mild intell. impairment	Severe delay	na
30	EPW 1111P1	m	CNVdel	7.7	BECTS	CTS	Rolandicsz., GTCS	Normal	Normal	Delayed	-
31	145	m	CNVdupl	na	CSWS	CSWS	No seizures	Normal	Intellectual impairment	Delayed	+

Abbreviations: FH = family history with respect to epilepsy, EEG abnormalities and/or febrile seizures, m = male, f = female, BECTS = benign epilepsy with centrotemporal spikes (rolandic epilepsy), Panyiot. = Panayiotopoulos syndrome, ABPE = atypical benign partial epilepsy, LKS = Landau-Kleffner syndrome, CSWS = epileptic encephalopathy with continuous spike-wave during sleep, CTS = centrotemporal spikes/sharp-waves, sw = sharp-waves, ssw = sharp-slow-waves, PPR = photoparoxysmal response, sz. = seizure, sec. GTCS = secondary generalized tonic-clonic seizure, na = unknown, phenotype underlined: patients who had either BECTS or ABPE and electrical status in sleep were subsumed under the phenotype of CSWS.

[1] Array-CGH analysis revealed a duplication at 8q11.23 and a duplication at 15q26.1q26.2. [2] Array-CGH analysis showed a *de novo* microduplication at 22q11.21 and small maternally inherited intronic deletion of *NRXN1*.

Supplementary Figure 1 Localization of novel *GRIN2A* mutations

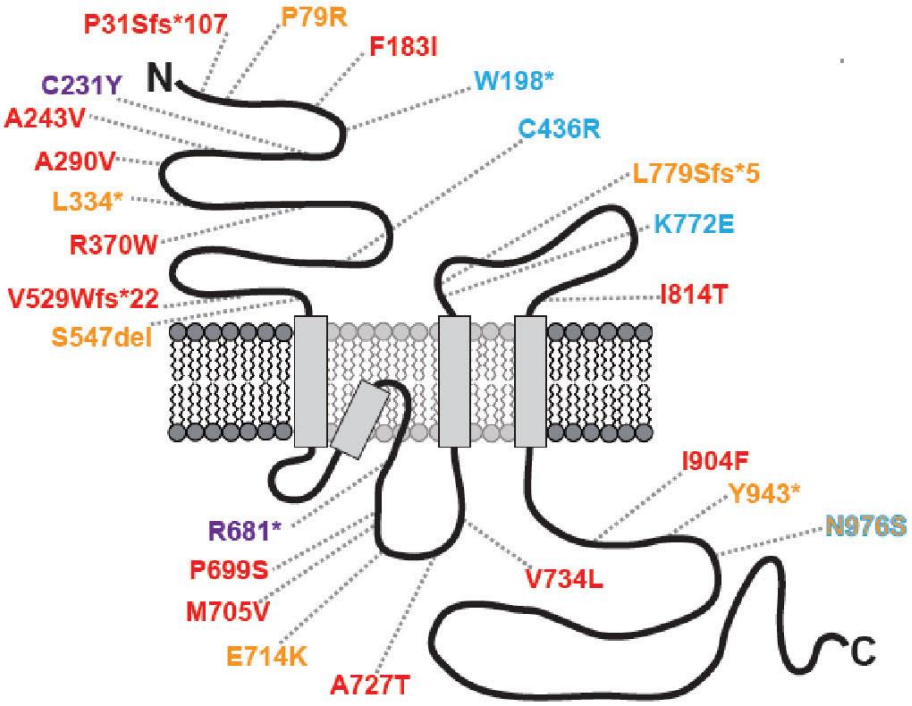


Figure Legend

Supplementary Figure 1 Localization of novel *GRIN2A* mutations

This figure illustrates the localization of novel *GRIN2A* mutations in a protein model of NR2A. The colors indicate the different phenotypes of mutation carriers: red = BECTS, blue = ABPE, purple = LKS, orange = CSWS. Mutation p.N976S (= p.Asn976Ser) occurs twice, in a patient with ABPE and a patient with CSWS.

Supplementary Figure 2 Distribution of phenotypes in cohorts II and III

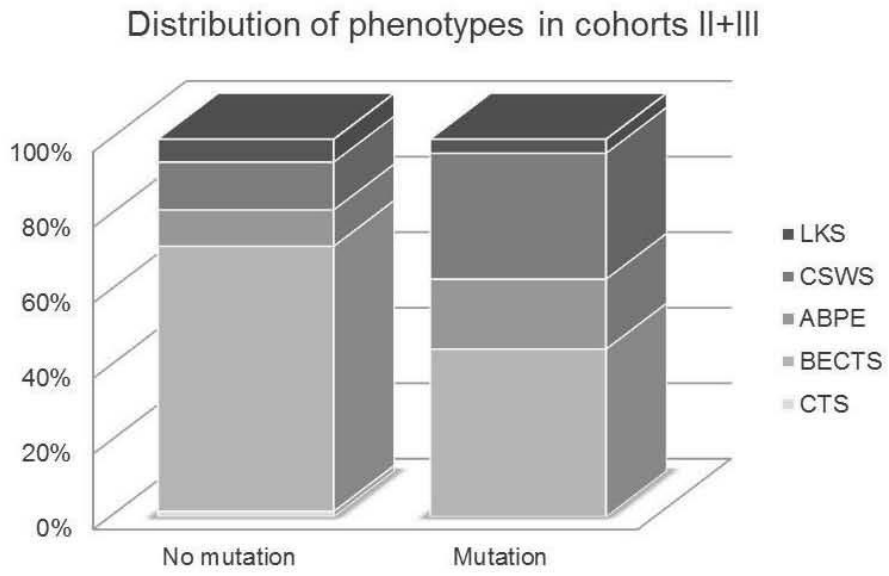
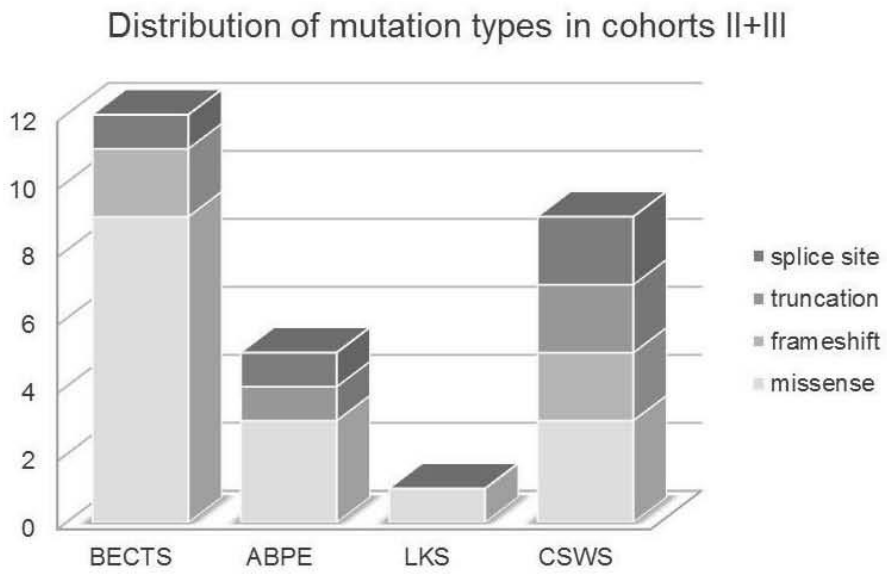


Figure Legend

Supplementary Figure 2 Distribution of phenotypes in cohorts II and III

The frequency of mutation carriers significantly increased trend towards more severe phenotypes (p=0.009; Cochran-Armitage test of trend).

Supplementary Figure 3 Distribution of mutation types in cohorts II and III



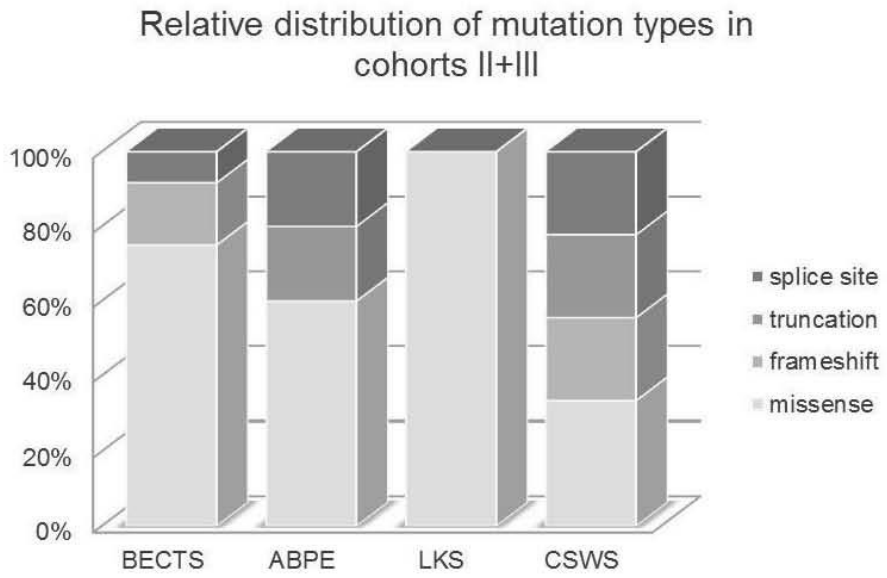


Figure Legend

Supplementary Figure 3 Distribution of mutation types in cohorts II and III

More severe phenotypes tended to co-occur with different types of mutations then less severe one. Pearson's corrected contingency coefficient C_{corr} equaled 0.52, evidencing substantial association between severity of phenotype and type of mutation.

Supplementary Figure 4 Copy Number Variations in cohort IV

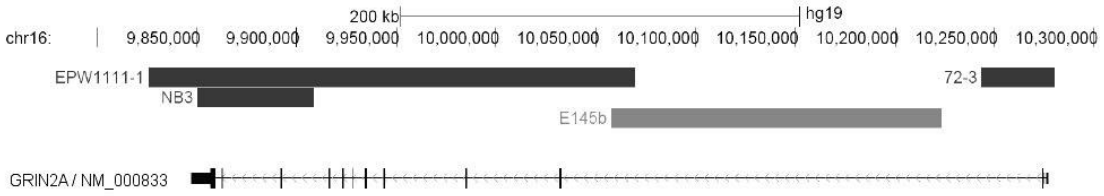


Figure Legend

Supplementary Figure 4 Copy Number Variations in cohort IV

Hg19 genomic localization and overview of transcript NM_000833. Dark grey bars represent size and location of microdeletions detected. The light grey bar represents size and location of an intronic duplication of unknown significance.

Supplementary material referring to methods section

Supplementary Table 4 Summary of cohorts I - IV

Cohort	Isolated CTS		BECTS		ABPE		LKS		CSWS		Total
	abs.	%	abs.	%	abs.	%	abs.	%	abs.	%	
Cohort I (*screening cohort*)	0	0	32	82.1	6	15.4	0	0	1	2.6	**39**
Cohort II (*confirmation cohort*)	3	2.5	48	40.3	17	14.3	17	28.6	34	14.3	**119**
Cohort III (*replication cohort*)	2	0.8	197	82.1	20	8.3	4	1.7	17	7.1	**240**
Total cohort for statistical analysis (= cohorts II+III)	5	1.4	245	68.2	37	10.3	21	5.8	51	14.2	**359**
Cohort IV (*CNV cohort*)	2	0.7	231	80.8	32	11.2	4	1.4	17	5.9	**286**

Supplementary Table 5a Primers for sequence analysis of *GRIN2A* in cohort 1

#PROBE	PROBE_ NAME	Localiza-tion	FORWARD_PRIMER	REVERSE_PRIMER	PRODUCT SIZE (bp)	START (NCBI Build 37)	STOP (NCBI Build 37)
Primer 3*		Promotor	CACTGGGGGAGGAGGAAG	GTTTGAGGGCGAGTGTGTGT	640	10276740	10277379
Pr001177516.1	RSA001422348	Exon1	tgtaaaacgacggccagtGAGGGAGGTGGATCTTTCCCA	caggaaacagctatgaccACCGCAGCAGAAAGACGCC	599	10276384	10276983
Pr001177476.1	RSA001422655	Exon2	tgtaaaacgacggccagtCTCCTGCACTTCGCCGCTG	caggaaacagctatgaccGGGCTCCCATTCTCCTGTGC	557	10275601	10276158
Pr001187950.1	RSA000647835	Exon3	tgtaaaacgacggccagtGGCTGTGACCCAGCATCACC	caggaaacagctatgaccTCCGTGATCCCAGCAGCCTA	279	10274139	10274418
Pr001177515.1	RSA001422346	Exon3	tgtaaaacgacggccagtTGGTTCTCACCAGGGCCAGT	caggaaacagctatgaccCACCTTGCAGGGACCGTCAG	592	10273704	10274296
Pr001177459.1	RSA001422955	Exon4	tgtaaaacgacggccagtCGCCTCCAGGCTGTAGTCCC	caggaaacagctatgaccTGCTCAGGAATGTCTGCAAATGA	579	10031953	10032532
Pr001177514.1	RSA001422345	Exon4	tgtaaaacgacggccagtGTGTGCATCGGGACCTCTGG	caggaaacagctatgaccGTGGGCTGGGACATGCAGAA	412	10031825	10032237
Pr001177510.1	RSA001422336	Exon4	tgtaaaacgacggccagtCAGACAGATCGATCAATCCCAGG	caggaaacagctatgaccCCTTGGCCTCACCGGGTATG	542	10031546	10032088
Pr001187948.1	RSA000647825	Exon5	tgtaaaacgacggccagtGCTCAACAGGAAATGCGTGGG	caggaaacagctatgaccGCAAGCAGCTTCCTTCAGGG	541	9984744	9985285
Pr001177508.1	RSA001422333	Exon6	tgtaaaacgacggccagtAAGGGTTGGGCACGTTCAGG	caggaaacagctatgaccGGCTGGGATGGAGAACTGCC	445	9943571	9944016
Pr001177469.1	RSA001422954	Exon6	tgtaaaacgacggccagtTGTCTGTGGAGTCAGTTGGACCAC	caggaaacagctatgaccCCGTGTGGCCCAGGTACAAG	511	9943267	9943778
Primer 3*		Exon7+8	TGAACAAACCTTCCAGTGCT	GTCCCATCCTCTGAGCAAAC	586	9934429	9935014
Primer 3*		Exon9	TGATTAGCTTCTTTTGAAGGATCA	CACCATGCCTGGTCTAGAGT	369	9927860	9928228
Pr001177513.1	RSA001422330	Exon10	tgtaaaacgacggccagtGGATTCTGGACAGGCACGGA	caggaaacagctatgaccTCCCGCTGAATTGCATGGTT	521	9923422	9923943
Pr001177512.1	RSA001422329	Exon10	tgtaaaacgacggccagtAGGAAACACGACCACTCGGG	caggaaacagctatgaccTCTGGCAACCTTCGGTGCTG	585	9923026	9923611
Pr001187899.1	RSA000651856	Exon11	tgtaaaacgacggccagtGGGCCCATCTGGACCACAGT	caggaaacagctatgaccTGCATGCATTTACCTCCTAACACCA	415	9915989	9916404
Pr001189738.1	RSA000412231	Exon12	tgtaaaacgacggccagtGGGAGCAAACTCATCATGCAAAGA	caggaaacagctatgaccGGGATGGAGAGGCCATGAGTC	428	9892028	9892456
Primer 3*		Exon13	TCACTGCAACAATATTAGGTAGGC	CCCAAGCGCTTTTCTAAACC	500	9862649	9863148
Pr001187898.1	RSA000651854	Exon13	tgtaaaacgacggccagtTTCTCATGTCCCGCCTGCTG	caggaaacagctatgaccTCCTCACTGCCTGTGAATGTTGTG	499	9862481	9862980
Primer 3*		Exon14	CCCTATGCTTTGCAACTTGTC	GAATCCACGGATTTCTTCCA	650	9858342	9858991
Pr001177529.1	RSA001422323	Exon14	tgtaaaacgacggccagtTTTCAGGTAGGTGCGCTCGAC	caggaaacagctatgaccCCCTCATCATGGACATGGTTTCA	535	9858080	9858615
Pr001177528.1	RSA001422321	Exon14	tgtaaaacgacggccagtAGGCAGGCATCGCACTTGAA	caggaaacagctatgaccGGACCCACTCCCTAAAGAGCCC	594	9857682	9858276
Pr001177524.1	RSA001422369	Exon14	tgtaaaacgacggccagtCGTTGGTCATCCCTGTGGGAG	caggaaacagctatgaccGGGCTTTCCAACAACGACCA	598	9857274	9857872
Pr001189794.1	RSA000389524	Exon14	tgtaaaacgacggccagtTGGACATCCAACATTTACCCTCCA	caggaaacagctatgaccCCTCAAGGACAGGGAACGGC	537	9856928	9857465
Pr001177523.1	RSA001422368	Exon14	tgtaaaacgacggccagtTCAGGTTTACATGCACACCATATTGC	caggaaacagctatgaccTCGGAGCATGTTATGCCTTATGC	565	9856563	9857128

For sequence analysis, primers of the NCBI Primer Set RSS000057426.1 were used. * Primers designed using Primer3 (www.primer3.sourceforge.net)

Supplementary Table 5b Primers for sequence analysis of *GRIN2A* in cohort 2

Localiza-tion	FORWARD_PRIMER	REVERSE_PRIMER	PRODUCT SIZE (bp)	START (NCBI Build 37)	STOP (NCBI Build 37)
Exon3	TGGCTCCTAGAGCAGGAAGCCAAG	GGGTTGGAGAGGCAAGACCTGGTT	692	10273685	10274376
Exon4	TGCTCAGGAATGTCTGCAAATGATTGA	CGATCAATCCCAGGTGTAGGTTTCCA	978	10031555	10032532
Exon5	TGGTCAACTAGCAATGGTTGCATGG	GAGAAAGCATTCCAATTGCACCTTACAGA	479	9984656	9985134
Exon6	TGCCCGTTTGTCTTAGCACAGAAGTAGA	GGGCAAAACTACTCACCTCATATTGCTGGT	594	9943382	9943975
Exon7+8	TGCTATAATTAAGGACACTTTCTGCTTTTGCTG	TGCCATGGCCAAACAGAGCTAAACA	752	9934392	9935143
Exon9	TGCAAATGGATGTCTGGGCTTCCT	CCATGCCTGGTCTAGAGTAATGTGTTCTAA	310	9927862	9928171
Exon10	GGTGGCTGCTGCGTGGTTGTC	TCAATGAGAGGCACCTGAATCTCTTCC	398	9923192	9923589
Exon11	TCCTGGAGCTCACAGACTGGCTGA	TCAGAAGGAAACCTGGGATGCTCA	552	9915937	9916488
Exon12	TGCTTTCAGGATCAGGCCTCAGGA	TCATGCAAAGATCCACTGGGAAGC	365	9892041	9892405
Exon13	TTTGTCCTCACTGCAACAATATTAGGTAGGC	GTGACATGCCCAAGAAAGGCTGGT	569	9862587	9863155
Exon14.1	CCAGGGCTCCTGCAAGAAGTGC	TGGGGTATTTGGAGGCCACTGA	805	9858113	9858919
Exon14.2	CCCAGAGGGATGAGGCAACAGC	GCTCCGGGAGGGCCTGCTAA	836	9857471	9858306
Exon14.3	CCAGCAGGACTGGGCACAGAA	TCCTCTTAGCAGGGCACTATTGGACA	693	9856908	9857600

Supplementary Figure 5 Study design

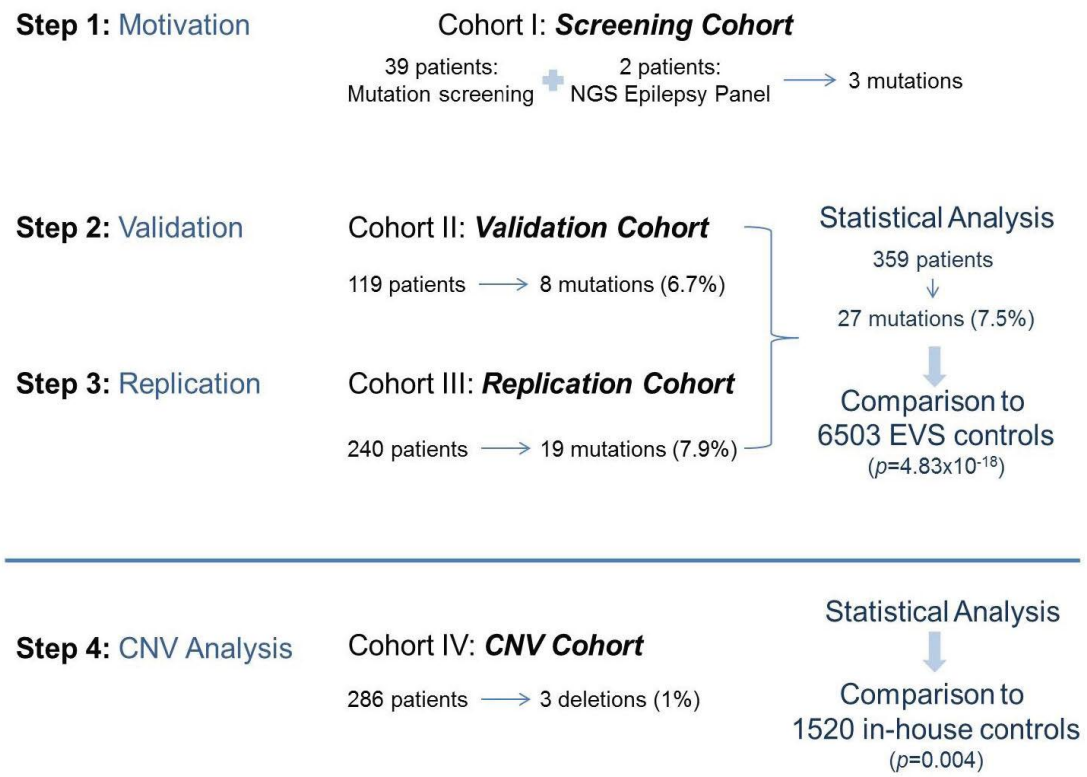


Figure Legend

Supplementary Figure 5 Study design

This flow-chart demonstrates the overall study design illustrating our three-step design of motivation, validation and replication. Additionally, a CNV analysis was performed.

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3.2.3 16p11.2 duplications in RE (submitted; revised manuscript)

Reinthaler EM*, **Lal D*,** Reymond A, Jacquemont et al. 16p11.2 duplications confer risk for typical and atypical Rolandic epilepsy. **BRAIN**

**These authors contributed equally to this work*

16p11.2 600 kb Duplications Confer Risk for Typical and Atypical Rolandic Epilepsy

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Abstract

Rolandic Epilepsy is the most common idiopathic focal childhood epilepsy. Its molecular basis is largely unknown though a complex genetic etiology is generally assumed. We demonstrate that genomic duplications between breakpoints 4 and 5 at the 16p11.2 locus, previously associated with neurodevelopmental disorders, are also associated with typical and atypical Rolandic epilepsy. In a cohort of 393 patients, we identified five patients with the typical recurrent 600 kb (29.5-30.1Mb) duplication and a sixth case with an atypical shorter rearrangement (in total 1.53%). This differed significantly from the prevalence observed in the general population of European origin [32/62403; (0.05%) Fisher's exact test $P = 1.4 \times 10^{-7}$, OR = 30.2, 95% CI: 10.3-73.9]. The duplication is not associated with all forms of epilepsies, as none was detected in 330 and 1408 patients with either temporal lobe epilepsy or genetic generalized epilepsies, respectively. In a subsequent screen among children carrying the 16p11.2 600 kb rearrangement we identified three patients in 117 duplication carriers (2.6%) but none in 202 carriers of the reciprocal deletion. The 16p11.2 duplication is the second recurrent structural variant after *GRIN2A* mutations and deletions identified for typical and atypical Rolandic epilepsy.

Keywords: rolandic epilepsy; microduplications; 16p11.2; genetics

Abbreviations: RE= rolandic epilepsy; ARE= atypical rolandic epilepsy; CTS= centrotemporal spikes; CNV= copy number variation; SNP= single nucleotide polymorphism

Introduction

Rolandic epilepsy (RE), also known as benign epilepsy with centrotemporal spikes (BECTS), is the most common childhood epilepsy with a prevalence of 0.2 – 0.73/1000 (i.e. about 1/2500) (Beilmann *et al.*, 1999; Sidenvall *et al.*, 1996; Waaler *et al.*, 2000). RE is related to rarer, and less benign epilepsy syndromes, including atypical benign partial epilepsy (ABPE), Landau-Kleffner syndrome (LKS) and epileptic encephalopathy with continuous spike-and-waves during sleep (CSWSS) (Doose *et al.*, 2001; Gobbi *et al.*, 2006; Guerrini and Pellacani, 2012; Hahn *et al.*, 2001), referred to as RE related syndromes, or atypical Rolandic epilepsy (ARE) by some authors (Fejerman, 2009). RE and ARE share blunt, high-voltage, characteristically shaped centrotemporal spikes (CTS) of characteristic morphology in the EEG. In ARE spikes become generalized during slow wave sleep, morphology, location and background activity may vary to some extend (Doose *et al.*, 2001; Fejerman, 2009). The CTS EEG trait is not entirely specific to RE, as it is also found in 2-4% of healthy children (Eeg-Olofsson *et al.*, 1971; Okubo *et al.*, 1994), in 10-28% of children with autism spectrum disorder (ASD) (Chez *et al.*, 2006; Lewine *et al.*, 1999; Tuchman and Rapin, 1997) and in the Fragile-X-Syndrome (Musumeci *et al.*, 1991; Musumeci *et al.*, 1999). RE is further characterized by focal (perioral sensorimotor) seizures and secondarily generalized seizures, which resolve spontaneously during adolescence. ARE denote more severe forms of focal childhood epilepsies with various additional seizure types, developmental language delay or regression, speech dyspraxia and variable neuropsychiatric deficits (Gobbi *et al.*, 2006; Hughes, 2011).

The underlying etiologies of RE remain largely unknown although a genetic basis has been postulated. Multiplex family studies have suggested an autosomal dominant inheritance of the EEG trait CTS (Bali *et al.*, 2007; Heijbel *et al.*, 1975). However several other arguments, amongst them the distribution of seizure-risk in relatives of patients with RE, argue for a complex mode of inheritance (Vears *et al.*, 2012). Linkage studies identified loci for CTS on 15q14 (LOD 3.56) and 11p13 (LOD 4.30) (Neubauer *et al.*, 1998; Strug *et al.*, 2009). Fine mapping of the latter locus revealed an association with the *ELP4* gene, but causative mutations have not yet been identified (Strug *et al.*, 2009). Markers linked to chromosome 16p12-11.2 (LOD 3.68) were found in one family affected by RE with paroxysmal exercise-induced dystonia and writer's cramp (Guerrini *et al.*, 1999), while disease-associated variants in the *SRPX2 gene* were found in a family with X-linked rolandic epilepsy, oral and speech dyspraxia and intellectual disability and in one patient with perisylvian polymicrogyria and rolandic seizures (Roll *et al.*, 2006). Rare variants in *KCNQ2* and *KCNQ3* were identified in a small number of patients with RE (Neubauer *et al.*, 2008). Very recently it was shown that mutations in *GRIN2A* are major genetic risk factors for idiopathic focal epilepsies with CTS, with and without language dysfunction (Carvill *et al.*, 2013; Lemke *et al.*, 2013; Lesca *et al.*, 2013).

Copy number variants (CNV) are an important source of structural genomic variation. Whereas many CNVs, especially non-recurrent ones, are not necessarily related to a clinical phenotype, six prominent recurrent CNVs (1q21, 15q11.2, 15q13.3, 16p11.2, 16p13.11 and 22q11.2) are strongly associated with seizures and a range of neurodevelopmental and neuropsychiatric disorders including autism (Cook and Scherer, 2008; Cooper *et al.*, 2011; de Kovel *et al.*, 2010; Helbig *et al.*, 2009; Sebat *et al.*, 2007; Stefansson *et al.*, 2008). One of the characteristic features

of these recurrent CNVs is their remarkable phenotypic variability suggesting a shared genetic basis of the above diseases. The 16p11.2 600 kb BP4-BP5 microdeletion (OMIM #611913) is associated with ASD, obesity and intellectual disability with and without epilepsy, whereas the reciprocal duplication (OMIM #614671) is linked to schizophrenia, microcephaly, intellectual disability and being underweight (Bijlsma *et al.*, 2009; Jacquemont *et al.*, 2011; McCarthy *et al.*, 2009; Shinawi *et al.*, 2010; Walters *et al.*, 2010; Weiss *et al.*, 2008; Zufferey *et al.*, 2012). Recurrent microdeletions at 15q11.2, 15q13.3 and 16p13.11 have been identified as important risk factors for genetic generalized epilepsy (GGE) accounting for about 1% of these patients but are also associated with a range of other neuropsychiatric symptoms (de Kovel *et al.*, 2010; Dibbens *et al.*, 2009; Helbig *et al.*, 2009). These pleiotropic effects and the associations between recurrent CNVs and GGE prompted us to investigate the frequency of these six recurrent CNVs and novel large CNVs in a cohort of children with RE or ARE.

Subjects and Methods

Study participants

In a multi-centre effort RE/ARE patients were recruited from Germany, Austria, Canada and Australia. 98 of the patients were ascertained via multiplex-families with the minimum criteria of at least two affected siblings. The index case had to be diagnosed with RE or ARE, and the affected sibling(s) either with RE, ARE, or the EEG trait only. All other 183 RE/ARE were patients recruited consecutively at the participating clinics, as soon as the diagnosis of RE/ARE was present, irrespective of their family history for seizures. Diagnosis of RE was performed according to the International Classification of Seizures and Epilepsies (Berg *et al.*, 2010). The various related types of atypical Rolandic epilepsy were diagnosed as specified

previously (Aicardi and Chevrie, 1982; Doose *et al.*, 2001; Hahn *et al.*, 2001). The discovery set included a total of 281 unrelated patients of Caucasian ancestry affected by RE (n = 230) and ARE (n = 51) (165 males and 116 females), and 1512 platform matched, unscreened German population controls (755 males and 757 females). The replication cohort consisted of 112 cases with RE (n = 109) and ARE (n = 3) (63 males and 49 females), from Australia (n = 78) and Austria (n = 34) ascertained with the diagnosis of RE/ARE irrespective of whether they have a family history of seizures. Additional SNP-array data were obtained from 1408 genetic generalized epilepsy (GGE) patients, 330 mesial temporal lobe epilepsy (mTLE) patients and 2256 German population controls (KORA: n = 1250, POGEN: n= 1006). We also studied 319 children carrying a 600kb 16p11.2 rearrangement. These patients were assembled by screening neurodevelopmental disorders cohorts through a network of cytogenetic centers (Jacquemont *et al.*, 2011; Zufferey *et al.*, 2012). For summarized description of all cohorts see Supplementary Table 1. Written informed consent was obtained from all participating patients and, when appropriate, from both parents and adolescents. This study was approved by all respective local institutional review boards.

Genotyping, copy number variation detection and validation

CNVs were detected by high-density SNP genotyping arrays (Illumina HumanOmniExpress BeadChip) and subsequent CNV calling was carried out with the PennCNV software (Wang *et al.*, 2007). CNVs were considered to match published recurrent CNVs if they overlapped at least 80% of the respective CNV-length. All called recurrent candidate CNVs were more than 350 kb in size and covered with 100 - 756 consecutive SNP-probes. Smaller CNVs of at least 50 kb nested within the recurrent candidate regions were considered if they included one

of the previously proposed candidate genes (de Kovel *et al.*, 2010; Dibbens *et al.*, 2009). Genome-wide CNV screening beyond the six candidate regions was restricted to CNVs with a segment size > 500 kb (Nicoletti *et al.*, 2012) and a minimum of 50 markers to achieve a high accuracy and reproducibility of CNV callings across different array platforms, laboratory sites and calling algorithms (Pinto *et al.*, 2011). CNVs were manually inspected in the Illumina Genome Viewer Software for the regional SNP heterozygosity state (BAF) and log2 ratios of the signal intensities (LRR). 1512 controls genotyped on IlluminaHumanOmniExpressBeadChip and 330 mTLE patients genotyped on IlluminaHap550 array were analyzed with PennCNV. Regions of CNVs detected in patients were manually inspected in the control samples for the presence of duplications and deletions. CNV calling for samples genotyped on Affymetrix 6.0 array were performed as previously reported (de Kovel *et al.*, 2010; Lal *et al.*, 2013). Recurrent CNV validation and segregation in families was performed with real-time quantitative PCR (qPCR) using TaqMan CNV probes (Supplementary Table 2; Life Technologies, Darmstadt, Germany) and custom array-CGH for the 16p11.2 duplication (Agilent Technologies, Santa Clara, CA). This custom array contains about 544 probes in the 16p11.2 region (chr16:29,500,000-30,200,000, hg19) with an average probe spacing of 1.3 kb.

Results

CNV detection

In our discovery cohort we identified CNVs at four of the six investigated recurrent candidate loci (15q11.2, 15q13.3, 16p11.2 and 22q11.2) (Table 1) as well as other CNVs (Table 2). We found a significant enrichment of 16p11.2 duplications in RE/ARE patients when compared with controls. Four out of 281 patients (1.42%)

carried a ~600 kb 16p11.2 microduplication compared to only one in 1512 controls (0.07%) [Fisher´s exact test, P =0.0026, OR = 21.8; 95% CI: 2.14-1068.36] (Table 1). We identified one additional patient with a smaller partial duplication (approximately 110kb of unique sequence; hg19 chr16:29,650,000-29,760,000). This atypical rearrangement starts at BP4 and encompasses the genes *SPN*, *QPRT*, and *C16orf54* within the unique sequence (Supplementary Fig. 1). Of note, we identified one additional individual carrying the classical 16p11.2 duplication. He was screened with the initial discovery cohort, but was finally not included as not all inclusion criteria were met (suspected focal cortical dysplasia). All six tentative 16p11.2 duplications, including the one later removed due to the uncertain MRI result, were validated by Taqman quantitative real-time PCR and custom array-CGH. We established the breakpoint boundaries at the classical BP4 and BP5 hotspots (Zufferey *et al.*, 2012) in all five 600 kb sized typical 16p11.2 duplications. To confirm our findings we subsequently screened the 16p11.2 locus in an independent replication cohort of 112 patients with RE/ARE. This screen revealed one further classical recurrent 600 kb-microduplication (0.89%) (Fig. 1) which could be validated by qPCR.

We also identified a nominally significant increase of duplications at the 15q11.2 locus (approximate genomic coordinates according to hg19 22,750,000-23,350,000) in our discovery cohort. Six patients displayed the duplication in comparison to 8 out of 1512 controls [Fisher´s exact test, P = 0.014, OR =4.1; 95% CI: 1.16-13.59]. This borderline association did not remain significant after Bonferroni correction for multiple comparisons.

To validate the previously reported prevalence of 16p11.2 duplications among the general population we examined array data from an additional cohort of 2256 healthy individuals from Germany (KORA, PopGen) unscreened for epilepsy (Lal *et*

al., 2013) but did not detect any new 16p11.2 duplications or deletions. This is in accordance with previous work setting the prevalence of the duplication at 0.05% in the European population (31 duplications out of 58,635 tested individuals) (Jacquemont *et al.*, 2011). In a joint analysis of the discovery and replication cohorts with both control cohorts, 16p11.2 duplications were seen at a frequency of 1.3 % among patients and were thus significantly associated with RE/ARE [5/393 cases vs. 1/3768 controls, Fisher´s exact test, $P = 4.1x10^{-5}$ OR = 48.4, 95% CI: 5.40-2264.08]. Inclusion of the atypical duplication at the 16p11.2 locus further increased the significance of this association [$P = 4.42x10^{-6}$ OR= 58.27].

To explore whether the 16p11.2 duplication is a general genetic risk factor for common epilepsies, we screened 1408 European patients affected by GGE and 330 German patients diagnosed with mesial temporal lobe epilepsy (mTLE) for 16p11.2 duplications using high-density SNP arrays, but detected none.

Clinical data and segregation analysis

Patients harboring the 16p11.2 duplication either suffered from typical RE (n = 5) or atypical RE (n = 1) (Table 3). Three patients (F1-II.1, F3-II.1, F5-II.1) had in addition a history of febrile seizures, which can be expected in about 20% of children with RE or ARE (Doose *et al.*, 1997). All patients diagnosed with typical RE showed normal development and their neuropsychological assessments fell within the normal range. Only patient F1-II.1, diagnosed with ARE/LKS, presented with severe developmental delay. For individuals F3-II.1 and F6-I.1 calculations of the Body Mass Index (BMI) were possible, revealing severely to moderately reduced BMIs (F3-II.1: 13.31; F6-I.1: 18.6) consistent with previous reports (Jacquemont *et al.*, 2011). No data were available on the BMIs of the remaining duplication carriers and on head circumferences of all patients. Duplications were inherited in all four cases

where testing was possible (Fig. 2) with a maternal transmission in three of them. The smaller atypical duplication was inherited from the father who himself had a history of febrile seizures (Family 5). While the classical duplication co-segregated with RE in one family (Family 2), two other families displayed reduced penetrance with reportedly unaffected mothers carrying the duplication (F1-I.2 and F4-I.2). We could not determine whether these mothers did exhibit RE-characteristic EEG trait during their childhood. Family 1 is interesting as it demonstrates the variability of the phenotypes in the duplication carriers, as the index case (F1-II.1) was affected by severe ARE/LKS, while his younger sister only displayed CTS in EEG (F1-II.3). Notably, the third sibling in this family (F1-II.2) presented the EEG trait without being a duplication carrier (Fig. 2). This family is not only affected by the 16p11.2 duplication but also recently reported carrying a novel p.C231Y *GRIN2A* mutation (Lemke *et al.*, 2013). Whereas the *GRIN2A* variation segregates with the CTS trait, the 16p11.2 duplication seems not to be necessary for the electroclinical component of the phenotype. In Family 4, we detected in addition to the 16p11.2 duplication a novel missense variation of unknown significance in the *DEPDC5,* a gene which was recently identified in focal epilepsies (Dibbens *et al.*, 2013; Ishida *et al.*, 2013). In this family the 16p11.2 duplication was inherited from the unaffected mother and the *DEPDC5* variant from the unaffected father, resulting in a mutational load in the child. Which of those two or if both genetic alterations synergistically contribute to the phenotype is not known.

Genotype-phenotype correlations in the 16p11.2 rearrangement cohort

To further specify the association between RE/ARE and 16p11.2 duplications, we examined a large cohort of 319 children (≤18yrs) with 600 kb BP4-BP5 rearrangements (202 deletions and 117 duplication carriers) from the 16p11.2

European consortium. We detected an equal rate of 18% of patients with epilepsies (combining unspecified, focal, generalized epilepsies and infantile spasms) among the duplication carriers (n=22/117) and deletion carriers (n=37/202) (Supplementary Table 3). In the duplication group, three individuals were diagnosed with RE/ARE (Supplementary Table 4). One patient presented with typical RE (short nocturnal motor facial seizures and centro-temporal spikes activated by sleep). He also suffered from reading and spelling difficulties and was diagnosed with an attention deficit disorder. The second patient displayed the same electro-clinical features but, in addition, also presented with a delay of language, a behavioral disorder, a borderline IQ (75) and an early age of onset (2 years of age). He was therefore diagnosed as atypical RE. The third patient displayed an ARE-epileptic encephalopathy (initially CSWSS, but eventually language regression and ASD traits which led to the diagnosis of LKS). An additional (fourth) patient had only CTS activated by sleep without seizures. In the deletion group, a single patient had CTS activated by sleep without seizures but none suffered from RE/ARE. Hence RE/ARE occurred only in duplication carriers (2.6% n=3/117) but not in deletion carriers (n=0/202) [Fisher´s exact test, P = 0.05]. Although it was not possible to assess the exact prevalence of RE/ARE in the complete cohorts screened (i.e. including cases without 16p11.2 rearrangements), we note that RE/ARE features are enriched approximately 30-fold in the 16p11.2 duplication cohort as compared to the general population given an RE-prevalence of 0.05% (Jacquemont *et al.*, 2011).

Other structural rearrangements

To investigate whether other large CNVs beyond the above six candidate regions were present in our RE/ARE patients we performed a genome-wide screen for CNVs larger than 500 kb in all 281 individuals of the discovery cohort. In particular,

we were interested whether 16p11.2 duplication carriers might display additional large CNVs. We found 23 additional CNVs in the 281 RE/ARE patients screened (Table 2). Six of these CNVs were partially overlapping with CNVs previously reported in patients with epilepsy (Heinzen *et al.*, 2010; Lesca *et al.*, 2012; Mefford *et al.*, 2007; Mefford *et al.*, 2010; Mefford *et al.*, 2011; Valvo *et al.*, 2012). With the exception of one duplication mapping to chromosome 12q24.33, which was identified in two patients and in two out of 1512 controls from the discovery cohort, all other CNVs occur as single events in our patient dataset. Interestingly, in one 16p11.2 duplication carrier (F3-II.1) we identified a second large duplication (1.03Mb) on chromosome 22q11.21. Of note, this duplication is shorter than the recurrent 22q11.21 duplication which is 3 MB in size (OMIM #608363) and it does not include the proposed candidate gene *COMT*.

Discussion

In summary, we report a highly significant association of the recurrent 16p11.2 microduplication with RE/ARE spectrum epilepsies. We detected the 16p11.2 duplication in 1.53% of RE-patients (six individuals including the smaller atypical rearrangement), which equals a more than 30-fold enrichment when compared to the prevalence in the general population (0.05%) (Jacquemont *et al.*, 2011).

Our cohorts partly included patients that were required to have a positive family history for RE/ARE or the EEG trait as an entry criterion (98 of 393 patients). As inherited genetic factors might be enriched in cases with a familial background a multiplex pedigree ascertainment method might lead to an overestimation of the frequency of such variants in the overall patient population. However, we believe that the bias in this study was small as the majority of patients (75%) were enrolled

irrespective of their family history and because the frequency of the duplication carriers was similar in the multiplex and singly ascertained cohorts (4/295 and 2/98 respectively). However, this potential sampling bias does not play a role for our conclusion that the 16p11.2 is a susceptibility factor for RE because sampling of unrelated cases with a positive family history has no influence on the expected prevalence of the 16p11.2 duplication in the patient cohort under the assumption of the alternative null hypothesis.

Although the effect size appears considerable the penetrance is incomplete as illustrated by the presence of clinically apparently unaffected duplication carriers in our families (with the caveat that mild childhood phenotypes might have been missed or not reported in the adult carriers). Likewise, the severity and expressivity of the phenotypes varied considerably between affected members of the same family. Similar observations have been made with other CNVs that are nevertheless firmly established as risk factors for epilepsies (de Kovel *et al.*, 2010).

One remarkable feature of the 16p11.2 rearrangements is the wide diversity of the associated phenotypes. The BP4-BP5 duplication and its reciprocal deletion were previously associated with schizophrenia/autism, micro-/macrocephaly and underweight/obesity mirror phenotypes as well as impairment in cognitive performance (Jacquemont *et al.*, 2011; McCarthy *et al.*, 2009; Shinawi *et al.*, 2010; Walters *et al.*, 2010; Weiss *et al.*, 2008). In this present report we extend the list of neurodevelopmental disorders associated with the recurrent 16p11.2 CNV even further to the full spectrum of the RE/ARE phenotype ranging from mild RE cases to severe forms of ARE with epileptic encephalopathies. Fitting with clinical observations which have long suggested a shared etiology between RE and a whole host of other neuropsychiatric syndromes and symptoms [e.g. cognitive deficits, language disorders, autism, ADHD (Clarke *et al.*, 2007; Danielsson and

Petermann, 2009; Kavros *et al.*, 2008; Tovia *et al.*, 2011) we have identified the 16p.11.2 duplication as one shared genetic susceptibility factor. In light of the phenotypic variability in two families additional modulating genetic variants are already identified and further are expected and will be the focus of future research.

The 600 kb long chromosomal region between BP4 and BP5 on 16p11.2 contains 29 genes (Fig. 1). Most of these genes are expressed in the brain and might thus contribute singly or jointly to the etiology of RE and related disorders. Several studies on model organisms have attempted to narrow down the dosage sensitive genes for the observed brain-related traits. Haploinsufficiency of *ALDOA* and *KIF22* have been related to change in brain morphology in zebrafish (Blaker-Lee *et al.*, 2012), while alterations in head size anticorrelated with levels of *KCDT13*, *MAPK3* and *MVP* in teleosts and rodents (Golzio *et al.*, 2012). One particularly interesting candidate is the *PRRT2* gene with a putative role in synaptic vesicle functioning, as it has been associated with a range of different childhood epilepsies. Mutations in *PRRT2* are, for instance, the main cause of benign familial infantile seizures (BFIS) and other infantile epilepsy syndromes as well as paroxysmal kinesigenic dyskinesia (Chen *et al.*, 2011; Heron *et al.*, 2012; Schubert *et al.*, 2012; Wang *et al.*, 2011; Wood, 2012).

Although atypical CNVs in the 16p11.2 interval are rare they are valuable in narrowing down the critical genomic interval. We detected one partial 110 kb duplication excluding *PRRT2* but encompassing *SPN, QPRT* and *C16orf54* in a patient who suffered from RE. The *QPRT* gene is of interest as it encodes an enzyme (quinolinate phosphoribosyltransferase), which converts quinolinic acid to NAD+ and thus detoxifies this excitotoxic compound (Foster and Schwarcz, 1985; Schwarcz *et al.*, 2012). Intracerebral injection of quinolinic acid causes seizures in

mice (Lapin, 1978; Vezzani *et al.*, 1989) and elevation of quinolinate levels in the human brain is associated with neurodegenerative disorders (Nemeth *et al.*, 2005; Vamos *et al.*, 2009). While dosage differences in *QPRT* might explain the observed phenotype, this single, isolated rearrangement does not allow us to draw any firm conclusions, especially as we do not have any information on the CTS status of the single previously reported additional individual, who carried a partially overlapping 136 kb duplication, encompassing the *QPRT* gene (Jacquemont *et al.*, 2011).

Of interest, the detected borderline association of the 15q11.2 duplication with RE/ARE is supported when the comparison is made with its previously reported frequency in the general population (Cooper *et al.*, 2011) [6/281 cases vs. 44/9841controls, Fisher´s exact test, P = 0.0025]. Hopefully these findings will encourage further studies on larger RE/ARE cohorts to assess whether the 15q11.2 duplication really confers any risk to RE/ARE.

In conclusion, we have identified the 16p11.2 duplication as an important genetic risk factor for RE/ARE with 1.53 % of patients carrying the variation. Our results suggest that the 16p11.2 duplication confers susceptibility mainly for RE/ARE but not for other common epilepsy syndromes, such as GGEs or TLE. However, one reservation that has to be made is that due to the ascertainment method our cohort was biased towards patients with a positive family history and a more severe phenotype. To a certain extent this limits conclusions on the exact frequency of the duplication and its distribution within the RE/ARE disease spectrum. Another open question that remains to be further clarified is the nature of the additional phenotypes associated with the duplication in patients with RE/ARE. And finally, an interesting but unresolved point concerns the role of additional genetic factors

modulating the penetrance and the variable clinical features of the disease. Future studies on other well phenotyped cohorts could shed more light on these open issues.

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Appendix

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Supplementary material

Supplementary material is available at Brain online.

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Figure legends

Figure 1 16p11.2 duplications

GRCh37/hg19 region on chromosome 16p11.2. The extent of the BP4-BP5 16p11.2 duplications found in the discovery cohort is compared with that of the small atypical duplication. The duplication is flanked by low-copy repeat segmental duplications depicted by yellow, orange and greys bars. Note that only the unique sequences of the duplications are shown.

Figure 2 Pedigrees of 16p11.2 microduplication carriers

Familial segregation of 16p11.2 microduplications. Index cases are depicted by the letter “P” and an arrow. Individuals diagnosed with RE/ARE, Centrotemporal spikes (CTS) and Febrile seizures (FS) are represented as filled black symbols, unaffected family members are indicated by open symbols. Question marks denote unaffected family members in whom a possible EEG-phenotype in childhood cannot be excluded; RE, Rolandic Epilepsy; ARE, Atypical Rolandic Epilepsy;

Tables

Table 1Recurrent CNVs in 281 RE/ARE probands and 1512 Controls

	Cases (n=281)			Controls (n=1512)					
CNV locus	**Position (Mb)[a]**	**CNV**	**n**	**PID**	**Phenotype (size)**	**n detected**	**OR**	**P value uncorrected**	**P value Bonferroni[b] corrected**
15q11.2	22.8-23.3	DEL	1	GGRE14	RE	0	-	n.s.	
		DUP	6	AVRE10 AVRE11 AVRE12 AVRE13 AVRE14 GGRE15	RE	8	4.1;	0.014*	0.17
15q13.3	30.9-32.5	DEL	1[c]	AVRE15	ARE	1		n.s.	
		DUP	2[d]	AVRE12 AVRE16	RE	7[c] 1		n.s.	
16p11.2	29.5-30.1	DUP	4	F1-II.1 F2-III.1 F4-II.1 F5-II.1	3xRE, 1xARE	1	21.8	0.0026*	0.03*
16p13.11	14.8-16.4	DEL	0			2	-	n.s.	
		DUP	0			1	-	n.s.	
22q11.2	19.1-22.2	DUP	1	AVRE04	RE	0	-	n.s.	

Table 1 Legend

Table 1 Recurrent candidate microdeletions/microduplications in the discovery cohort

[a] approximate genomic coordinate according to hg19

[b] corrected for 12 comparisons (for six loci with two CNV states each)

[c] deletion is greater than the classical recurrent 1.4Mb deletion

[d] duplications are smaller (~ 430 kb) than the classical recurrent 1.4 Mb duplication but encompass the *CHRNA7* gene

Abbreviations: ARE: atypical Rolandic Epilepsy, RE: Rolandic Epilepsy,

n.s. = not significant

Table 2 Heterozygous CNVs greater than 500kb in 281 RE/ARE patients

Case ID	Gender	Cytoband	Coordinates[a] (hg19/Build37)	Size (kb)	CNV	Included Genes[b]	Controls N=1512	Previously reported[c]
AVRE01	Female	1q31.2-q31.3	Chr1:193694880-195909031	2214	dup	*None*	0	
GGRE01	Male	1p36.32	Chr1:4529544-5043734	514	del	*AJAP1, BC037321*	0	
GGRE02	Male	2p24.1	Chr2:21683285-23585786	1903	del	*AK090620*	0	
GGRE03	Male	2p22.3	Chr2:32659489-33235324	576	dup	*BIRC6, MIR558, TTC27, LOC285045, LOC100271832, LTBP1*	4	
GGRE04	Male	3q24	Chr3:143178525-145790803	2612	dup	*SLC9A9, NHE9, C3orf58, PLOD2*	0	
AVRE02	Male	3q29	Chr3:195460549-196096451	636	dup[d]	*MUC20, MUC4, TNK2, AK127609, SDHALP1, TFRC, BC151150, BC144580, ZDHHC19, OSTalpha, AF088041, PCYT1A, TCTEX1D2,*	0	Recurrent Hotspot 3q29duplication

						TM4SF19, AK124973, OCTM4, UBXN7		
AVRE03	Male	5p15.2	Chr5:12543792-13312470	769	del	*TAG1*	0	
GGRE05	Female	5p14.3	Chr5:20886878-21437412	551	dup	*AK093362, LOC728411*	0	
GGRE06	Female	5q15	Chr5:96507017-97053083	546	del	*RIOK2*	0	(Heinzen et al., 2010)
AVRE04	Male	7p11.2	Chr7:57256128-57878853	623	dup	*DQ598473, ZNF716, L37717*	1	
GGRE07	Female	8q13.3	Chr8:70931383-71738054	807	del	*PRDM14, NCOA2, TRAM1, LACTB2, XKR9*	0	
AVRE04	Male	8q21.13-q21.2	Chr8:82517136-85021548	2504	del	*IMPA1, SLC10A5, ZFAND1, CHMP4C, SNX16, BC038578*	0	(Mefford *et al.*, 2010)
AVRE05	Female	10q21.1	Chr10:56288865-56840255	551	dup	*PCDH15*	0	(Lesca *et al.*, 2012)
GGRE08	Male	11q11-q12.1	Chr11:54695706-55849535	1154	dup	*TRIM48, AB231737, OR4A16, OR4A15, OR4C15, OR4C16, OR4C11, OR4P4,*	0	

						OR4S2, OR4C6, OR5D13, OR5D14, OR5L1, OR5D18, OR5L2, OR5D16, SPRYD5, OR5W2, OR5I1, OR10AG1, OR7E5P, OR5F1, OR5AS1		
GGRE09;GGRE10	Male	12q24.33	Chr12:129806980-130479794	673	dup	*TMEM132D*	2	
AVRE06	Male	14q22.2-q22.3	Chr14:54629941-56027815	1398	del	*CDKN3, AY257479, UNQ155, CNIH, GMFB, CGRRF1, SAMD4A, AK096898, KIAA1053, GCH1, WDHD1, SOCS4, MAPK1IP1L, LGALS3, DLGAP5, FBXO34, KIAA0831, TBPL2, C14orf33*	0	
GGRE11	Male	16q12.1	Chr16:4888917	580	dup	*CBLN1,*	0	

			7-49469134			*C16orf78*		
GGRE12	Female	17q12	Chr17:34815551-36182400	1367	dup	*ZNHIT3, MYO19, PIGW, GGNBP2, DHRS11, MRM1, BC084573, LHX1, AATF, ACACA, C17orf78, TADA2A, TADA2L, DUSP14, SYNRG, AP1GBP1, DDX52, HNF1B*	0	Recurrent Hotspot 17q12 duplication (Mefford *et al.*, 2007)
AVRE07	Female	17q25.1	Chr17:71834171-72622965	789	dup	*RPL38, MGC16275, TTYH2, Z49982, DNAI2, BX648926, KIF19, BTBD17, GPR142, GPRC5C, AK126429, CD300A, CD300LB, CD300C, CD300LD, C17orf77, CD300E*	2	
AVRE08	Male	18p11.21	Chr18:13760290-14988113	1228	dup	*RNMT, MC5R, MC2R,*	0	

						ZNF519, BC034578, ANKRD20A5, AX747360, DQ578597, DQ587539, DQ590589, DQ583161, DQ596563, DQ596206, LOC284233, CXADRP3, POTEC, ANKRD30B		
F3.II.1	Male	22q11.21	Chr22:18892575-19921640	1029	dup[d]	*DGCR6, PRODH, KIAA1647, DGCR5, DGCR9, DGCR10, DGCR2, DGCR14, TSSK2, GSC2, SLC25A1, CLTCL1, HIRA, MRPL40, C22orf39, BX648073, CR625276, UFD1L, UFD1, U84523, CDC45L, CLDN5, BC042984, LOC150185, SEPT5,*	0	Recurrent Hotspot (Valvo *et al.*, 2012)

						GP1BB, TBX1, GNB1L, C22orf29, KIAA1645, TXNRD2, TRXR2A		
GGRE13	Male	22q13.32-q13.33	Chr22:49396413-49988815	592	dup	*BC033837*	0	
AVRE09	Male	Xp22.31	ChrX:6470011-8135053	1665	dup	*HDHD1A, STS, VCX, PNPLA4, MIR651*	0	(Mefford *et al.*, 2010)

Table legend

[a] Start and end positions of the CNVs were assessed with PennCNV.

[b] Genes are based by PennCNV-boundaries and ENSEMBL database. Genes which are partially affected are included.

[c] CNV overlaps partly with CNV previously reported in epilepsy CNV studies. Reference is given when CNVs are from the same type.

[d] CNV overlaps with hotspot but does not contain the proposed candidate gene

Table 3 Individuals with 16p11.2 duplication and electro-clinical features

PID	Phenotype	Gender	Age at onset	Seizures	EEG	MRT/ CT	Neuropsychological development	Language development	Family History/ Relationship	16p11.2 duplication/Size
Discovery cohort										
F1-II.1	ABPE/LKS	M	1.0 yrs/3.0yrs	FS/GTCS	ESES	normal	Severe delay	Severe delay, speech regression	Sibling of F1-II.3	~600kb
F1-II.3	EEG only	F	7yrs	No seizures	CTS	normal	Mild delay	Mild delay	Sibling of F1-II.1	~600kb
F2-II.2	RE	F	?	nocturnal TCS	CTS	normal	normal	normal (dyslexia)	mother of F2-III.1	~600kb
F2-III.1	RE	M	2.5yrs	nocturnal TCS, Rolandic seizures	CTS	normal	normal	normal	positive/ son of F2-II.2	~600kb
F3-II.1	RE	M	0.5/5yrs	FS/nocturnal TCS	CTS	normal	normal	normal	negative	~600kb
F4-II.1	RE	F	5yrs	TCS	CTS/ transient ESES	normal	normal	normal	negative	~600kb
F5-I.1	FS	M	?	FS	?	?	?	?	father of F5-II.1	~110kb
F5-II.1	RE	F	1.6/5.5yrs	FS/ TCS, Rolandic seizures	CTS	normal	normal	normal	positive / daughter of F5-I.1	~110kb
Confirmation cohort										
F6-I.1	RE	F	6yrs	Nocturnal TCS, starring	CTS	normal	normal	normal	negative	~600kb

Clinical characteristics of 16p11.2 duplication carrier. RE: Rolandic Epilepsy, ABPE: Atypical benign partial epilepsy of childhood, FS: Febrile seizures, TCS: Tonic clonic seizures, ESES: Electrical status epilepticus during slow sleep, CTS: Centrotemporal spikes LKS: Landau-Kleffner syndrome

Figures

Figure 1

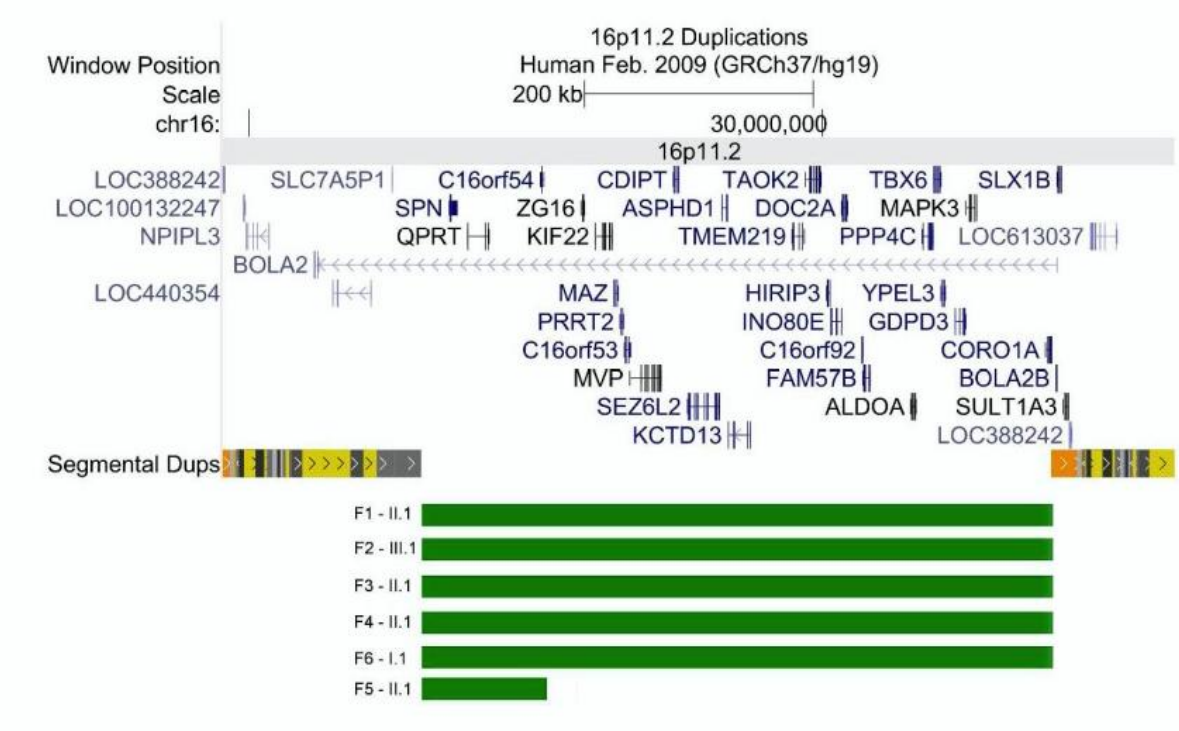
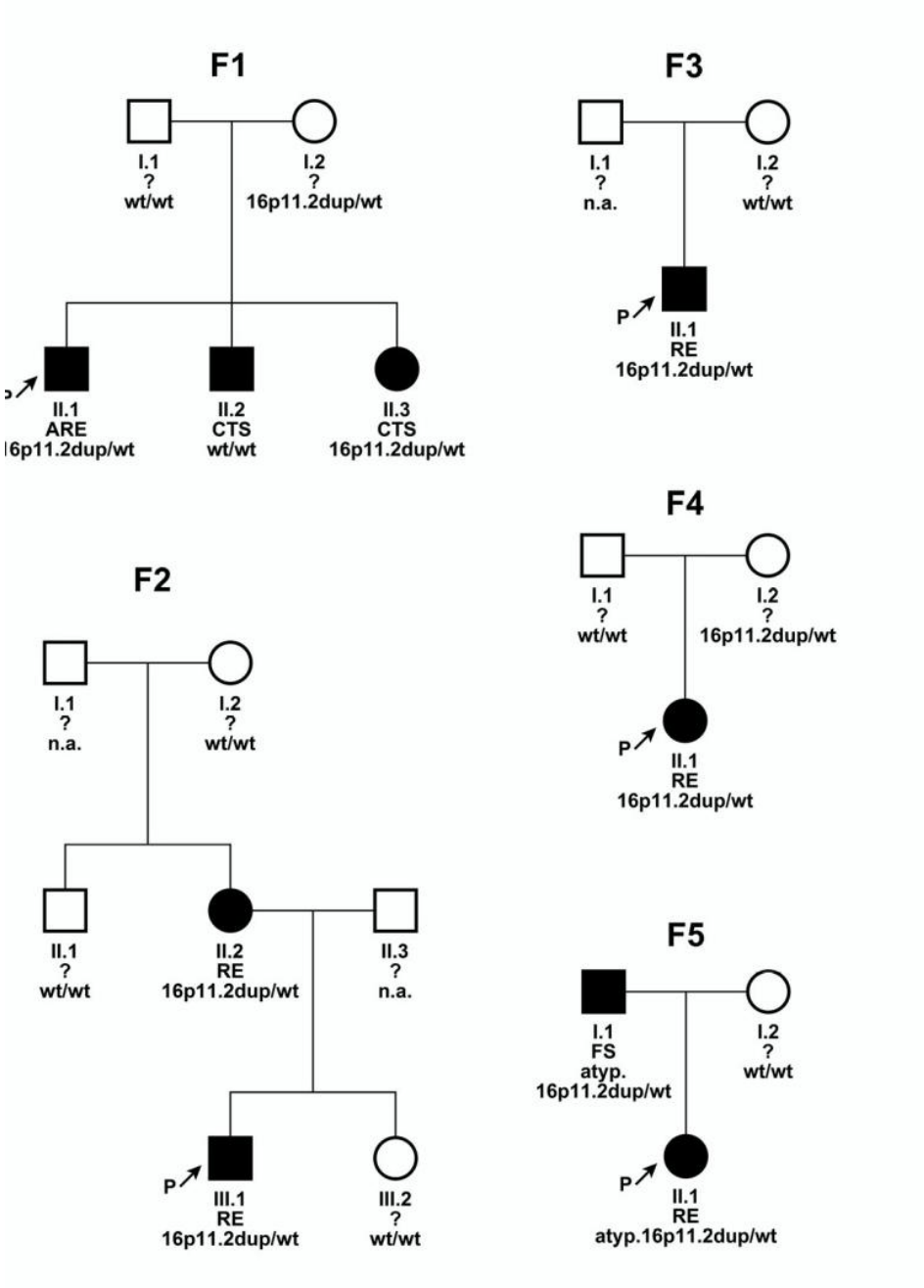


Figure 2


F1
I.1
?
wt/wt
I.2
?
16p11.2dup/wt
II.1
ARE
16p11.2dup/wt
II.2
CTS
wt/wt
II.3
CTS
16p11.2dup/wt
F3
I.1
?
n.a.
I.2
?
wt/wt
P
II.1
RE
16p11.2dup/wt
F2
I.1
?
n.a.
I.2
?
wt/wt
II.1
?
wt/wt
II.2
RE
16p11.2dup/wt
II.3
?
n.a.
P
III.1
RE
16p11.2dup/wt
III.2
?
wt/wt
F4
I.1
?
wt/wt
I.2
?
16p11.2dup/wt
P
II.1
RE
16p11.2dup/wt
F5
I.1
FS
atyp.
16p11.2dup/wt
I.2
?
wt/wt
P
II.1
RE
atyp.16p11.2dup/wt

Supplementary information

16p11.2 600 kb Duplications Confer Risk for Typical and Atypical Rolandic Epilepsy

Figures

Figure Legend

Supplementary Fig. 1 GRCh36/hg18 region on chromosome 16p11.2. Green box highlights genomic region of the atypical CNV detected in patient F5-II.1 inherited by her father F5-I.1. The CNV covers the genes *SPN*, *QPRT* and *C16orf54* but not the epilepsy associated gene *PRRT2*.

Tables

Supplementary Table 1 Summary of the Samples Used in This Study

Cohort	Phenotype	Sample Size	Gender m/f	Method
RE/ARE discovery cohort[a]		**281**	162/116	IlluminaHumanOmniExpressExomeBeadChip
	RE	*230*		
	ARE	*51*		
German inhouse controls[b]		**1512**	755/757	IlluminaHumanOmniExpressBeadChip
RE/ARE confirmation cohort[c]		***112***	63/49	IlluminaHumanOmniExpressBeadChip MLPA & qPCR
	RE	109		
	ARE	3		
Population controls[d]		**2256**	1179/1077	
	PopGen	1006		Affymetrix 6.0
	KORA	1250		Affymetrix 6.0
Generalized Epilepsy cohort[e]	*GGE*	**1408**		Affymetrix 6.0
Focal Epilepsy cohort[f]	*mTLE*	**330**		IlluminaHap550 array
16p11.2 CNV cohort[g]	*Children ascertained for ND*	**319**		Various techniques[33]
Deletion cases	*with epilepsy*	37/202		
Duplication cases	*with epilepsy*	22/117		

Table Legend

Supplementary Table 1 Summary of the Samples Used in This Study

a) Patients were recruited by collaborating centers of the EuroEPINOMICS-COGIE initiative. Samples were collected at the Department of Neurology, Medical University of Vienna, Austria and at the Department of Neuropediatrics, University Medical Clinic Giessen, Germany. 98 Samples were collected via multiplex pedigrees the remaining 183 samples were all single patients. Rolandic Epilepsy (RE); Landau-Kleffner syndrome (LKS), atypical benign partial epilepsy (ABPE) and electrical status epilepticus in slow wave sleep (CSWS) are subsumed under atypical Rolandic Epilepsy (ARE). **b)** In-house controls matched for analysis platform and geographic region. Population-based epidemiological controls drawn from the HNR (Heinz Nixdorf RECALL) study consisting of males and females aged 45 to 75 from the Ruhr area in Germany (n=1317) and additional in-house German control population (n=195). **c)** Additional RE/ARE patients recruited by collaborating centers of the EuroEPINOMICS-COGIE initiative and collected at the Department of Neurology, Medical University of Vienna, Austria (n=34). RE patients collected at the Epilepsy Research Centre, Melbourne Brain Centre, Department of Medicine, University of Melbourne, Australia (n=78) **d)** German control subjects available from the PopGen biobank (Population-Based Recruitment for Genetics Research) and KORA (Cooperative Health Research in the Region of Augsburg) **e)** genetic generalized epilepsy (GGE) patients collected by the EPICURE Project, for further description see (Lal *et al.*, 2013) **f)** patients with mesial temporal lobe epilepsy (mTLE) provided by the Department of Neuropathology, University of Bonn Medical Center, Bonn, Germany **g)** 16p11.2 rearrangement carriers recruited for neurodevelopmental disorders (ND) including : developmental delay (DD), intellectual disability (ID) and severe learning disorders (LD) through a network of more than 30 European cytogenetic centers.

Supplementary Table 2 CNV TaqMan Assay probes for the validation of the 16p11.2 and 15q11.2 duplications.

3.2.3.1 **TaqMan CNV Assays**		
3.2.3.2 Gene	**3.2.3.3 AssayID**	**3.2.3.4 CNV**
3.2.3.5 QPRT	3.2.3.6 Hs02383672_cn	3.2.3.7 16p11.2
3.2.3.8 SEZ6L2	3.2.3.9 Hs01431215_cn	3.2.3.10 16p11.2
3.2.3.11 MAPK3	3.2.3.12 Hs02001681_cn	3.2.3.13 16p11.2
3.2.3.14 CYFIP1	3.2.3.15 Hs02065338_cn	3.2.3.16 15q11.2

Supplementary Table 3. Frequency of different type of epilepsies in the 16p11.2 European consortium

	Partial	**Generalized**	**Infantile spasms**	**Unspecified**
DUP N=117 (%)	9 (7.7)	3 (2.5)	3 (2.5)	7 (6)
DEL N=202 (%)	12 (6)	7 (3.4)	2 (1)	16 (8)

Supplementary Table 4. Individuals with RE/ARE from the 16p11.2 European consortium

ID	Phenotype	Gender	W/H/HC (Zscore)	Age at seizures onset	Seizures	EEG	MRI	Neuropsychological development	Language development	Family history
526	RE	M	NA/-1/-0.3	7yrs	Rolandic seizures	CTS	normal	VIQ 95, NVIQ 86, FSIQ 85,[a] Attention deficit, reading difficulties	normal	Brother with RE
815	ARE	M	-1.3/-1.5/-2	2yrs	Rolandic seizures	CTS	normal	FSIQ 75,[b] behavioral impairment	delayed	No
567	ARE/LKS	M	NA	1.6yrs	Atypical absences	ESES	normal	severe delay, autistic signs	speech regression	No

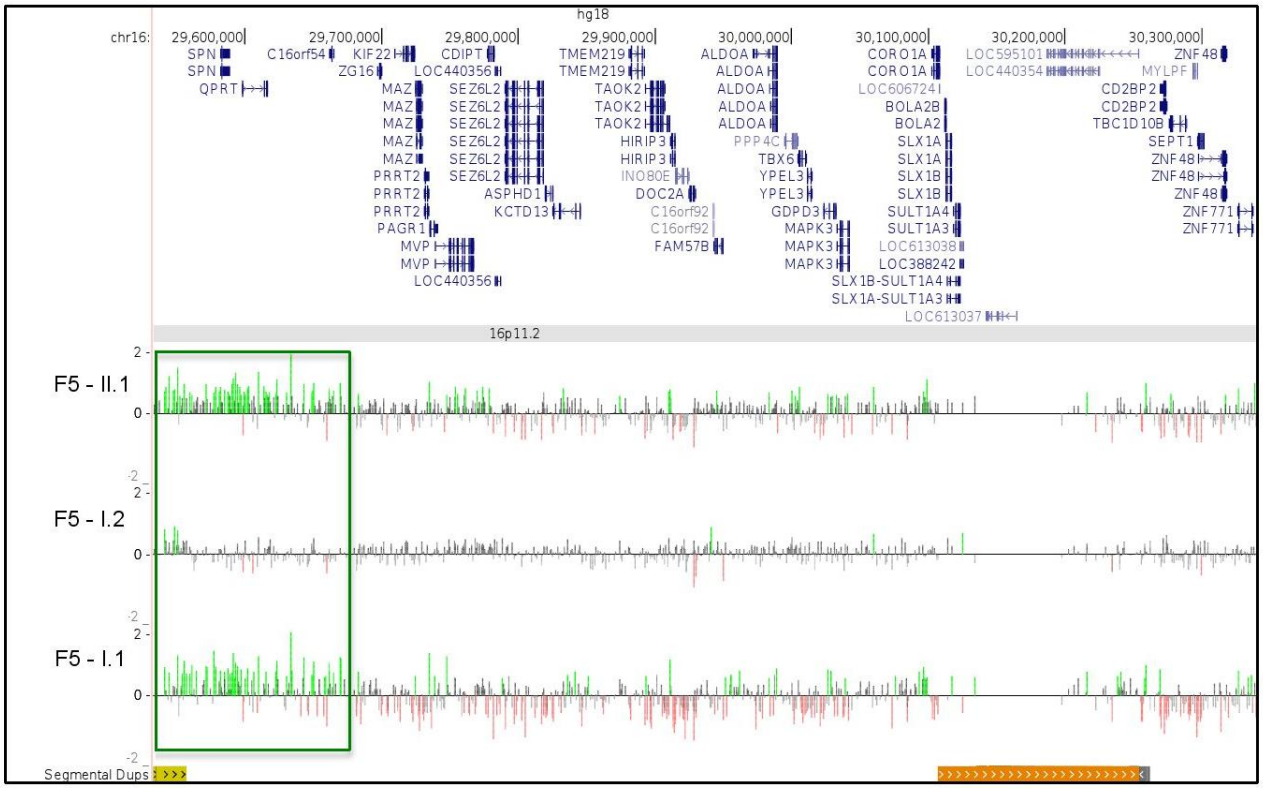
Table legend

Supplementary Table 4 Clinical features, MRI and EEG of individuals with RE/ARE from the 16p11.2 European consortium

PID: Patient Identity; RE: Rolandic Epilepsy; ARE: Atypical Rolandic Epilepsy; LKS: Landau-Kleffner Syndrome; W/H/HC: Weight, Height, Head Circumference; CTS: Centro-Temporal Spikes; ESES: Electrical Status Epilepticus during Sleep ; VIQ: Verbal Intelligence Quotient; NVIQ: Non Verbal Intelligence Quotient; FSIQ: Full Scale Intelligence Quotient; NA: Not Available; a) Wechsler Intelligence Scale Children-Third Edition performed at 10 years old; b) Wechsler Preschool and Primary Scale of Intelligence-Third Edition performed at 5,6 years old.

Reference

Lal D, Trucks H, Moller RS, Hjalgrim H, Koeleman BP, de Kovel CG, et al. Rare exonic deletions of the RBFOX1 gene increase risk of idiopathic generalized epilepsy. Epilepsia 2013; 54: 265-71.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30,100,000
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KIF22
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ALDOA
CORO1A
LOC595101
ZNF48
SPN
ZG16
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CORO1A
LOC440354
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QPRT
MAZ
SEZ6L2
TAOK2
ALDOA
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PPP4C
SLX1A
SEPT1
MAZ
SEZ6L2
HIRIP3
TBX6
SLX1A
ZNF48
PRRT2
SEZ6L2
INO80E
YPEL3
SLX1B
ZNF48
PRRT2
ASPHD1
DOC2A
YPEL3
SLX1B
ZNF48
PRRT2
KCTD13
C16orf92
GDPD3
SULT1A4
ZNF771
PAGR1
C16orf92
MAPK3
SULT1A3
ZNF771
MVP
FAM57B
MAPK3
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MVP
MAPK3
LOC388242
LOC440356
SLX1B-SULT1A4
SLX1A-SULT1A3
LOC613037
16p11.2
F5 - II.1
F5 - I.2
F5 - I.1
2
0
-2
Segmental Dups

3.2.4 *DEPDC5* in RE (in revision)

Lal D*, Reinthaler EM*, Schubert J*, Muhle M, et al., (2013) *DEPDC5* mutations in Rolandic epilepsy spectrum. **Annals of Neurology** (submitted)

DEPDC5 mutations in Rolandic epilepsy spectrum

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Abstract

Two recent studies have identified *DEPDC5* loss-of-function mutations in familial focal epilepsy with variable foci, autosomal dominant nocturnal frontal epilepsy and familial temporal lobe epilepsy. We report on four patients with classic Rolandic epilepsy and novel variants in *DEPDC5*, one with a truncating effect. In addition, we identified three families with atypical phenotypes carrying truncating mutations in *DEPDC5*. The detected variants were all novel, inherited and present in all family members exhibiting the epilepsy trait clinically or electroencephalographically. Altogether, our findings suggest that mutations of *DEPDC5* contribute to the genetic variance of genetic focal childhood epilepsy syndromes including Rolandic epilepsy.

Key words:

*DEPDC5, R*olandic epilepsy, focal genetic epilepsy, focal idiopathic epilepsy, mutation, gene

Introduction

Rolandic epilepsy (RE), or benign epilepsy with centrotemporal spikes (BECTS), is a common focal epilepsy syndrome of childhood.[1,2] RE is considered related to much rarer, less benign epilepsy syndromes, referred to as atypical Rolandic epilepsy (ARE) by some authors.[3] Notably, “atypical benign partial epilepsy” (ABPE) with astatic seizures and atypical absences,[4,5,6] Landau-Kleffner syndrome (LKS) with acquired speech dyspraxia, epileptic encephalopathies with continuous spike-and-wave during sleep (CSWS), and “epilepsy aphasia spectrum” with language delay and autistic features[7,8] may belong to a spectrum with RE on its benign and CSWS/LKS on its severe end.[3,6,8]

Mutations in *DEPDC5* encoding the Dishevelled, Egl-10 and Pleckstrin [DEP] domain-containing protein 5, have recently been identified as the major cause of inherited focal epilepsies including familial focal epilepsy with variable foci (FFEVF), autosomal dominant nocturnal frontal epilepsy (ADNFLE) and familial temporal lobe epilepsy (FTLE).[9,10] DEPDC5 is a member of the GATOR2 complex that interacts with the mTOR complex1 which is potentially accessible by mTOR inhibitors.[11]

The present study tested whether *DEPDC5* mutations contribute to genetic focal childhood epilepsies.

Patients and Methods

DEPDC5 screening cohort

To investigate an involvement of *DEPDC5* in the etiology of focal epilepsies in childhood, two cohorts comprising 290 European patients were included in the *DEPDC5* mutation screen:

Patients - RE cohort

Patients of European ancestry were recruited at several centers from Germany, Austria and Canada. The study was approved by all respective local institutional review boards. The investigated cohort consisted of index patients selected from 88 multiplex families with at least two affected siblings. In 88, families at least one of the affected probands suffered from RE or ARE, the second affected sibling presented either with RE, ARE or the EEG trait only. Of all 88 familiar index patients tested (male=54, female=34), 73 presented with RE, 12 with ARE, and 3 with ESES. In addition we included 120 non-familial patients (69 males, 51 females). Of these sporadic patients 111 suffered from RE, and nine from ARE.

Sequencing - RE cohort

For all 208 patients, exome data were generated which featured an average coverage >30x for 77% of the target sequences (for details see[12]). Variants were filtered for high-quality rare variants in *DEPDC5* by comparison to an in-house variation database, dbSNP build 137 (www.ncbi.nlm.nih.gov/projects/SNP/), 1000Genomes database (www.1000genomes.org/), and the Exome Variant Server (http://evs.gs.washington.edu/EVS/). Variant validation and segregation analyses were performed by Sanger sequencing following standard protocols.

Screening for *DEPDC5* mutations in non-Rolandic genetic focal epilepsy syndromes

Furthermore, mutations in *DEPDC5* were screened in a cohort of 82 families comprising a broad spectrum of genetic focal epilepsy phenotypes, with variable degrees of intellectual disability. This cohort was analyzed using a targeted next generation sequencing (NGS) panel approach covering 285 epilepsy-associated genes as described previously.[13]

Results

Detection and inheritance testing of rare variants in *DEPDC5*

Mutational screening of *DEPDC5* in 208 RE patients identified a total of two novel, heterozygous truncating mutations (Fig 1; Fig 2, Table 1), including a frameshift variant p.I1139Mfs*24 (c.3417delA; according to reference transcript NM_001242896.1, reference protein NP_001229825.1) and a splice site variant c.59-1G>C . Furthermore, we detected two novel nonsense variants p.R865* (c.2593C>T) and p.R243* (c.727C>T). In addition, we identified three missense mutations (p.V272L, c.814G>T; p.V90I, c.268G>A; p.S1153G, c.3457A>G) of uncertain significance in the RE cohort (Figure 1). The p.V272L and the p.S1153G mutations are predicted to be disease causing whereas the p.V90I exchange is predicted to effect splicing by *in silico* analysis using the Mutation T@ster Tool (http://www.mutationtaster.org). The variants were distributed through the whole gene and protein (Fig 2). None of the seven *DEPDC5* variants was present in dbSNP137, the 1000 Genomes Project database or in the 6,503 exomes for which variants are currently listed in the National Heart, Lung, and Blood Institute (NHLBI) exome variant server (EVS, accessed 06/2013) database. One stop codon generating variant and one frameshift variant in *DEPDC5* have been annotated in the 6,503 exomes listed in the EVS database, whereas no deleterious mutation was observed in >450 exomes of our in-house database fulfilling the same exome sequencing quality filters (various non-epilepsy projects; about 80% Caucasian ancestry). *DEPDC5* mutations occurred significantly more often in the RE cohort of affected individuals (2/208; P = 0.005 Fisher´s exact test).

Familial Segregation analysis

The segregation of *DEPDC5* variants identified in the RE index patients were tracked in seven families (Fig 1). In Family 3 the mutation was introduced by the grandfather and transmitted to the index patient by the mother. In the remaining five families the variants were inherited, once maternally and four times paternally. Four out of 18 variant carriers were affected by RE, one by Rolandic epilepsy persisting to adulthood (REPA), two by ABPE and two by unclassified epilepsies as well as one affected by unclassified cryptogenic epilepsy. Therefore, the penetrance for the epilepsy is incomplete. All tested individuals exhibiting seizures carried mutations in *DEPDC5*. Notably, we cannot rule out that seizure-free family members might have expressed the RE-characteristic

CTS EEG-trait at younger age, considering its age-related expression with maximum manifestation during childhood.

Clinical features of individuals with deleterious *DEPDC5* mutations

Family 1 (p.I1139Mfs*24; c.3417delA)

The index patient (F1-II.1) was diagnosed (classic) RE.

Family 2 (c.59-1G>C, splice site)

The male index patient (F2-II.1; c.59-1G>C) suffered from sensorimotor seizures of his right arm and revealed a characteristically shaped fronto-central sharp wave focus. He was diagnosed with RE and treated successfully with carbamazepine. After weaning the medication seizures reoccurred. The epilepsy was tentatively labeled “RE persisting to adulthood” (REPA in fig.1). His younger sister (F2-II-2; c.59-1G>C) presented with atypical absences (starring attacks) at age 3.5 years. Centrotemporal spikes were recorded bilateral at age 3.5 years, and left sided only at age 5.5 years. At age 8 years she suffered from repeated exercise-induced atypical absences preceded by an unspecific aura. She is seizure-free under ongoing oxcarbazepine therapy. The epilepsy was classified as ABPE (RE with atypical absences).

Family 3 (p.R865*; c.2593C>T)

The mother (F3-II.1; p.R865*) presented atonic seizures, starting at age 3 years, without any intellectual deficits. First documented EEG at 9 years and later at 9.5 years revealed temporo-posterior sharp waves bilateral. Since age 10 years she remained seizure-free without medication. Repeated EEG recordings were normal. The benign epilepsy is consistent with ABPE (RE with astatic seizures). Her son (F3-I.1, p.R865*) expressed myoclonic, atonic, and generalized tonic-clonic seizures started already at age 12 weeks. The epilepsy proved therapy-resistant and the child´s development remained severely impaired. The epilepsy was labeled unclassified cryptogenic focal epilepsy. The grandfather in this family (F3-III.2) suffered from a unclassified epilepsy (no more details available).

Family 4 (p.R243* ; c.727C>T)

The father (F4-II.2, DNA not available for testing) of our index patient reportedly had a focal epilepsy that resolved spontaneously during adolescence, possibly consistent with

RE. The available index patient (F4-I.3, p.Arg243*) however, did not present with a benign course. The patient´s seizures started at age 8 month. At age 2 years he showed myoclonic seizures with centro-temporal spikes, later during adulthood focal tonic seizures and multifocal spikes were documented. The epilepsy syndrome remained unclassified.

Clinical features of individuals with *DEPDC5* missense mutations

Family 5 (pV272L; c.814G>T), Family 6 (p.V90I; c.268G>A), Family 7 (p. S1153G; c.3457A>G)

For all index patients in these families (F5-II.1, F6-II.1, F7-II.1) the epilepsy was diagnosed as (classic) RE. In Family 5 the mutation carrying healthy brother (F5-II.2; pV272L) also displayed a characteristic EEG focus at age 4.9 years.

Discussion

Our *DEPDC5* screen revealed in total seven rare mutations, of those four are predicted to be severely damaging due to the insertion of a stop codon or splice site alterations, whereas the missense variations are of uncertain significance. However, i*n silico* analysis predicted two out of three missense variants as disease causing and one modifying splicing. Five families were part of our initial RE screening cohort (Fam. 1,2,5,6 and 7). Two families (Fam. 3 and 4) were members of the second cohort comprising 82 families with unclassified genetic focal epilepsies.

The observed penetrance in the analyzed families is like in previous reports incomplete.[9,10] One truncating mutation (Family 1) and three missense variants (Families 5-7) were found in individuals that were diagnosed as typical RE. The observed frequency of 2% (4/208) is lower compared to the previous reports on familial focal epilepsy with variable foci, autosomal dominant nocturnal frontal epilepsy and familial temporal lobe epilepsy.[9,10] In Family 2 carrying the tentative diagnosis “Rolandic epilepsy persisting to adulthood” a splice site variant was detected. In today’s view this family might also receive the diagnosis of “familial focal epilepsy with variable foci”. However, EEG records in this family repeatedly reported EEG findings characteristic for RE. Two additional families (Fam. 3 and 4) revealed a more or less private phenotype that differs greatly between affected individuals including intractable epilepsy and cognitive impairment.

In summary, our results strengthen *DEPDC5* as a focal epilepsy gene and extend the *DEPDC5*-related phenotypic spectrum to RE and related childhood epilepsies.

Appendix

Table RE patients with *DEPDC5* variants

PID	Phenotype	Gender	Age at onset	Seizures	EEG	MRT/ CT	Neuropsychologic development	Language development	Family History/ Relationship	DEPDC5 Variant
F1-II.1	RE	M	4.8 years	Nocturnal seizures	CTS	normal	normal	normal	negative	c.3417delA p.I1139Mfs*24
F2-II.1	RE persisting to adulthood (REPA)	M	8 years	Sensorimotor seizures	CTS bilateral and frontal	normal	normal	normal	Sibling of F2-II.2	c.59-1G>C splice site
F2-II.2	ABPE	F	3.5 years	Atypical absences, Sensorimotor seizures with aura, exercise induced	CTS Multifocal	normal	normal	normal	Sibling of F2-II.1	c.59-1G>C splice site
F3-II.1	ABPE	F	3 years	Atonic seizures, seizure free since puberty	ShW occipital	n.d	normal	normal	Mother of F3-I.1	c.2593C>T p.R865*
F3-I.1	Cryptogenic focal epilepsy	M	12 weeks	Atonic seizures, GTCS, myoclonic seizures	Mulitfocal and generalized SW	normal	Mental retardation	Severely delayed	Son of F3-II.1	c.2593C>T p.R865*
F3-III.2	Unclassified epilepsy "during childhood only"	M	Childhood	Possible GTCS	Not available	unknown	normal	normal	Grandfather of F3-I.1	c.2593C>T p.R865*

Clinical characteristics of *DEPDC5* variation carriers. RE= Rolandic Epilepsy, CTS= Centrotemporal spikes; ABPE= atypical benign partial epilepsy of childhood; REPA= Rolandic epilepsy persisting to adulthood; ShW= Sharpe Wave; GTCS=generalized tonic clonic seizure

Acknowledgments

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Potential Conflicts of Interest:

Authors must state explicitly whether potential conflicts do or do not exist. Authors should do so in the manuscript in a separate section following any Acknowledgements and preceding the References. Please provide additional detail, if applicable, to any potential conflicts of interest in your cover letter that accompanies the manuscript.

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Figure 1 Segregation of *DEPDC5* affecting variants

Segregation analyses could be performed for which DNA samples of family members were available. The respective *DEPDC5* mutations (NM_001242896.1, NP_001229825.1) co-segregated with a variable phenotype of either seizures or pathologic EEG patterns in most family members. Only a few individuals carried the respective familial mutation but did not present any clinical features, indicating incomplete penetrance of the mutations. However, subclinical phenotypes (e.g. EEG patterns) have not been investigated in these individuals (indicated by question mark). Abbreviations: n.a = DNA was not available for testing; RE = rolandic epilepsy; CTS = centrotemporal spikes; ABPE= atypical benign partial epilepsy of childhood, REPA = Rolandic epilepsy persisting to adulthood.

Figure 2 Overview of *DEPDC5* variants

Top: Genomic localization and structure of the identified *DEPDC5* variants (NM_001242896.1, hg19). Black dashes highlight the variant position. Bottom: Schematics of the DEPDC5 protein (NP_001229825.1), alterations are indicated.

Figures

Figure 1

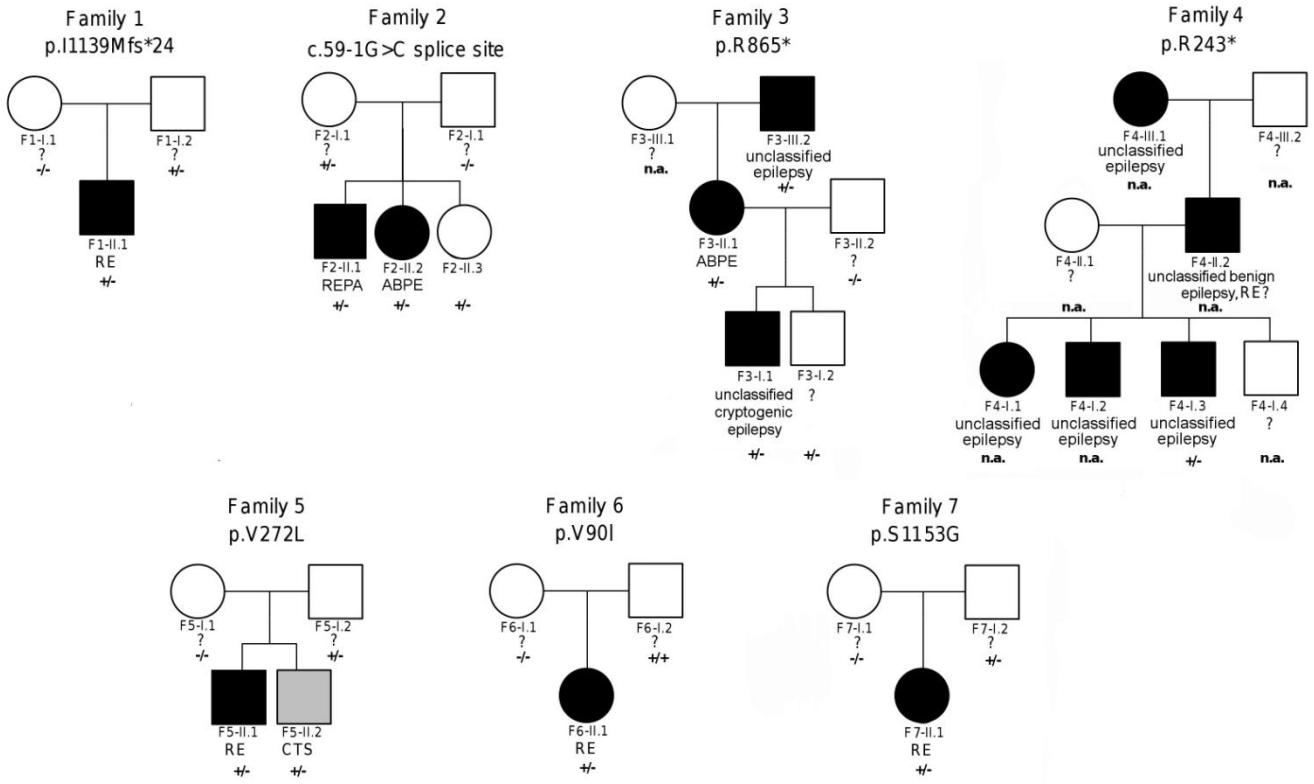
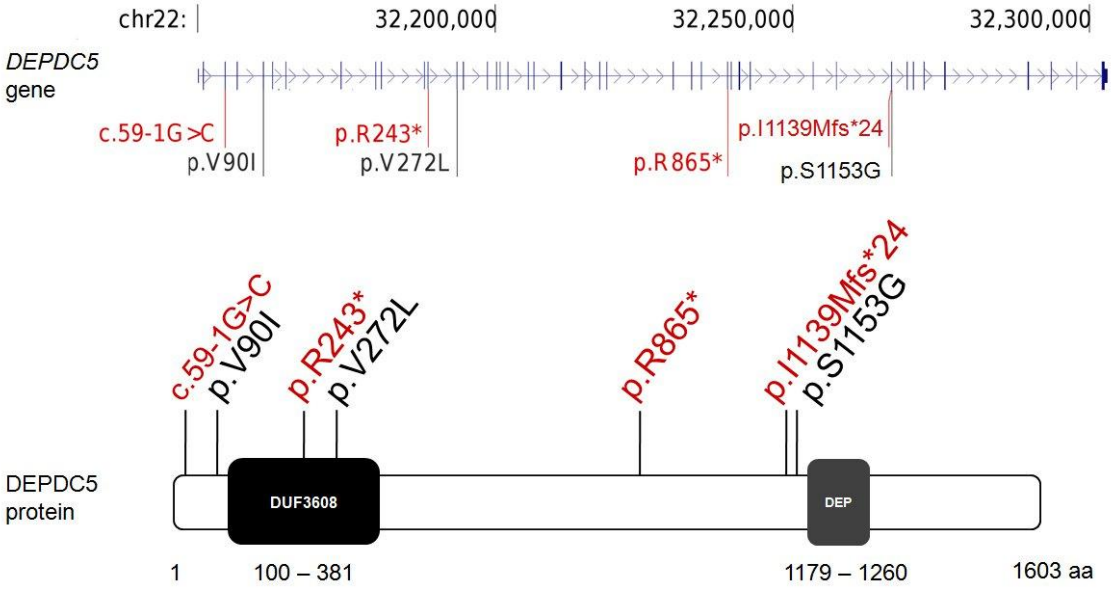


Figure 2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32,200,000
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32,300,000
DEPDC5
gene
c.59-1G>C
p.V90I
p.R243*
p.V272L
p.R865*
p.I1139Mfs*24
p.S1153G
c.59-1G>C
p.V90I
p.R243*
p.V272L
p.R865*
p.I1139Mfs*24
p.S1153G
DEPDC5
protein
DUF3608
DEP
1
100 – 381
1179 – 1260
1603 aa

3.3 Publications derived from additional projects

3.3.1 *NDUFV1* in IBSN (published)

Lal D, Becker K, Motameny S, Altmüller J, Thiele H, et al. (2013) Homozygous missense mutation of NDUFV1 as the cause of infantile bilateral striatal necrosis. **Neurogenetics** 14: 85–87. doi:10.1007/s10048-013-0355-z.

Neurogenetics (2013) 14:85–87
DOI 10.1007/s10048-013-0355-z

LETTER TO THE EDITORS

Homozygous missense mutation of *NDUFV1* as the cause of infantile bilateral striatal necrosis

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Infantile bilateral striatal necrosis (IBSN) includes a heterogeneous group of disorders, characterized by symmetrical degeneration predominantly of the caudate nucleus and the putamen [1]. The clinical features encompass pyramidal and extrapyramidal signs such as choreoathetosis, dystonia, spasticity, dysarthria, and dysphagia in conjunction with developmental regression or mental retardation, and additional neurological symptoms [1]. The differential diagnosis has to take into account a variety of toxic, infectious, metabolic, and neurodegenerative disorders [1]. In familial cases, mutations in the mitochondrial genes *ATP6* and *ND6*, and in the nuclear encoded genes nucleoporin 62 (*NUP62*) and mitochondrial thiamine pyrophosphate transporter (*SLC25A19*), have been described [2]. We report two siblings with IBSN and a mutation in *NDUFV1*, not yet linked to this condition.

The patients' parents are unrelated, but belong to a population of German settlers immigrating to Russia about 250 years ago (Fig. 1c). In both children, gait and speech difficulties were noticed from age 4 years. Neurological examination at age 9 (F3) and 5 years (F5) revealed dysarthria, muscular hypertonia with cogwheel rigidity, exaggerated deep tendon reflexes, and bilateral Babinski signs, while MRI demonstrated symmetric cystic lesions of the putamen (Fig. 1a, b). The patients were treated with biotin and thiamine before the definite diagnosis was established, and additionally, with creatine, riboflavin, and other vitamins thereafter. This resulted in a distinct and sustained improvement of their neurological status until last examination at age 11 and 7 years, respectively. Both still walk and attend regular schools.

Biochemical analysis of a muscle biopsy from patient F3 revealed isolated complex I deficiency (0.08 U/U citrate synthetase; normal 0.17–0.56). Therefore, we sequenced the entire mtDNA including the genes *ATP6* and *ND6* without detecting pathogenic variants [3, 4].

Electronic supplementary material The online version of this article (doi:10.1007/s10048-013-0355-z) contains supplementary material, which is available to authorized users.

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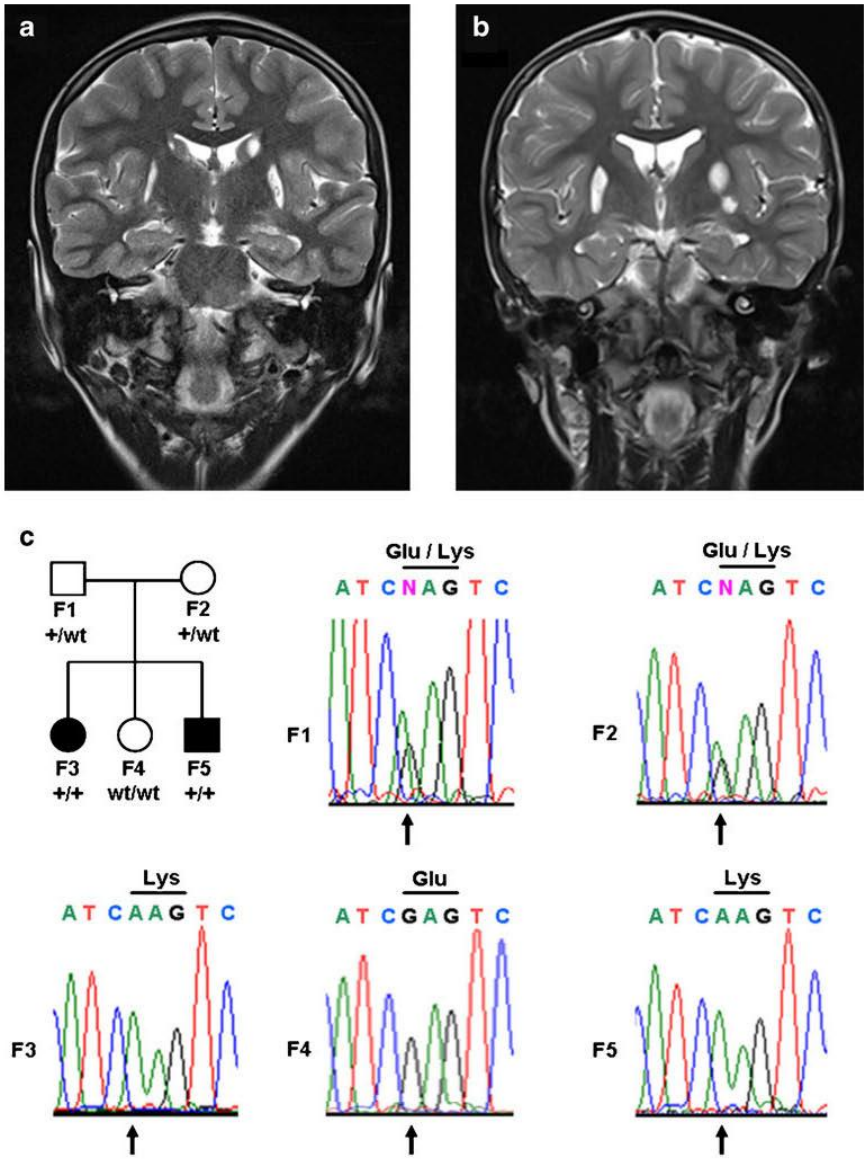

Fig. 1 MRI findings, pedigree chart, and mutation analysis of the family. **a** T2-weighted coronal MR image of patient F3 at age 9 years, demonstrating symmetric cystic lesions of the putamen and the caudate head. **b** T2-weighted coronal MR image of patient F5 showing bilateral liquor isotense lesions of the putamen at age 5 years, closely resembling those of his older sister. **c** Pedigree chart and Sanger sequencing electropherograms of all family members demonstrating a homozygous G>A transition at position c.640 of *NDUFV1* (*arrows*) in patients F3 and F5, a heterozygous transition in both parents (*F1* + *F2*), and two wild-type (*wt*) alleles in the healthy sister (F4) [5]

Because sequencing of the nuclear IBSN candidate genes *NUP62* and *SLC19A3* also yielded normal results, we sequenced the whole exome of both patients by applying a filter strategy that assumed autosomal recessive inheritance [3, 4]. Stringent filtering resulted in a list of six top variants that included the homozygous mutation c.640G>A (p.E214K) in *NDUFV1* (Fig. 1c and Supplementary material) [3 5].

The NDUFV1 protein is part of respiratory chain complex I and is directly involved in electron transfer from NADH. At least 17 subjects with *NDUFV1* mutations are on record, making it one of the most frequently reported genetic defects in complex I deficiency [6]. The majority of these patients suffered from Leigh and Leigh-like syndromes or was affected by progressive leukencephalopathies [5, 6].

Individuals with IBSN vary arbitrarily with respect to age at onset, clinical features, and course of disease [1]. Hitherto, nonprogressive course and discrepancy between distinct extrapyramidal signs and well-preserved mental functions were prominent features in our patients.

The mutation p.E214K is located in a highly conserved region of the NDUFV1 protein within the flavin mononucleotide binding site and has been reported once in a compound heterozygous state in a patient with a Leigh phenotype [5]. This argues for the pathogenicity of this variant, thereby expanding the phenotype associated with *NDUFV1* mutations and extending the list of genes associated with IBSN.

Ethical standards All examinations were done after obtaining written consent from the parents, comply with the current law, and a regional ethical board approved the study.

Conflict of interest The authors declare that they have no conflict of interest.

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Supplementary material to

Homozygous missense mutation of *NDUFV1* as the cause of infantile bilateral striatal necrosis

NEUROGENETICS

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Whole-exome sequencing

Genomic DNA was prepared according to Illumina's TruSeq Sample Preparation v2 (Illumina, CA, USA). Capture was performed with Illumina's TruSeq Exome Enrichment according to the manufacturer's instructions, targeting 62 megabases of functional sequences annotated, that include 20,794 target genes and 201,121 target exons. Sequencing was done on Illumina's HiSeq2000 using 2x100 bp paired-end reads. Sequence data were mapped using MAQ.[3] Duplicate reads were removed. Variant sites (single nucleotide variants and indels) were called using SAM tools.[4] 7.9Gb and 6,5Gb target aligning sequence data was obtained and led to a coverage of 30 reads or more for 87.2% and 84.3% of the exome target sequences for individuals F3 and F5, respectively. In total, 13533 (F5) and 13559 (F3) synonymous; and 13052 (F5) and 13185 (F3) non-synonymous variants were identified. Sequence data were filtered against dbSNP136, the 1000 Genomes Project data, variants annotated at the Exome Variant Server database (http://evs.gs.washington.edu/EVS/) and our in-house database of exome variants (with data from > 400 exomes of individuals affected by different disorders). Due to their impact on protein structure and function the analysis was focused on rare missense, nonsense, frameshift and splice-site variants. The filters applied to narrow down the list of variants and the 6 top variants identified are presented in table 1 and 2, respectively.

Table 1: Applied exome data filter strategy

Step	Applied Filter
1	Removal of all variants of known population frequency >0.001
2	Removal of all variants which are found more than 4 times in the in-house database
3	Removal of all variants which are not shared by both affected (F3,F5)
4	Base quality filter (Removal of all variants PHRED <14)
5	Coverage Filter (Removal of all variants TOTAL_DEPTH <6)
6	Removal of all heterozygous variants: Removal of variants with read frequency <75%
7	Removal of synonymous, intergenic, UTR and intronic variants Removal of all X and Y chromosome variants because of recessive model affecting male and female siblings

Table 2 List of exome variants shared by both patients when using the recessive inheritance model and applying stringent filter criteria shown in table 1.

Chromosome	Base pair position	Reference allele	Alternative allele	Gene	Protein change
11	**67377981**	**G**	**A**	**NDUFV1**	**GluLys**
1	110235888	T	A	GSTM1	SerThr
11	61017160	A	G	PGA4	ThrAla
1	13368530	G	C	PRAMEF5	ValLeu
11	64852639	A	G	ZFPL1	AsnSer
1	146246790	G	A	WI2-3658N16.1	GlnX

4 Overall discussion of the studies presented

4.1 Identified variants associated with IGE

We have previously shown that recurrent microdeletions at 15q11.2, 16p13.11 and especially 15q13.3 are genetic risk factors in up to 3% of common IGE syndromes and other neurodevelopmental disorders (Helbig et al., 2009; De Kovel et al., 2010; Mefford et al., 2010; Mulley et al., 2011). Our previous SNP-array CNV studies were limited to analysis of large recurrent microdeletions (>500 kb). Advances in CNV calling algorithms allowed us here to perform a deletion screening at single gene resolution. With regard to the low statistical power and CNV size variability, we focused on candidate microdeletions affecting genes involved in neuronal excitability.

We were able to detect microdeletions in two genes, the first encoding the neuronal splicing regulator *RBFOX1* (Lal et al., 2013a) and the second encoding the postsynaptic scaffolding protein gephyrin (*GPHN*; Dejanovic et al., in review). We demonstrated for the first time a significant association with deletions affecting *RBFOX1* with a disease (Lal et al., 2013a), thereby supporting previous single case reports relating *RBFOX1* deletions with a broad range of neurodevelopmental disorders including epilepsy (Bhalla et al., 2004; Martin et al., 2007; Gallant et al. 2011). All *RBFOX1* deletions identified were located within the highly conserved 5'-untranslated exons and are predominantly expressed in brain, suggesting that the 5'-terminal *RBFOX1* region contains important regulatory elements (Damianov et al., 2010), which when lost might alter translation efficiency and mRNA stability (Wang et al., 2005). Consistent with our findings, the structural genomic variations of the *RBFOX1* gene reported previously in three single patients with neurodevelopmental disorders, including epilepsy, also disrupted the 5'-terminal *RBFOX1* exons (Bhalla et al., 2004; Martin et al., 2007; Gallant et al. 2011). Furthermore, a recent study published after our IGE study, could identify additional 12 deletions in *RBFOX1* all affecting the 5'-terminal *RBFOX1* exons in a cohort of 2124 pediatric patients with neurodevelopmental diseases (Zhao 2013). Six out of the 12 deletion carriers had epilepsy.

In our study on *GPHN*, we identified two patients with deletions and none in 2792 controls (Dejanovic et al., in revision). These findings are in line with a recent study that demonstrated significant association of rare *GPHN* microdeletions with

neurodevelopmental disorders (*P* = 0.009; 6/8775 cases versus 3/27019 controls) and found that three out of five *GPHN* microdeletions tested for inheritance arose as *de novo* events (Lionel et al., 2013). In our study, we furthermore present additional functional data elucidating the molecular mechanisms by which the N-terminal *GPHN* deletions cause dysfunctional GABAergic synaptic inhibition and thereby increase susceptibility of IGE (Dejanovic et al., in revision). Both *GPHN* deletions affected exons of the gephyrin G-domain, alike previously reported *GPHN* deletions (Lionel et al., 2013). We were able to show that one of our truncated gephyrin transcripts is stably expressed in human cells and perturbs the clustering of wild-type gephyrin at inhibitory synapses in cultured mouse hippocampal neurons in a dominant-negative manner. This effect is consistent with previous observations where stress-induced irregular splicing of *GPHN* resulted in curtailed postsynaptic gephyrin and $GABA_A$ α2 receptor scaffolds (Förstera et al., 2010). In contrast, the other *GPHN* deletion causes a frameshift resulting in a premature stop codon (p.V22Gfs*7; NP_001019389.1) and hence translating into a short non-functional protein, which most likely leads to haplo-insufficiency of the gene. Our *in vitro* analysis showed a reduced amount of the synaptic γ-aminobutyric acid type A receptor ($GABA_AR$) in the presence of the truncated Δ5-9 gephyrin (Dejanovic et al., in review). This pathogenic alteration results in a functionally convergent effect with a reduced GABAergic synaptic inhibition similar to that observed with mutations of genes encoding $GABA_AR$ subunits (*GABRA1, GABRB3, GABRG2* and *GABRD*) in rare families with dominantly inherited IGE syndromes (for reviews see Macdonald et al., 2013; Cossette et al., 2012).

In summary, we detected a significant statistical association of *RBFOX1* deletions with common idiopathic epilepsies (Lal et al., 2013a; Lal et al., 2013b). In addition, we provided genetic, functional and neurophysiological evidence that structural exonic microdeletions affecting the gephyrin G-domain can increase neuronal excitability by an impairment of GABAergic synaptic inhibition, and thereby confer susceptibility to IGE (Dejanovic et al., in revision). Taken together with our previous findings (Helbig et al., 2009; De Kovel et al., 2010), our results demonstrate that also partial genomic deletions of genes collectively account for a significant fraction of the heritability of common IGE syndromes. This observation is in line with studies on other neurodevelopmental diseases like e.g. schizophrenia where all of the currently

known risk CNVs are rare and confer substantial effects on risk (odds ratios 3–30; Kirov et al., 2013).

4.2 Identified variants associated with RE-spectrum epilepsies

Despite the strong genetic contribution to the CTS-EEG trait, as shown in several family studies, the etiology of RE/ARE is largely unknown (Neubauer et al., 1998; Bali et al., 2007; Strug et al., 2009). In the past, a significant genetic etiology of RE was questioned because of low concordance rates in monozygous twins (Vadlamudi et al., 2004, 2006). In contrast, a recent study suggests that RE has a genetic component consistent with complex inheritance based on the increased frequency of epilepsies in relatives of RE patients (Vears et al., 2012). To clarify this question we performed a comprehensive genomic screen for exonic coding mutations in 242 RE/ARE patients as well as CNVs in 308 RE/ARE patients representing the largest cohort of RE-spectrum patients investigated up to date.

We demonstrate that mutations and CNVs in the *GRIN2A* gene encoding the NMDA-receptor NR2A subunit represent a major genetic risk factor in 7.5% of children with idiopathic focal epilepsies including RE (Lemke et al., 2013). This is of particular interest as NMDA receptors are promising treatment targets. It has been shown that NMDAR antagonists have antiepileptic effects in both clinical and preclinical studies (Ghasemi et al., 2011). At least 20 NMDAR antagonists are marketed or in development for a broad range of neurology indications. (Osherovich et al., 2013). There is some evidence that conventional antiepileptic drugs may also affect NMDAR function (Ghasemi et al., 2011). Based on our (Lemke et al., 2013) and convergent results of other groups (Lesca et al., 2013; Carvill et al., 2013) commercial companies (e.g. Naurex, Inc.) started physiological characterization of epilepsy-associated mutations in cell culture and animal models (Osherovich et al., 2013; http://www.naurex.com/).

We also found that exonic deletions and truncating mutations of *RBFOX1* as well as *RBFOX3* contribute to the genetic variance of common idiopathic epilepsy syndromes (Lal et al., 2013a; Lal et al., 2013b). In addition, our present results demonstrate that mutations in *DEPDC5* predispose specifically to focal epilepsies including RE (Dibbens et al., 2013; Ishida et al., 2013; Lal et al., in revision). Moreover, genome-wide CNV screening identified 16p11.2 duplications as an

important genetic risk factor for the RE-spectrum epilepsies (Reinthaler et al., in revision), thereby further supporting the associations between 16p11.2 duplications and early-onset neurodevelopmental disorders (Bijlsma et al., 2009; Jacquemont et al., 2011; McCarthy et al., 2009; Shinawi et al., 2010; Walters et al., 2010; Weiss et al., 2008; Zufferey et al., 2012). In summary, we were able to identify genetic risk factors in a significant fraction (~10%) of RE and ARE patients investigated in our study cohort. These results prove a significant genetic contribution to RE/ARE that was challenged previously (Vadlamudi et al., 2004, 2006).

4.3 Epilepsy genetics

Epilepsy is one of the most common neurological disorders, characterized by recurrent unprovoked seizures due to neuronal hyperexcitability and abnormal synchronization (Hauser et al., 1993). We have genetically investigated two common major subtypes of idiopathic epilepsies (i) idiopathic generalized epilepsies (IGEs) and (ii) the RE-spectrum epilepsies. Both groups are distinguishable by their leading seizure types, age-dependent onset and electroencephalographic criteria. For IGE a whole string of literature supports a strong genetic etiology (reviewed by Helbig et al., 2008) whereas for RE the genetic impact in disease has been challenged previously (Vadlamudi et al., 2004, 2006). Both idiopathic epilepsies were expected to have a complex genetic architecture (Figure 3).

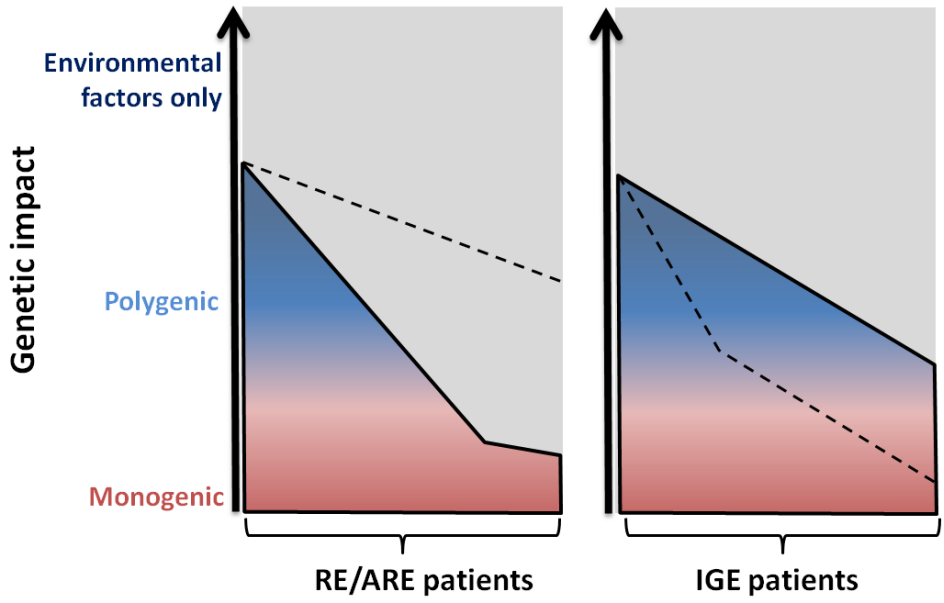

Figure 3 Expected genetic factors for idiopathic epilepsies. Dashed lines indicate the expected genetic contribution to disease prior to our studies and solid lines indicate the current notion of the contributing genetic factors.

We have identified genetic risk factors in *RBFOX1*and *RBFOX3* (Lal et al., 2013a; Lal et al., 2013b), *GRIN2A* (Lemke et a., 2013), *GPHN* (Dejanovic et al., in revision), *DEPDC5* (Lal et al., in revision) as well as at the locus 16p11.2 (Reinthaler et al., in revision). However, these variants are not prevalent in a similar frequency in all epilepsy types. As an example, we detected *GRIN2A* mutations (Lemke et al., 2013) in a broad range of focal childhood epilepsies but we did not observe these in epilepsies with age-of-onset in adulthood (data not shown). Furthermore, *DEPDC5* mutations are likely to be responsible for a fraction of focal epilepsies regardless of age of onset (Dibbens et al., 2013; Ishida et al., 2013; Lal et al., in revision) but not for generalized epilepsies (data not shown). The 16p11.2 duplication is absent in temporal lobe epilepsies as well as IGE syndromes but constitutes, besides the RE-spectrum (Reinthaler et al., 2013), an important genetic risk factor for autism (Weiss et al., 2008) and obesity (Jacquemont et al., 2011). Otherwise, exonic deletions of *RBFOX1* are present in both types of common idiopathic epilepsies. Our identified CNVs and gene mutations are not specific to epilepsy. Notably, the variants are also implicated in other epilepsy types and a broad range of neurodevelopmental diseases including autism, schizophrenia and intellectual disability (Coe et al., 2012; Helbig et al., 2009; De Kovel et al., 2013; Mefford et al., 2010; Mulley et al., 2011; Reutlinger et al., 2010; Lesca et al., 2012; Lionel et al., 2013; Förstera et al., 2010; Bhalla et al., 2004; Martin et al., 2007; Gallant et al., 2011, Dibbens et al., 2013; Ishida et al., 2013).

The penetrance for all identified variants is incomplete as illustrated by the presence of apparently clinically unaffected variant carriers in the families. This is in agreement with observations from large recurrent microdeletions previously identified in IGE (Helbig et al., 2009; Dibbens et al., 2009; De Kovel et al., 2010) and a range of neurodevelopmental disorders (reviewed by Carvill et al., 2013). Most likely, many identified variants impair basic neurodevelopmental processes that affect a variety of physiologic functions. However, a close relation between severe childhood epilepsies and autism has been expected (reviewed in Coe et al., 2012). Likewise, the severity and expressivity of the phenotypes varied considerably in all our studies between affected members of the same family. Thus, the individual epilepsy phenotype is probably further specified by the interplay of environmental influences with genetic background following an oligo-/polygenic inheritance model with substantial genetic heterogeneity (Mulley et al., 2011).

Based on our small cohort sizes and the large amount of rare variants per individual we had insufficient statistical power to analyze our data at a genome-wide level. Extrapolation of effect sizes and frequencies from published studies show that thousands of individuals are required to reach acceptable statistical power in WES studies (Kiezun et al., 2012). Therefore, the presented studies are based on *a priori* assumptions of a candidate gene/locus hypothesis. Consequently, a potentially large part of the individual genetic disease predisposition is not explored. In the future, the constantly increasing study sizes through worldwide collaborations may allow well-powered screens for detection of mutations with major to modest effect sizes.

Furthermore, the individual epilepsy-causing genetic predisposition is also likely to be influenced by common variants (Mullen et al., 2009). Studies on common genetic variants like our recently published genome-wide association study for IGE (3020 IGE patients and 3954 controls) have identified common susceptibility loci with minor effects (EPICURE Consortium et al., 2012). The translation of these results into biological context is challenging (EPICURE et al., 2012, reviewed by Edwards et al., 2013). In general, our ongoing WES-based mutation screening studies solely focus on the CCDS-defined protein-coding regions. However, recent publications by the ENCODE project have assign biochemical functions to 80% of the genome, especially in genomic regions outside of the well-studied protein-coding regions (ENCODE Project Consortium et al., 2012), opening new avenues for disease research. Thus, studies on candidate genes, novel epilepsy associated genes, non-protein coding regions of the genome and the epigentic landscape are warranted to elucidate the complex genetic interplay in the etiology of idiopathic epilepsies.

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6 Summary / Zusammenfassung

6.1 Summary

Epilepsy is one of the most common neurological disorders characterized by recurrent unprovoked seizures due to increased neuronal hyperexcitability and abnormal synchronization. I have explored the genetic architecture of the most common types of idiopathic epilepsies, the idiopathic generalized epilepsies (IGEs) and the spectrum of idiopathic focal epilepsies related to rolandic epilepsies (REs). Both groups are distinguishable by the leading seizure types, age-dependent onset and electroencephalographic criteria. For IGEs a complex genetic contribution is indicated from family and twin studies, whereas the genetic basis of REs has been a matter of debate. At present, the majority of genetic factors predisposing to common idiopathic epilepsies remain elusive. The aim of the present studies was the molecular genetic dissection of genetic risk factors with strong epileptogenic effects in idiopathic epilepsies and the elucidation of their molecular pathways in epileptogenesis. Therefore, our group conducted copy number variation (CNV) analyses using high-resolution SNP arrays in two case-control cohorts comprising: i) 1582 IGE and 2795 populations controls, and ii) 308 patients with RE-spectrum epilepsies and 1512 controls of European descent. In addition, we performed candidate gene sequence analysis in 242 patients with RE-spectrum epilepsies. With regard to the low power of our study samples for genome-wide scans, our current strategies focused on a candidate gene/locus approach of genes and CNVs that have been implicated in the pathogenesis of epilepsies and related neurodevelopmental disorders, as well as candidate genes with a presumed impact on neuronal excitability. In the present cumulative thesis, I report the results of our current CNV and sequence analyses of highly plausible candidate genes based on three peer-reviewed publications and another three manuscripts that are currently in revision.

We are the first to show a significant statistical association for deletions affecting the neuronal splicing regulator *RBFOX1* with idiopathic epilepsies (Lal et al., 2013a). Moreover, we also detected deletions and truncating mutations in *RBFOX1* as well as in the neuronal splicing regulator *RBFOX3* in the RE cohort (Lal et al., 2013b). We provide genetic, functional, and neurophysiological evidence that structural exonic microdeletions affecting the *GPHN* gene, which codes for the synaptic scaffolding

protein gephyrin, may increase neuronal excitability by an impairment of GABAergic synaptic inhibition and thereby confer susceptibility for IGE (Dejanovic et al., in revision). We also found that missense and truncating exonic mutations and microdeletions of the *GRIN2A* gene encoding the NMDA-receptor NR2A subunit are a major genetic risk factor in 7.5% of children with idiopathic focal epilepsies including RE-spectrum epilepsies (Lemke et al., 2013). Notably, mutations in the *DEPDC5* gene, encoding DEP domain-containing protein 5, preferentially predispose to focal idiopathic epilepsies (Lal et al., in revision). Finally, screening for recurrent CNVs revealed 16p11.2 duplications as an important genetic risk factor for the spectrum of REs (Reinthaler et al., in revision), thereby further supporting the associations between 16p11.2 duplications and early onset neurodevelopmental disorders.

In conclusion, our studies identified several genetic risk factors in a significant fraction of idiopathic epilepsy patients. Moreover, the present study is the first that clearly proves a genetic impact on the etiology of the common REs at the molecular genetic level. We are aware that our small samples sizes provide insufficient power for exome-wide rare-mutation or genome-wide rare-CNV association testing. Future large-scale studies are warranted to identify additional pathogenic variants to better understand the complex genetic architecture of the most common types of idiopathic epilepsies.

6.2 Zusammenfassung

Epilepsie ist eine der häufigsten neurologischen Erkrankungen, charakterisiert durch wiederkehrende, nicht provozierte epileptische Anfälle aufgrund neuronaler Übererregbarkeit sowie gesteigerter kortikaler Synchronisation. In der vorliegenden Arbeit wurde die genetische Architektur der beiden häufigsten Formen von idiopathischen Epilepsien, den idiopathisch generalisierten Epilepsien (IGE) und der idiopathisch fokalen Epilepsie (sog. Rolando Epilepsie, RE) untersucht. Beide Gruppen unterscheiden sich hinsichtlich ihrer charakteristischen Anfallstypen, dem altersabhängigen Auftreten und dem elektroenzephalographischen Muster. Familien- und Zwillingsstudien belegen eine vorwiegend genetische Disposition der häufigen IGE Syndrome, während die Beteiligung von genetischen Einflüssen an der Ätiologie der RE bislang umstritten war. Bei den häufigen idiopathischen Epilepsien ist die Mehrzahl der beteiligten genetischen Faktoren noch unbekannt. Das Ziel der vorliegenden Arbeit war die molekulargenetische Identifikation von genetischen Risikofaktoren. Aufgrund der geringen Größe unserer Kollektive war nur die Identifikation von Varianten mit starken epileptogenen Effekten möglich. Hierzu haben wir *copy number variation* (CNV)-Analysen mit hochauflösenden SNP-Arrays in zwei Patienten-Kontroll-Kohorten durchgeführt. Die Kollektive bestanden aus: i) 1582 IGE-Patienten und 2795 Kontrollen mit europäischer Herkunft und ii) 308 Epilepsie-Patienten aus dem RE-Spektrum und 1512 Populationskontrollen europäischer Herkunft. Darüberhinaus haben wir Kandidatengen Sequenzanalysen bei 242 Patienten mit RE durchgeführt. Im Hinblick auf die geringe statistische Größe unserer Studienkohorten waren unsere Strategien auf Kandidatengen/-lokus-Ansätze ausgelegt. Dafür wurden Kandidatengene und -CNVs ausgewählt, die eine Funktion in der synaptischen Transmission aufweisen oder bei anderen neurologischen Erkrankungen bereits als Risikofaktoren identifiziert wurden. In der vorliegenden kumulativen Dissertation berichte ich über die Ergebnisse unserer aktuellen CNV und Sequenzanalysen und beziehe mich auf drei veröffentlichte Manuskripte, sowie auf drei weitere Artikel, die sich derzeit in Revision befinden.

Unsere Arbeiten belegen erstmals eine signifikante statistische Assoziation von Mikrodeletionen, die den neuronalen Spleiß-Regulator *RBFOX1* betreffen, mit idiopathischen Epilepsien (Lal et al., 2013a). Ferner konnten wir nachweisen, dass Deletionen und *loss-of-function*-Mutationen von *RBFOX1* und *RBFOX3* in der RE-

Kohorte auftreten (Lal et al., 2013b). Wir fanden genetische, funktionale und neurophysiologischen Belege dafür, dass exonische Mikrodeletionen in *GPHN* die neuronale Erregbarkeit durch eine Beeinträchtigung der GABAergen synaptischen Hemmung steigern und dadurch das Risiko für IGE erhöhen (Dejanovic et al., in Revision). Wir konnten ebenso belegen, dass *missense*- und *loss-of-function*-Mutationen sowie Mikrodeletionen von *GRIN2A*, das für eine Untereinheit des NMDA-Rezeptors NR2A kodiert, einen genetischen Risikofaktor bei 7,5 % der Kinder mit idiopathischen fokalen Epilepsien darstellen (Lemke et al., 2013). Schließlich konnten wir zeigen, dass Mutationen in *DEPDC5* präferentiell zu fokalen idiopathischen Epilepsien prädisponieren (Lal et al., in Revision). Unser Screening für rekurrente CNVs identifizierte 16p11.2-Duplikationen als signifikanten genetischen Risikofaktor für das Spektrum der RE-assoziierten Epilepsien (Reinthaler et al., in Revision). Dadurch konnten wir die Assoziation zwischen 16p11.2-Duplikationen und früh einsetzenden neurologischen Entwicklungsstörungen erhärten und das phänotypische Spektrum um die RE erweitern.

Zusammenfassend haben unsere Studien mehrere genetische Risikofaktoren bei einem bedeutenden Anteil der idiopathischen Epilepsie-Patienten identifiziert. Die Ergebnisse unserer Untersuchungen belegen nunmehr das Vorliegen einer genetischen Disposition für die RE eindeutig. Große, multizentrische Studienkollektive werden die Vorrausetzung schaffen, um systematisch seltene Mutationen und CNVs zu identifizieren. Die Identifikation von weiteren genetischen Risikofaktoren wird die Grundlage zu einem besseren Verständnis der komplexen genetische Architektur der Epilepsien bilden.

7 Appendix

7.1 Declaration of contribution as co-author

I am first author or shared first author in all studies included in this dissertation. I contributed majorly or equally (if shared first author) in the following processes in the studies listed in chapter 2:

- Study design
- Exome Sequencing
- Mutation identification and analysis
- Genotyping, CNV calling, analysis and validation
- Transcript analysis
- Statistical analysis
- Data interpretation
- Manuscript writing

Statutory declaration and statement

Ich versichere, dass ich die von mir vorgelegte Dissertation selbständig angefertigt, die benutzten Quellen und Hilfsmittel vollständig angegeben und die Stellen der Arbeit einschließlich Tabellen, Karten und Abbildungen , die anderen Werken im Wortlaut oder dem Sinn nach entnommen sind, in jedem Einzelfall als Entlehnung kenntlich gemacht habe; dass diese Dissertation noch keiner anderen Fakultät oder Universität zur Prüfung vorgelegen hat; dass sie - abgesehen von Teilpublikationen - noch nicht veröffentlicht worden ist sowie, dass ich eine solche Veröffentlichung vor Abschluss des Prüfungsverfahrens nicht vornehmen werde.

Die Bestimmungen dieser Promotionsordnung sind mir bekannt.

Die von mir vorgelegte Dissertation ist von Prof. Dr. Peter Nürnberg betreut und von Herr Prof. Dr. Neubauer sowie von Herr Prof. Dr. Nöthen begutachtet worden.

Teilpublikationen sind unter "List of publications" aufgeführt.

Ich versichere, dass ich alle Angaben wahrheitsgemäß nach bestem Wissen und Gewissen gemacht habe und verpflichte mich, jedmögliche, die obigen Angaben betreffenden Veränderungen, dem Dekanat unverzüglich mitzuteilen.

Datum Unterschrift

7.2 Curriculum Vitae

Personal Data

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Education and Training

since 08/2011:	PhD Thesis on genetics of idiopathic epilepsy syndromes (Supervised by Prof. Nürnberg, Prof. Neubauer, Dr. Sander)
since 10/2011:	Cluster of excellence CECAD Graduate School fellow
4/2009 - 8/2011:	Master of Science program (University of Cologne)
10/2005 - 3/2009:	Bachelor of Science program (University of Cologne)

International internships

11/2012 - 12/2012:	Visiting scientist for collaborative work during PhD project (Prof. Mefford) (Division of Genetic Medicine, University of Washington, USA)
09/2010 - 12/2010:	Research project in Immunology (The John Curtin School of medical Research, Australian National University, Canberra, Australia)

Advanced courses qualified and attended

06/2012:	Wellcome Trust Advanced Courses: Human Genome Analysis: Genetic Analysis of Multifactorial Diseases (7 days; Hinxton, Cambridge, UK)
05/2012:	EMBO Practical course: Bioinformatics and Comparative Genome Analyses (14 days; Naples, Italy)

Service to scientific community and honors

11/2013:	1st place poster award Annual EuroEPINOMICS Consortium meeting 2013
since 11/2013:	Author & editor of http://channelopathist.net (Scientific blog about the genetics of the epilepsies)
since 8/2013:	Member of the Genetics Commission of the International League against Epilepsy
06/2013 - 11/2013	Member of the organizing committee for the 2nd CECAD Graduate Symposium
since - 1/2013:	Reviewer for human genetics section: Neuropediatrics

7.3 List of publications

Lemke JR*, **Lal D***, Reinthaler EM*, Steiner I, Nothnagel M, et al. (2013) Mutations in GRIN2A cause idiopathic focal epilepsy with rolandic spikes. **Nat Genet** 45: 1067–1072. doi:10.1038/ng.2728. **(IF: 35.2)**

Lal D, Trucks H, Møller RS, Hjalgrim H, Koeleman BPC, et al. (2013) Rare exonic deletions of the RBFOX1 gene increase risk of idiopathic generalized epilepsy. **Epilepsia** 54: 265–271. doi:10.1111/epi.12084. (IF: 3.9)

Lal D, Reinthaler EM, Altmüller J, Toliat MR, Thiele H, et al. (2013) RBFOX1 and RBFOX3 Mutations in Rolandic Epilepsy. **PLoS ONE** 8: e73323. doi:10.1371/journal.pone.0073323. **(IF:3.7)**

Lal D, Becker K, Motameny S, Altmüller J, Thiele H, et al. (2013) Homozygous missense mutation of NDUFV1 as the cause of infantile bilateral striatal necrosis. **Neurogenetics** 14: 85–87. doi:10.1007/s10048-013-0355-z. **(IF:3.6)**

Mullen SA., Carvill GL, Bellows S, Bayly MA, Trucks H, **Lal D**, Sander T, Berkovic SF, Dibbens LM, Scheffer IE, and Mefford HC (2013) Copy number variants are frequent in genetic generalized epilepsy with intellectual disability. Neurology 10.1212/WNL.0b013e3182a95829. **(IF:8.3)**

Yabas M, Teh CE, Frankenreiter S**, Lal D,** Roots CM, et al. (2011) ATP11C is critical for the internalization of phosphatidylserine and differentiation of B lymphocytes. **Nat Immunol** 12: 441–449. doi:10.1038/ni.2011. **(IF:26.2)**

Reinthaler EM*, **Lal D***, Reymond A, Jacquemont et al. 16p11.2 duplications confer risk for typical and atypical Rolandic epilepsy. **BRAIN** (in revision) **(IF:9.9)**

Dejanovic B*, **Lal D***, Catarino BC*, Arjune S et al. Exonic microdeletions of the gephyrin gene exert a dominant-negative effect on GABAergic synaptic inhibition in two patients with idiopathic generalized epilepsy. **Neurobiology of Disease** (in revision) **(IF:5.4)**

Lal D*, Reinthaler EM*, Schubert J*, Muhle M, et al., (2013) DEPDC5 mutations in Rolandic epilepsy spectrum. **Annals of Neurology** (in revision) **(IF:11.3)**

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