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De novo mutations in the classic epileptic encephalopathies

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Abstract

Epileptic encephalopathies (EE) are a devastating group of severe childhood epilepsy disorders for which the cause is often unknown. Here, we report a screen for *de novo* mutations in patients with two classical EE: infantile spasms (IS, n=149) and Lennox-Gastaut Syndrome (LGS, n=115). We sequenced the exomes of 264 probands, and their parents, and confirmed 329 *de novo* mutations. A likelihood analysis showed a significant excess of *de novo* mutations in the ~4,000 genes that are the most intolerant to functional genetic variation in the human population ($p=2.9 \times 10^{-3}$). Among these are *GABRB3* with de novo mutations in four patients and *ALG13* with the same de novo mutation in two patients; both genes show clear statistical evidence of association. Given the relevant site-specific mutation rates, the probabilities of these outcomes occurring by chance are $p=4.1 \times 10^{-10}$ and $p=7.8 \times 10^{-12}$, respectively. Other genes with de novo mutations in this cohort include: *CACNA1A*, *CHD2*, *FLNA*, *GABRA1*, *GRIN1*, *GRIN2B*, *HDAC4*, *HNRNPU*, *IQSEC2*, *MTOR*, and *NEDD4L*. Finally, we show that the *de novo* mutations observed are enriched in specific gene sets including genes regulated by the Fragile X protein ($p<10^{-8}$), as was reported for autism spectrum disorders (ASD)[1].

Genetics is believed to play an important role in many epilepsy syndromes; however, specific genes have been discovered in only a small proportion of cases. Genome-wide association studies for both focal and generalized epilepsies have revealed few significant associations, and rare copy number variants explain only a few percent of cases[2–5]. An emerging paradigm in neuropsychiatric disorders is the major impact of *de novo* mutations on disease risk[6,7]. We searched for *de novo* mutations associated with EE, a heterogeneous group of severe epilepsy disorders characterized by early onset of seizures with cognitive and behavioral features associated with ongoing epileptic activity. We focused on two "classic" forms of EE: IS and LGS, recognizing that some patients with IS evolve to LGS.

Exome sequencing of 264 trios (Additional Methods) identified 439 putative *de novo* mutations. Sanger sequencing confirmed 329 *de novo* mutations (Supplementary Table 2), and the remainder were either false positives, a result of B cell immortalization, or in regions where the Sanger assays did not work (Supplementary Table 3).

Across our 264 trios, we found nine genes with *de novo* SNV mutations in two or more probands (*SCN1A* n=7, *STXBP1* n=5, *GABRB3* n=4, *CDKL5* n=3, *SCN8A* n=2, *SCN2A n=2*,

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ALG13 n=2, *DNM1 n=2*, and *HDAC4* n =2). Of these, *SCN1A*, *STXBP1*, *SCN8A*, *SCN2A*, and *CDKL5* are generally considered known EE genes.[8–13] To assess whether the observations in the other genes implicate them as risk factors for EE, we determined the probability of seeing multiple mutations in the same gene given the sequence specific mutation rate, size of the gene, and the number and gender of patients evaluated in this study (Additional Methods). The number of observed *de novo* mutations in *HDAC4* and *DNM1* are not yet significantly greater than the null expectation. However, observing four unique *de novo* mutations in *GABRB3* and two identical *de novo* mutations in *ALG13* were found to be highly improbable (Table 1, Figure 1). We performed the same calculations on all the genes with multiple *de novo* mutations observed in 610 control exomes and found no genes with a significant excess of *de novo* mutations (Supplementary Table 4). While mutations in *GABRB3* have previously been reported in association with another type of epilepsy[14], and through *in vivo* studies in mice *GABRB3* haploinsufficiency has been suggested to be one of the causes of epilepsy in Angelman's syndrome[15], our observations implicate it, for the first time, as a single gene cause of EE and provide the strongest evidence yet available for any epilepsy. Likewise, *ALG13*, an X-linked gene encoding a subunit of the uridine diphosphate-N-acetylglucosamine transferase, was previously shown to carry a novel *de novo* mutation in a male patient with a severe congenital glycosylation disorder with microcephaly, seizures, and early lethality[16]. Furthermore, the exact same *ALG13 de novo* mutation identified in this study was observed as a *de novo* mutation in an additional female patient with severe intellectual disability (ID) and seizures[17].

Each trio harbored on average 1.25 confirmed *de novo* mutations, with 181 probands harboring at least one. Considering only *de novo* SNVs, each trio harbored on average 1.17 *de novo* mutations (Supplementary Figure 1). Seventy-two percent of the confirmed *de novo* SNV mutations were missense and 7.5% were loss-of-function (splice donor, splice acceptor, or stop-gain mutations). Compared to rates of these classes of mutations previously reported in controls (69.4% missense and 4.2% loss of function mutations)[18–20], we observed a significant excess of loss-of-function mutations in patients with IS and LGS (Exact binomial p=0.01), consistent with data previously reported in ASD[7,18–20].

Neale *et al.*[7] recently established a framework for testing whether the distribution of *de novo* mutations in affected individuals differs from the general population. Here, we extend their simulation-based approach by developing a likelihood model that characterizes this effect and describes the distribution of *de novo* mutations among affected individuals in terms of the distribution in the general population, and a set of parameters describing the genetic architecture of the disease. These parameters include the proportion of the exome sequence that can carry disease-influencing mutations (η) and the relative risk (γ) of the mutations (Supplementary Methods). Consistent with what was reported in ASD[7], we found no significant deviation in the overall distribution of mutations from expected (γ=1 and/or η=0). It is, however, now well-established that some genes tolerate protein-disrupting mutations without apparent adverse phenotypic consequences, while others do not. To take this into account, we employed a simple scoring system that uses polymorphism data in the human population to assign a tolerance score to every considered gene (Additional Methods). We then found that known EE genes rank amongst the most intolerant genes using this scheme

(Supplementary Table 8). We therefore evaluated the distribution of *de novo* mutations within these 4,264 genes that are within the 25th percentile for intolerance and found a significant shift from the null distribution ($p=2.9\times10^{-3}$). The maximum likelihood estimates of η (percentage of intolerant genes involved in EE) was 0.021 and γ (relative risk) was 81, suggesting there are 90 genes amongst the intolerant genes that can confer risk of EE and that each mutation carries substantial risk. We also found that putatively damaging *de novo* variants in our cohort are significantly enriched in intolerant genes compared with control cohorts (Supplementary Methods).

We next evaluated whether the *de novo* mutations were drawn preferentially from six gene sets (Additional Methods, Supplementary Table 10), including ion channels[21], genes known to cause monogenic disorders with seizures as a phenotypic feature[22], genes carrying confirmed *de novo* mutations in patients with ASD[7,18–20] and in patients with ID[17,23], and FMRP-regulated genes. Taking into account the size of regions with adequate sequencing coverage to detect a *de novo* mutation (Additional Methods), we found significant over-representation for all gene lists in our data (Supplementary Table 10), and no over representation in controls [17–20,23].

To determine possible interconnectivity among the genes carrying a *de novo* mutation, we performed a protein-protein interaction analysis and identified a single network of 71 connected proteins (Figure 2 and Supplementary Figure 7). These 71 proteins include six encoded by Mendelian Inheritance in Man (MIM, http://www.omim.org/) EE genes that have one or more *de novo* mutation in an EE patient in this study Genes in this protein-protein network were also found to far more likely overlap with the ASD[77],[18,20,24] and severe ID[17,23] exome sequencing study genes, and with FMRP-associated genes, than the genes not in this network (Supplementary Table 11).

In support of a hypothesis that individual rare mutations in different genes may converge on pathways, we draw attention to the six mutations that all affect subunits of the gamma-aminobutyric acid (GABA) ionotropic receptor (four in *GABRB3*, and one each in *GABRA1* and *GABRB1*), and highlight two interactions: *HNRNPU* interacting with *HNRNPH1* and *NEDD4L* (identified here) binding to *TNK2*, a gene previously implicated in EE[25] (Figure 2). Although the *HNRNPU* mutation observed here is an indel in a splice acceptor site, and therefore likely functional, the *HNRNPH1 de novo* mutation is synonymous and thus of unknown functional significance (Supplementary Table 2). Importantly, a minigene experiment suggests that this synonymous mutation induces skipping of exon 12 (Supplementary Methods).

Evaluation of the clinical phenotypes among patients revealed significant genetic heterogeneity underlying IS and LGS, and begins to provide information about the range of phenotypes associated with mutations in specific genes (Supplementary Table 13). We identified four genes, *SCN8A*, *STXBP1*, *DNM1*, and *GABRB3*, with *de novo* mutations in both patients with IS and patients with LGS. Although IS may evolve to LGS, in three of these cases, the patients with LGS did not initially present with IS, suggesting phenotypic heterogeneity associated with mutations in these genes yet supporting the notion of shared genetic susceptibility. Interestingly, in multiple patients we identified *de novo* mutations in

genes previously implicated in other neurodevelopmental conditions, and in some cases with very distinctive clinical presentations (Supplementary Table 12). Most notably, we found a *de novo* mutation in *MTOR*, a gene recently found to harbor a causal variant in mosaic form in a case with hemimegalencephaly[26]. Our patient however showed no detectable structural brain malformation. Similarly, we found one patient with a *de novo* mutation in *DCX* and another with a *de novo* mutation in *FLNA*, previously associated with lissencephaly and periventricular nodular heterotopia (PVNH), respectively[27,28]; neither patient had cortical malformations detected on magnetic resonance imaging.

In addition to *de novo* variants, we also screened for highly penetrant genotypes by identifying variants that create newly homozygous, compound heterozygous, or hemizygous genotypes in the probands that are not seen in parents or controls (Supplementary Methods). No inherited variants showed significant evidence of association. Additional studies evaluating a larger number of EE patients will be required to establish the role of inherited variants in the risk IS and LGS.

In summary, we have identified novel de novo mutations implicating at least two genes, and also describe a genetic architecture that strongly suggests we have identified additional causal mutations in genes intolerant to functional variation. Given that our sample size already shows many genes with recurrent mutations, it is clear that even modest increases in sample sizes will confirm many new genes now seen in only one of our trios. Our results also emphasize that it may be difficult to predict with confidence the responsible gene, even among known genes, based upon clinical presentation. This makes it clear that the future of genetic diagnostics in EE will focus on the genome as a whole as opposed to single genes or even gene panels. In particular, several of the genes with *de novo* mutations in our cohort have also been identified in patients with ID or ASD. Finally, and perhaps most importantly, this work suggests a clear direction for both drug development and treatment personalization in the epileptic encephalopathies, as many of these mutations appear to converge on specific biologic pathways.

Additional Methods

Study subjects

IS and LGS patients evaluated in this study were collected through the Epilepsy Phenome/Genome Project (EPGP, www.epgp.org). Patients were enrolled across 23 clinical sites. Informed consent was obtained for all patients in accordance with the site specific Institutional Review Boards. Phenotypic information has been centrally databased and DNA specimens stored at the Coriell Institute – NINDS Genetics Repository (Supplementary Table 1). IS patients were required to have hypsarrhythmia or a hypsarrhythmia on EEG. LGS patients were required to have EEG background slowing or disorganization for age and generalized spike and wave activity of any frequency or generalized paroxysmal fast activity (GPFA). Background slowing was defined as <8Hz posterior dominant rhythm in patients over 3 years of age, and <5Hz in patients over 2 years of age. EEGs with normal backgrounds were accepted if the generalized spike and wave activity was 2.5 hertz or less and/or if GPFA was present.

All patients were required to have no evidence of moderate-to-severe developmental impairment or diagnosis of autistic disorder or pervasive developmental disorder prior to the onset of seizures. Severe developmental delay was defined by 50% or more delay in any area: motor, social, language, cognition, or activities of living; or global delay. Mild delay was defined as delay of less than 50% of expected milestones in one area, or less than 30% of milestones across more than one area. All patients had no confirmed genetic or metabolic diagnosis, and no history of congenital TORCH infection, premature birth (before 32 weeks gestation), neonatal hypoxic-ischemic encephalopathy or neonatal seizures, meningitis/encephalitis, stroke, intra-cranial hemorrhage, significant head trauma, or evidence of acquired epilepsy. All IS and LGS patients had an MRI or CT scan interpreted as normal, mild diffuse atrophy or focal cortical dysplasia. (Our case with the mutation in *HNRNPU* had had a reportedly normal MRI but on review of past records, a second more detailed MRI was found showing small regions of PVNH.) In order to participate, both biological parents had to have no past medical history of seizures (except febrile or metabolic/toxic seizures).

A final diagnosis form was completed by the local site EPGP principal investigator based on all collected information. A subset of cases was reviewed independently by two members of the EPGP Data Review Core to ensure data quality and consistency. All EEGs were reviewed by a site investigator and an EEG core member to assess data quality and EEG inclusion criteria. EEGs accepted for inclusion were then reviewed and scored by two EEG core members for specific EEG phenotypic features. Disagreements were resolved by consensus conference among two or more EEG core members before the EEG data set was finalized. MRI scans were reviewed by local investigators and an MRI core member to exclude an acquired symptomatic lesion.

Exome sequenced unrelated controls (n=436) used to ascertain mutation frequencies were sequenced in the Center for Human Genome Variation as part of other genetic studies.

Exome sequencing, alignment and variant calling

Exome sequencing was carried out within the Genomic Analysis Facility in the Center for Human Genome Variation (Duke University). Sequencing libraries were prepared from primary DNA extracted from leukocytes of parents and probands using the Illumina TruSeq library preparation kit following the manufacturer's protocol. Illumina TruSeq Exome Enrichment kit was used to selectively amplify the coding regions of the genome according to the manufacturer's protocol. Six individual barcoded samples (two complete trios) were sequenced in parallel across two lanes of an Illumina HiSeq 2000 sequencer. Alignment of the sequenced DNA fragments to Human Reference Genome (NCBI Build 37) was performed using the Burrows-Wheeler Alignment Tool (BWA) (version 0.5.10). The reference sequence we use is identical to the 1000 Genomes Phase II reference and it consists of chromosomes 1–22, X,Y, MT, unplaced and unlocalized contigs, the human herpesvirus 4 type 1 (NC_007605), and decoy sequences (hs37d5) derived from HuRef, Human Bac and Fosmid clones and NA12878.

After alignments were produced for each individual separately using BWA, candidate *de novo*, recessive, and compound heterozygous genotypes were jointly called with the GATK Unified Genotyper for all family members in a trio. Loci bearing putative *de novo* mutations

were extracted from the VCFs that met the following criteria: (1) the read depth in both parents should be greater than or equal to 10; (2) the depth of coverage in the child should be at least one tenth of the sum of the coverage in both parents; (3) for *de novo* variants, less than 5% of the reads in either parent should carry the alternate allele; (4) at least 25% of the reads in the child should carry the alternate allele; (5) the normalized, phred-scaled likelihood (PL) scores for the offspring genotypes AA, AB, and BB, where A is the reference allele and B is the alternate allele, should be >20, 0, and >0, respectively; (6) the PL scores for both parents should be 0, >20, and >20; (7) at least three variant alleles must be observed in the proband; and (8) the *de novo* variant had to be located in a CCDS exon targeted by the exome enrichment kit. PL scores are assigned such that the most likely genotype is given a score of 0, and the score for the other two genotypes represent the likelihood that they are not the true genotypes. SnpEff (version 3.0a) was used to annotate the variants according to Ensembl (version 69) and consensus coding sequencing (CCDS release 9, GRCh37.p5) and limited analyses to exonic or splice site (2 bp flanking an exon) mutations. All candidate *de novo* mutations that were absent from population controls, including a set of 436 internally sequenced controls and the ~6500 individuals whose single nucleotide variant data is reported in the Exome Variant Server, NHLBI Exome Sequencing Project (ESP), Seattle, WA (URL: http://evs.gs.washington.edu/EVS/) [date (August, 2012)] were also visually inspected using Integrative Genomics Viewer (IGV). All candidate *de novo* mutations were confirmed with Sanger sequencing of the relevant proband and parents. For comparison, we also called *de novo* variants from probands and parents individually for a subset of trios. Using this individual calling approach we identified and confirmed an additional 46 *de novo* mutations. These were included in all the downstream *de novo* mutation analyses.

Calculation of gene specific mutation rate

Point mutation rates were scaled to per base-pair, per generation, based on the human genome sequences matrix[30] (kindly provided by Drs. Shamil Sunyaev and Paz Polak), and the known human average genome *de novo* mutation rate (1.2×10^{-8})[31]. The mutation rate (M) of each gene was calculated by adding up point mutation rates in effectively captured CCDS regions in the offspring of trios, and then dividing by the total trio number (S = 264). The p-value was calculated as [1 – Poisson cumulative distribution function (x-1, λ)], where x is the observed *de novo* mutation number for the gene, and λ is calculated as $2S*M$ for genes on autosome or $(2f + m)*M$ for genes on chromosome X (f and m are the number of sequenced female and male probands, respectively). Genes on Y chromosome were not part of these analyses. Two *de novo* mutations in gene *ALG13* are at the same position, likewise in gene *SCN2A*. We calculated the probability of this special case as [1 – Poisson cumulative distribution function (1, $(2f + m)*r$)], where r reflects the point mutation rate on that specific *de novo* mutation position. Further investigations indicated that it is unlikely for these *de novo* mutations, which occur at the same site across distinct probands, to have been caused by sequencing or mapping errors (Supplementary Methods).

Calculation of mutation tolerance for HGNC genes

To quantitatively assign a mutation tolerance score to genes in the human genome (HGNC genes), we calculated an empirical penalty based on the presence of common functional

variation using the aggregate sequence data available from the 6,503 samples reported in the Exome Variant Server, NHLBI Exome Sequencing Project (ESP), Seattle, WA (URL: http://evs.gs.washington.edu/EVS/) [date (August, 2012) accessed]). We first filtered within the EVS database and eliminated from further consideration, genes where the number of 10-fold average covered bases was less than 70% of its total extent. In calculating a score, we focused on departures from the average common functional variant frequency spectrum, corrected for the total mutation burden in a gene. We construct this score as follows. Let *Y* be the total number of common, MAF>0.1%, missense and nonsense (including splice) variants and let *X* be total number of variants (including synonymous) observed within a gene. We regress *Y* on *X* and take the studentized residual as the score (*S*). Thus the raw residual is divided by an estimate of its standard deviation and thus account for differences in variability that comes with differing mutational burdens. *S* measures the departure from the average number of common functional mutations found in genes with a similar amount of mutational burden. Thus, when $S = 0$ the gene has the average number of common functional variants given its total mutational burden. Genes where $S < 0$ have less common functionals than average for their mutational burden and thus, would appear to be less tolerant of functional mutation, indicating the presence of weak purifying selection. We further investigated how different 'intolerance' thresholds of *S* captured known EE genes (Supplementary Table 8). Supplementary Figure 6 illustrates how different percentiles of *S* lead to the classification of different proportions of the known EE genes as 'intolerant'. Note that *ARX* is not in these analyses as this gene did not meet a 70% of gene coverage threshold. The dashed vertical line in Supplementary Figure 6 illustrates the 25th percentile of *S* and shows that using this threshold results in 12 out of the 14 assessed known genes being considered 'intolerant'. On the basis of this analysis, we used this 25th percentile threshold in classifying genes as intolerant in all subsequent analyses. Supplementary Table 9 lists the 25th percentile of most intolerant genes that had Sanger confirmed *de novo* mutations amongst the IS/LGS probands.

Defining the CCDS opportunity space for detecting *de novo* mutations

For each trio, we defined callable exonic bases, that had the opportunity for identification of a coding *de novo* mutation, by restricting to bases where each of the three family members had at least 10-fold coverage, obtained a multi-sampling (GATK) raw phred-scaled confidence score of ≥20 in presence or absence of a variant, and were within the consensus coding sequence (CCDS release 9, GRCh37.p5) or within the two base-pairs at each end of exons to allow for splice acceptor and donor variants. Using these three criteria, the average CCDS-defined *de novo* mutation opportunity space across 264 trios was found to be 28.84Mb ± 0.92Mb (range of 25.46Mb – 30.25Mb).

To explore at the gene level, we similarly assessed the *de novo* calling opportunity within any given trio for every gene with a CCDS transcript. For genes with instances of non-overlapping CCDS transcripts, we merged the corresponding regions into a consensus summary of all CCDS-defined bases for that gene. Using these criteria, over 85% of the CCDS-defined exonic regions were sequenced, to at least 10-fold coverage across the three family members, in over 90% of trios. All 264 trios covered at least 79% of the CCDS-defined regions under the CCDS opportunity space criteria. Calculations of CCDS

opportunity space for calling a *de novo* mutation, aside from the Y chromosome, were used in both the gene-list enrichment and architecture calculations.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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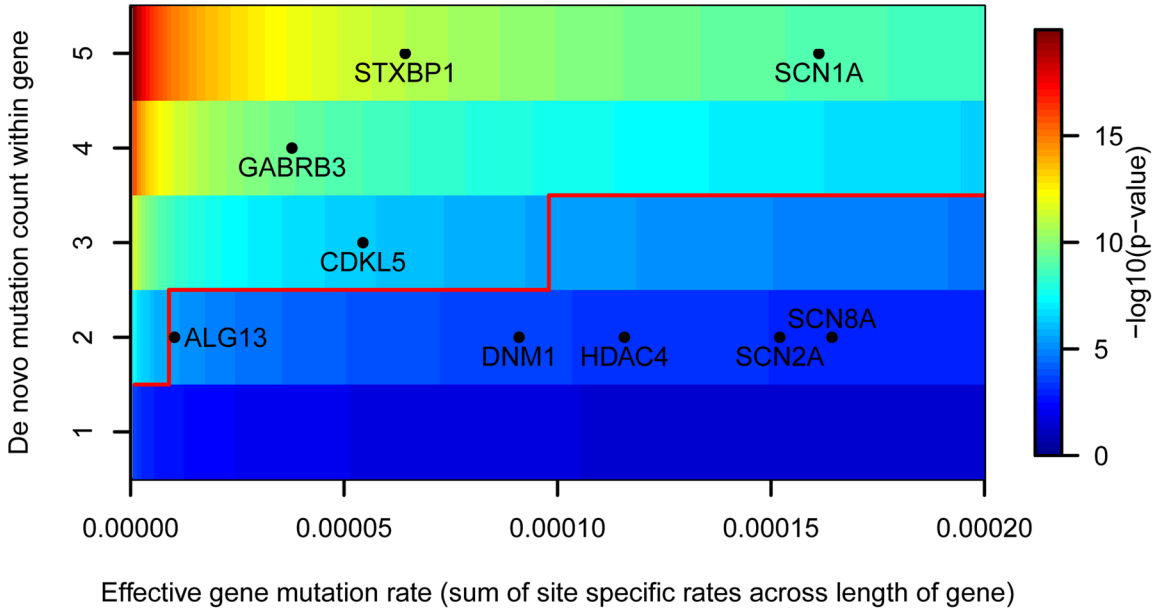

Figure 1. Heat map illustrating the probability of observing the number of *de novo* mutations in genes with an estimated gene mutation rate

The number of *de novo* mutations required to achieve significance is indicated by the solid red line. The superimposed black dots reflect positions of all genes found to harbor multiple *de novo* mutations in our study. *GABRB3*, *SCN1A*, *CDKL5*, *STXBP1* have significantly more *de novo* mutations than expected. The positions indicated for *ALG13* and *SCN2A* reflect only the fact that there are two mutations observed, not that there are two mutations affecting the same site (Additional Methods).

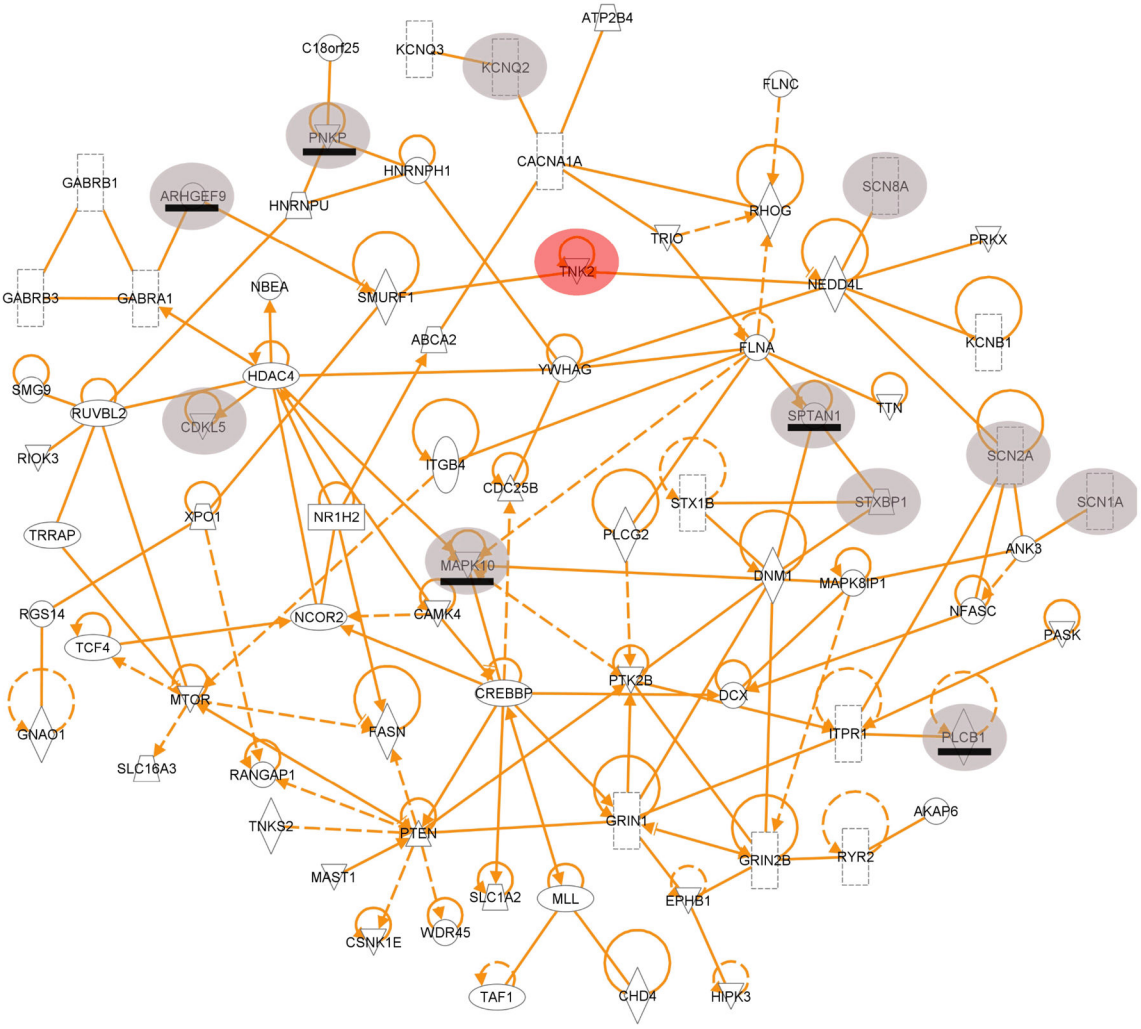

Figure 2. A protein-protein interaction network of genes with *de novo* mutations found in IS and LGS patients studied

Six of the genes found to harbor *de novo* mutations in an IS or LGS patient are known MIM EE genes (shaded circles). Five additional known MIM EE genes that were not found to be mutated in the 264 EE patients, but are involved in this network, are also shown (shaded circles with the gene underlined). The previously identified severe infantile epilepsy gene *TNK2* is superimposed into this network (red circle).

Table 1

Genes with greater than one *de novo* SNV mutation in this cohort of 264 trios, and the probabilities of getting greater than or equal observed *de novo* mutation tally by chance.

Gene	Chr	Average effectively captured length (bp)	Weighted mutation rate	*De novo* mutation number	p-value[†]	
SCN1A	2	6063.70	1.61×10^{-4}	5[*]	1.12×10^{-9}	***
STXBP1	9	1917.51	6.44×10^{-5}	5	1.16×10^{-11}	***
GABRB3	15	1206.86	3.78×10^{-5}	4	4.11×10^{-10}	***
CDKL5	X	2798.38	5.44×10^{-5}	3	4.90×10^{-7}	**
ALG13[#]	X	475.05	1.03×10^{-5}	2	7.77×10^{-12}	***
DNM1	9	2323.37	9.10×10^{-5}	2	2.84×10^{-4}	
HDAC4	2	2649.82	1.16×10^{-4}	2	4.57×10^{-4}	
SCN2A[#]	2	5831.21	1.52×10^{-4}	2	1.14×10^{-9}	***
SCN8A	12	5814.48	1.64×10^{-4}	2	9.14×10^{-4}	

[†] Adjusted α is equivalent to 0.05/18,091 = 2.76×10 (*), 0.01/18,091 = 5.53×10 (**) and 0.001/18,091 = 5.53×10^{-8} (***).

[*] Counts exclude three additional patients with an indel or splice site mutation as these are not accounted for in the mutability calculation.

[#] Two *de novo* mutations occur at the same position. The probability of these special cases obtain P = 7.77×10^{-12} and P = 1.14×10^{-9} for *ALG13* and *SCN2A*, respectively (Additional Methods).