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Mutations in *STX1B*, encoding a presynaptic protein, cause fever-associated epilepsy syndromes

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Febrile seizures affect 2–4% of all children¹ and have a strong genetic component². Recurrent mutations in three main genes (SCN1A, SCN1B and GABRG2)³⁻⁵ have been identified that cause febrile seizures with or without epilepsy. Here we report the identification of mutations in STX1B, encoding syntaxin-1B⁶, that are associated with both febrile seizures and epilepsy. Whole-exome sequencing in independent large pedigrees^{7,8} identified cosegregating STX1B mutations predicted to cause an early truncation or an in-frame insertion or deletion. Three additional nonsense or missense mutations and a de novo microdeletion encompassing STX1B were then identified in 449 familial or sporadic cases. Video and local field potential analyses of zebrafish larvae with antisense knockdown of stx1b showed seizure-like behavior and epileptiform discharges that were highly sensitive to increased temperature. Wild-type human syntaxin-1B but not a mutated protein rescued the effects of stx1b knockdown in zebrafish. Our results thus implicate STX1B and the presynaptic release machinery in fever-associated epilepsy syndromes.

We first performed genetic studies to identify new genes for feverassociated epilepsy syndromes in two large German pedigrees in which roles for known genes and loci had been excluded^{7,8}. Family F1 (Fig. 1a) showed high phenotypic variability, including simple febrile seizures and afebrile myoclonic-astatic, absence and generalized tonic-clonic seizures with onset in early childhood (Supplementary Note and **Supplementary Table 1**)⁷. Linkage analysis identified a single locus on chromosome 16p11.2 with a significant logarithm of odds (LOD) score of 4.27 (Supplementary Figs. 1-4). Phenotypes for family F2 (Fig. 1b) were more homogeneous, with febrile seizures and afebrile atonic, dyscognitive or generalized tonic-clonic seizures occurring between 10 months and 9 years of age⁸. There was suggestive linkage to the same centromeric region as in family F1 (16p12-q12)⁸, but no PRRT2 (NM_145239.2) mutation was detected as recently described for infantile seizures or paroxysmal dyskinesia^{9–14}. To identify the underlying genetic defects, we then performed whole-exome sequencing on both families. In three distant core members of F1 (IV.5, IV.40 and V.4; Fig. 1a),

we identified a truncating mutation in *STX1B* (NM_052874.3;

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Figure 1 New variants detected in STX1B in six independent families. (a) Pedigree of family F1 (ref. 7). The mutation c.166C>T (p.GIn56*) found in STX1B cosegregated well in affected individuals with a combination of afebrile and febrile seizures with some exceptions. (b) Pedigree of family F2 (ref. 8). Mutations (c.133_134insGGATGTGCATTG; p.Lys45delinsArgMetCysIleGlu and c.135_136AC>GA; p.Leu46Met) were found in STX1B that cosegregated with all affected family members and two unaffected probands. (c) Pedigree of family F3. The mutation c.140C>A (p.Ser47*) was found in 2009D11137 but not in the father with unclassifiable seizures. (d) The nonsynonymous variant c.657T>A (p.Val216Glu) was identified in the Swiss family F4. (e) The *de novo* mutation c.676G>C (p.Gly226Arg) was identified in the proband of family F5 with an MAE phenotype. (f) A de novo microdeletion was identified in the proband of family F6 with an MAE-related phenotype. Individuals who tested positive for a heterozygous STX1B mutation are denoted as M/+, whereas those carrying two wild-type alleles are denoted as +/+. The heterozygous microdeletion in the proband of F6 is indicated as Del/+. No DNA was available for individuals who are not labeled with one of these three notations.



c.166C>T; p.Gln56*), as the only shared putatively disease-causing mutation within the linkage interval. The mutation was confirmed by Sanger sequencing and was not detected in existing databases (National Heart, Lung, and Blood Institute (NHLBI) Exome Variant Server (EVS) database; dbSNP135 and 1000 Genomes Project) nor in 188 healthy German controls. Pyrosequencing showed cosegregation of the mutation in all six core family members (IV.5, IV.6, IV.40, V.4, V.5 and V.8; **Fig. 1a**), most affected individuals, including the obligate carriers, and one further unaffected individual (II.5), whom we regarded as showing non-penetrance for the mutation. Four

individuals not carrying the mutation had previously been suggested to have experienced febrile or afebrile seizures⁷. These occurrences can be explained by the following phenotypic features: (i) IV.19 and III.14 suffered from classical idiopathic/genetic generalized epilepsy without febrile seizures and thus did not have characteristics matching the febrile seizure–associated seizure phenotype of the family⁷, (ii) III.26 experienced a single febrile seizure⁷ and could be regarded as representing a phenocopy given the high prevalence of febrile seizures in the general population and (iii) III.27 had a few falls and one episode with head movements in childhood, which we first interpreted as

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	н _А	H _B H _C Q56* Q52 <i>R1s*2</i> S47*	r	SNARE TMR notif G226R V216E	
	1	<45delinsRCMIE/L46M			
b	Species		Seque	nce	Identity (%)
	H. sapiens (human)	EEIRGCIE KLS EDVE Q VKK Q HSAILAAPNP		$\texttt{TSIRELHDMF} \mathbf{v}$ DMAMLVESQ \mathbf{G} EMIDRIEYNV	
	P. troglodytes (common chimpanzee)				100
	<i>M. mulatta</i> (rhesus macaque)				100
	<i>F. catus</i> (domestic cat)				96.9
	<i>M. musculus</i> (house mouse)				100
	<i>D. rerio</i> (zebrafish)			N	97.2
	D. melangonaster (fruit fly)	BQDNEKS-PQT		KH-	68.1
	C. elegans (roundworm)	SVDIIANNEKSN-VN		S <u>M</u> <u>G</u> V	60.7
	X. tropicalis (Western clawed frog)			N	95.8

Figure 2 Overview of the location of variants and conservation of the mutated protein regions in syntaxin-1B. (a) Putative domain structure of syntaxin-1B derived from that for syntaxin-1A²⁴, as the isoforms share 83.6% of their amino acid sequences (using the alignment program Clustal 2.1). The structure is represented by a Habc domain, a SNARE motif and a transmembrane region (TMR). The different variants found in this study are indicated in black according to amino acid position. The p.GIn52Argfs*2 variant is shown in *italics*; this variant was already described in another study²⁰. (b) Sequence alignment of the syntaxin-1B homologs from different species. The last column shows identity to the human protein. The gray boxes highlight the amino acids mentioned in the text. Protein sequences were taken from the Ensembl database: Homo sapiens, ENSP00000215095: Pan troglodytes, ENSPTRP00000013711; Macaca mulatta, ENSMMUP0000004316; Felis catus, ENSFCAP0000002394; Mus musculus, ENSMUSP00000101874; Danio rerio, ENSDARP00000076389; Drosophila melangonaster, FBpp0292260; Caenorhabditis elegans, F56A8.7b; Xenopus tropicalis, ENSXETP00000059878.

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Figure 3 Spontaneous electrographic activity recorded from larvae with stx1b or control morpholino at 5 d.p.f. (a) Representative recording configuration of an agar-immobilized zebrafish larva at 5 d.p.f. Note the recording electrode placed in the optic tectum. OT, optic tectum; FB, forebrain. Scale bar, 125 µm. (b) Extracellular recordings from the optic tectum of larvae with control morpholino (ctrl-MO) at 5 d.p.f. (c) Representative epileptiform activities of larvae with stx1b morpholino (stx1b-MO) at 5 d.p.f. displaying polyspiking discharges (20-100 Hz) (left) or HFOs (100-200 Hz) (right). Top traces represent typical epileptiform patterns as seen in gap-free recordings. Bottom traces show high-resolution magnification of the epileptiform events. Color maps represent the LFP frequency content of polyspiking discharges (left) and HFOs (right) in the timefrequency domain. The color scale varies from blue (low magnitude) to red (high magnitude). The scales for the recording magnification and color maps are the same. The colored curves show power spectral density (PSD) normalized to its maximum peak value. Each curve corresponds to one larva with stx1b knockdown and represents an average PSD for all recorded events, including polyspiking discharges (blue, n = 39; red, n = 9; black, n = 17) (left) and HFOs (blue, n = 11; red, n = 9; black, n = 5) (right). (d) Mean duration of epileptiform events at room temperature in larvae with stx1b knockdown (total epileptiform activities, 112.8 ± 4.3 ms; polyspiking discharges, 159.7 ± 10.1 ms; HFOs, 90.4 ± 2.5 ms; *n* = 220 total events, 71 polyspiking discharge and 149 HFOs analyzed from 38 larvae; **** P < 0.0001, Mann-Whitney test). Control embryos (n = 10 larvae) did not exhibit any seizure events. Error bars, s.e.m.

b 5 d.p.f. ctrl-MO 2 mV 2 s С Polyspiking discharges High-frequency oscillations 5 d.p.f. stx1b-MO 5 d.p.f. stx1b-MO 0.5 mV 0.5 mV 2 s 2 s 0.2 mV 0.5 mV 0.1 s 0.1 s 400 400 (Hz) (Hz) 300 300 Frequency Frequency 200 200 100 100 0 1.2 1.2 Normalized power spectral Vormalized power spectral 1.0 1.0 0.8 0.8 density (a.u.) density (a.u.) 0.6 0.6 04 0.4 0.2 0.2 0 6 50 100 150 200 250 300 350 400 0 50 100 150 200 250 300 350 400 Frequency (Hz) Frequency (Hz) d 200 Mean duration of events 150

afebrile seizures⁷ but might have represented as non-epileptic events. Accordingly, the STX1B mutation segregated with the main phenotype of febrile and afebrile seizures in F1. To confirm that the STX1B mutation was disease causing, we subjected all six core family members and the clinically discordant family member IV.19 to whole-genome sequencing (Supplementary Fig. 5 and Supplementary Tables 2-4). Only one haplotype block was shared by all core members across the whole genome (but not by IV.19), containing the aforementioned nonsynonymous STX1B mutation (Supplementary Fig. 6).

Whole-exome sequencing in three members of F2 (III.1, III.5 and IV.1) identified a second new mutation in STX1B (complex indel; c.133_134insGGATGTGCATTG; p.Lys45delinsArgMetCysIleGlu and c.135_136AC>GA; p.Leu46Met) located in the described candidate region⁸. The mutation was not found in the aforementioned databases and cosegregated in the family with the seizure phenotype (Fig. 1b). Two unaffected family members (I.2 and II.4) carried the mutation, suggesting incomplete penetrance. The identified variant was the only plausible disease-relevant variant shared by all three sequenced individuals with the familial disease phenotype (Supplementary Table 5).

We then sequenced STX1B in further familial and sporadic cases with fever-associated familial epilepsies or epileptic encephalopathies for which roles for known genes had been excluded (including STXBP1 (ref. 15), which is functionally related to $STX1B^{6}$). In the first cohort of 299 independent probands (Supplementary Table 6), we detected 1 additional nonsense mutation in a Dutch proband with febrile seizures

and epilepsy (Fig. 1, F3), also predicted to result in early truncation of syntaxin-1B (c.140C>A; p.Ser47*). The proband's father had unclassifiable seizures but did not carry the mutation. The proband's mother was not available for testing. Second, targeted

1⁴⁶O 10tal Room temperature next-generation sequencing¹⁶ in 81 adult cases of Swiss origin with various forms of epilepsy and intellectual disability identified a new nonsynonymous STX1B mutation (c.657T>A; p.Val216Glu; Fig. 1d, F4). The parents of the mutation carrier were reported as healthy but were not available for testing. Third, we searched for STX1B mutations in 68 parent-offspring trios with fever-associated epileptic encephalopathies who underwent whole-exome sequencing (30 with Dravet syndrome and 38 with myoclonic-astatic epilepsy (MAE)). We detected one de novo missense mutation (c.676G>C; p.Gly226Arg) in a German proband with MAE (F5; Fig. 1e, Supplementary Table 7 and Supplementary Note). Fourth, a new 0.8-Mb de novo microdeletion encompassing STX1B was detected by array comparative genomic hybridization (CGH) and FISH analyses performed for diagnostic purposes in a German proband whose phenotype was consistent with MAE (F6; Fig. 1f, Supplementary Fig. 7 and Supplementary Note). The location of all alterations in syntaxin-1B and the strong conservation of the corresponding protein regions are presented in Figure 2. Additional clinical information is given in Supplementary Table 1 and the Supplementary Note. Sanger reads are shown in Supplementary Figure 8.

(ms) 100

50

2⁰

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Figure 4 Electrographic activity recorded from larvae with stx1b or control morpholino at 5 d.p.f. under hyperthermic conditions. (a) Representative epileptiform activities of larvae with stx1b knockdown at 5 d.p.f. displaying polyspiking discharges (PDs) (20–100 Hz) (left), HFOs (100–400 Hz) (middle) or isolated spikes (right). Color maps represent LFP frequency in the time-frequency domain. Color scale: blue (low magnitude) to red (high magnitude). Heat shock and temperature conditions are shown below the color maps. (b) Occurrence of epileptiform events in larvae with control or stx1b morpholino under heat shock. Sample sizes: ctrl-MO, n = 10; stx1b-MO, n = 14. (c) Mean duration of epileptiform events in heat-shocked larvae. n = 82 HFO events for larvae with ctrl-MO; total number of events, polyspiking discharges and HFOs: n = 381, 98 and 283 events analyzed, respectively. (d) Comparison of the mean number of epileptiform events in larvae with stx1b knockdown at room temperature and under heat shock, conditions. n = 17 and 14 larvae with stx1b-MO at room temperature and under heat shock, respectively. In **b**, a Student's unpaired *t* test with equal variances was used, and, in **c** and **d**, a Mann-Whitney test was used: **** $P \le 0.0001$, *** $P \le 0.0005$. Error bars, s.e.m.

We investigated the functional consequences of an STX1B defect *in vivo* in an *stx1b* knockdown model using morpholino antisense oligonucleotides in zebrafish¹⁷. The human and zebrafish syntaxin-1B proteins are 97% identical (Supplementary Fig. 9). Syntaxin-1B was detected at day 1 of embryonic development, with gradually increasing expression up to 4 days post-fertilization (d.p.f.) (Supplementary Fig. 10a). We titrated the concentration for the *stx1b* morpholino (*stx1b*-MO) to achieve knockdown (**Supplementary Fig. 10b**). We observed an early reduction in syntaxin-1B levels to about 14% of the levels seen with control morpholino, and the levels gradually increased as development progressed, reaching stable protein levels of 50% of control levels by 5 d.p.f. The control morpholino (ctrl-MO) did not have any effect on *stx1b* translation (Supplementary Fig. 10c,d). Larvae from *stx1b*-MO injections had a relatively normal appearance and only exhibited minor morphological abnormalities, such as lack of a swim bladder and, occasionally, slight tail curvature or pericardial edema (Supplementary Fig. 10e). At 4 d.p.f., about 40% of the larvae with stx1b-MO showed no touch response. This phenotype was transient, however (presumably as a result of transient protein knockdown), decreasing in frequency over the course of development (Supplementary Movies 1 and 2). By 5 d.p.f., larvae with stx1b-MO also displayed abnormal episodic behavior, such as repetitive pectoral fin fluttering, increased orofacial movements and myoclonus-like jerks (Supplementary Movies 3-5).

To investigate abnormal brain activity, we recorded local field potentials (LFPs) on larval optic tecta for larvae at 5 d.p.f. following injection with *stx1b*-MO or ctrl-MO (Fig. 3a)¹⁸. Epileptiform paroxysmal events consisted of multispike bursts with amplitudes exceeding baseline by at least threefold (Fig. 3c). Such recurrent spontaneous epileptiform events occurred in 17 of 38 larvae with stx1b-MO at a mean frequency of 12.9 ± 2.4 events/10 min, whereas controls only displayed baseline activity (Fig. 3b). Larvae with *stx1b* knockdown displayed two types of spontaneous epileptiform activity: polyspiking discharges and high-frequency oscillations (HFOs) (Fig. 3c). The spectral components of the LFP signal drastically increased in (i) the 20- to 100-Hz frequency band characterizing polyspiking discharges and (ii) the 100- to 200-Hz frequency band characterizing HFOs¹⁹. Polyspiking discharges lasted significantly longer than HFOs $(P \le 0.0001;$ Fig. 3d) but were two times less frequent (data not shown). These results could be reproduced with a second, nonoverlapping *stx1b* morpholino (*stx1b*-MO2) (**Supplementary Fig. 11**).

Elevation of temperature resulted in epileptiform discharges in larvae following injection with either stx1b-MO or ctrl-MO, consisting of polyspiking discharges, HFOs and isolated spikes with a duration of 10–30 ms (**Fig. 4a**). Larvae with stx1b-MO were more sensitive to an acute elevation in temperature, with much more frequent and longer HFOs than control larvae, and polyspiking discharges only occurred in larvae with stx1b knockdown (**Fig. 4a–c**). The duration of HFOs was significantly



Figure 5 Expression of wild-type and mutated human syntaxin-1B in larvae with stx1b knockdown. (**a**,**b**) Occurrence of epileptiform events in larvae with stx1b knockdown alone (black) versus those also expressing wild-type (WT) human (**a**) or Val216Glu (**b**) syntaxin-1B (white). In **a**, **P = 0.0011 (Mann-Whitney test). In **b**, for all larvae, P = 0.8494 (Mann-Whitney test) and, for seizure-positive larvae, P = 0.6836 (Student's unpaired *t* test with Welch correction). (**c**,**d**) Cumulative duration of epileptiform events in larvae with stx1b knockdown alone (black) versus those also expressing wild-type (**c**) or Val216Glu (**d**) syntaxin-1B (white). In **c**, **P = 0.001, ***P = 0.0008, Mann-Whitney test (33 larvae per group were analyzed). In **d**, for all larvae, P = 0.6573 and, for seizure-positive larvae, P = 0.5573 (Student's unpaired *t* test; n = 36 larvae analyzed per group). In **a**-**d**, error bars represent s.e.m. (**e**,**f**) Representative protein blots of syntaxin-1B protein levels at 5 d.p.f. in larvae with stx1b knockdown expressing wild-type (**e**) or Val216Glu syntaxin-1B (**f**). Gapdh was used as a loading control. (**g**) Representative images of larvae coinjected with stx1b-MO and human cDNA encoding Val216Glu syntaxin-1B selected for tectal field recordings and protein blot experiments. Green fluorescence shows the distribution of FAM-labeled stx1b-MO, and red fluorescence depicts the brain-specific expression of Val216Glu syntaxin-1B. Scale bar, 500 µm.

longer in larvae with *stx1b* knockdown that were subjected to hyperthermia than in controls ($P \le 0.0001$; **Fig. 4c**). Directly comparing larvae with *stx1b*-MO under normal and heat shock conditions, we found that hyperthermia induced a significantly higher occurrence of epileptiform events, specifically HFOs and not polyspiking discharges (**Fig. 4d**).

To confirm the specificity of the phenotype with the *stx1b* antisense morpholino and to determine the functional consequences of one of the identified mutations, we performed rescue experiments through central nervous system (CNS)-specific transgene expression of either wild-type or mutant human STX1B in zebrafish knockdown larvae (Fig. 5). We chose the p.Val216Glu alteration for analysis because it showed the least genetic evidence of causing disease. As expected, expression in knockdown larvae of wild-type syntaxin-1B led to significant rescue by greatly decreasing both the number of larvae with epileptiform activity (from 18 of 33 in larvae with stx1b-MO alone to 8 of 33 in larvae also expressing wild-type syntaxin-1B; P = 0.0011) and the number and cumulative duration of epileptiform discharges in larvae with *stx1b* knockdown (Fig. 5a,c,e). In contrast, expression of syntaxin-1B Val216Glu did not lead to rescue, as the number of larvae with seizure-like behavior was the same for larvae with stx1b-MO alone and those also expressing syntaxin-1B Val216Glu (21 of 36 larvae in each group) and the number and cumulative duration of seizures was not significantly different for the two groups, thereby confirming a pathological role and loss-of-function mechanism for this alteration, even though protein levels increased back to normal (Fig. 5b,d,f).

Our study identifies *STX1B* as a new gene in which mutations cause fever-associated epilepsy syndromes with a remarkably wide phenotypic spectrum, ranging from simple febrile seizures to severe epileptiform encephalopathies (including reduced penetrance without clinical signs). A causative role for *STX1B* mutations is suggested by their cosegregation with pathology and positive linkage analysis in our large families, the presence of two *de novo* mutations in severe cases and the possible existence of *de novo* mutations in two further cases. In addition, the mutations are predicted to be protein dam-

aging (Supplementary Table 8). Deleterious STX1B variants were absent in EVS and other coding variants were rare (5 nonsynonymous variants and 1 splice-site variation; 6/6,496 in total), showing a significant mutation load in our cohort of affected individuals (5/450; $P = 3.9 \times 10^{-4}$, Fisher's exact test). A single *de novo* frameshift mutation, located between the two truncating mutations from our study (encoding p.Gln52Argfs*2; Fig. 2), has recently been described in another cohort of 264 cases with epileptic encephalopathy²⁰. Furthermore, we provide functional proof that *stx1b* knockdown in a vertebrate in vivo system causes abnormal seizure-like behavior and epileptiform-like discharges increasing in number under hyperthermia. The lack of a touch response in larvae with *stx1b* knockdown supports the hypothesized role for syntaxin-1B in modulating γ -aminobutyric acid (GABA) and glutamate release²¹, as glutamate mainly drives this response in zebrafish²². The specificity of the knockdown experiment could be proven by rescue with wild-type syntaxin-1B, and the loss-of-function character of the STX1B point mutation encoding p.Val216Glu was demonstrated by the inability of the mutant to ameliorate spontaneous epileptiform discharges in stx1b knockdown larvae. To our knowledge, this is the first report describing the successful rescue of a morpholino-mediated knockdown phenotype using the expression of tissue-specific transgenesis at a relatively late stage of development (5 d.p.f.).

Jointly with synaptobrevin and SNAP25, syntaxin-1 forms the soluble *N*-ethylmaleimide–sensitive factor attachment receptor (SNARE) complex, mediating synaptic vesicle fusion to the presynaptic membrane. Syntaxin-1 comprises two highly conserved isoforms (syntaxin-1A and syntaxin-1B), composed of (i) two functionally important N-terminal domains (the N-peptide and H_{abc} domains) that bind to Munc18-1 and serve different functions in vesicle fusion, (ii) a C-terminal SNARE motif mediating assembly of the SNARE complex and (iii) a transmembrane region for membrane anchoring (**Fig. 2a**)²³. Syntaxin-1A and syntaxin-1B can rescue each other with respect to presynaptic functions in neuronal cultures²³.

Whereas *Stx1a* knockout mice develop and behave normally, without hints of epileptic events, mice carrying the so-called LE mutation promoting the open state of syntaxin-1B develop epileptic seizures at 2 weeks of age and severe ataxia, dying prematurely after 1–2 months, independent of the presence of syntaxin-1A²⁴. Thus, both isoforms can partially replace each other, but syntaxin-1B seems to be more important, possibly owing to its broader expression pattern²⁵. Hence, there is ample evidence for an important role of syntaxin-1B in presynaptic function and neuronal excitability.

The six mutations detected here truncate syntaxin-1B in the Habe domain, introduce an insertion or deletion in the same highly conserved region, introduce positive or negative charges into the conserved SNARE motif or delete one copy of the gene (Fig. 2). It is therefore conceivable that both truncating and in-frame mutations severely change the conformation and function of crucial parts of the protein and that all deleterious mutations lead to non-functional proteins, as shown for the p.Val216Glu substitution. There was no correlation between the apparent severity of the protein alterations and the severity of the clinical phenotypes, including intellectual disability. However, the deletion of other genes on chromosome 16 could have influenced the phenotype of the F6 proband (Supplementary Note). We hypothesize that haploinsufficiency for STX1B is a shared mechanism of pathogenesis in febrile seizures and epilepsy and that other unknown effects can modify the phenotype. In conclusion, our results link STX1B and the presynaptic release machinery to fever-associated epilepsy syndromes and establish 'synaptopathies' alongside channelopathies as a relevant group among neurological hyperexcitability syndromes.

URLs. NHLBI EVS, http://evs.gs.washington.edu/EVS/; dbSNP135, http://www.ncbi.nlm.nih.gov/SNP/; 1000 Genomes Project, http:// www.1000genomes.org/; Varbank, http://varbank.ccg.uni-koeln.de; Database of Genomic Variants, http://dgv.tcag.ca/dgv/app/home; USCS Genome Browser, http://genome.ucsc.edu/.

METHODS

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

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

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AUTHOR CONTRIBUTIONS

H.L., Y.G.W., C.V.E. and A.D.C. designed the study. FB., H.M., J.R.L., C.G.F.d.K., I.N., B.K., S.W., T.D., E.H.B., S. Baulac, R.S.M., H.H., B.P.C.K., P.D.J., I.H., Y.G.W., H.L. and the EuroEPINOMICS RES Consortium recruited and phenotyped subjects. Y.G.W., T.S., F.R., K.J.-R. and F.L.-H. performed or supervised wholegenome linkage analysis. J.S., P.M., A. Suls, J.R.L., H.T., K.K., A. Kawalia, E.R., J.C.R., G.G., L.H., D.J.G., S. Biskup, P.D.J., I.H., R.B., P.N., Y.G.W. and H.L. performed or supervised whole-exome, whole-genome or targeted panel sequencing. J.S. and M.R.T. performed segregation analysis and Sanger validation. J.S. and K.H. performed cohort screening by Sanger sequencing. A.C. and I.N. performed FISH and array CGH experiments. A. Siekierska, A. Kecskés and M.J. performed zebrafish knockdown studies. M.L., A. Siekierska, C.H. and B.M. performed zebrafish LFP and spectral analysis. P.A.M.d.W., A.D.C. and C.V.E. supervised all zebrafish studies. J.S., A. Siekierska, M.L., P.M., F.B., H.M., J.R.L., T.S., I.H., A.D.C., C.V.E., Y.G.W. and H.L. wrote the manuscript. All authors revised the manuscript.

COMPETING FINANCIAL INTERESTS

The authors declare competing financial interests: details are available in the online version of the paper.

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ONLINE METHODS

Cohorts and DNA extraction. The study was approved by the local ethics committees (198/2010BO1) and was performed in accordance with the ethical standards laid down in the 1964 Declaration of Helsinki and its later amendments. The subjects and their relatives analyzed in the study gave their written informed consent before inclusion in the study. Clinical information was obtained from medical charts and from direct contact with the subjects or their responsible physicians. EDTA blood samples were taken from each available family member, and DNA was extracted using standard procedures.

Linkage analysis. SNP genotyping was performed in family F1 using the Affymetrix Genome-Wide Human SNP Array 6.0 in all the family members indicated in the **Supplementary Note** and **Supplementary Figure 1**. In total, 37,415 high-quality SNPs (complete call rate, lack of mendelian errors and linkage disequilibrium (LD) pruning) were selected for linkage analysis. The map position of each SNP was obtained from the Affymetrix annotation file GenomeWideSNP_6.na32.annot.csv (hg19). Data management and quality control were performed with the program Alohomora²⁶. Pedigree relationships were validated by the graphical representation of relationship errors (GRR) program²⁷. Errors in the mendelian inheritance of alleles were detected with the Pedcheck program²⁸, and corresponding markers were removed.

The linkage program MERLIN²⁹ was applied to detect unlikely recombination events, and unlikely genotypes were set to missing. Linkage analysis of the complete family was carried out using the program SimWalk2 (refs. 30,31). Mega2 was used to convert data into the SimWalk2 format³². The Perl program HaploPainter was used for the visualization of haplotype information³³. LD pruning was performed with the program PLINK³⁴ using the SNP Array 6.0 genotype data of 60 HapMap CEU individuals (Utah residents of Northern and Western European ancestry). The pedigree is shown in **Supplementary Figure 11**.

Exome sequencing. *Exome sequencing of individuals from family F1.* DNA was fragmented by sonication (Covaris). The fragments were end repaired, and adaptors were ligated. Exome sequencing was performed for individuals IV.5, IV.40 and V.4. The DNA from individual V.4 was enriched using the NimbleGen Whole Exome kit (Roche NimbleGen), whereas DNA from individuals IV.5 and IV.40 was enriched using the Agilent SureSelect 40 Mb kit (Agilent Technologies). The samples were sequenced on an Illumina Genome Analyzer IIx instrument as paired-end 95-bp reads. Individuals IV.40 and V.4 were sequenced on two lanes, whereas individual IV.5 was sequenced on only one lane. Alignment and variant calling were performed using MAQ for SNP detection version 0.7.1 (ref. 35) and BWA-short version 0.5.7 (ref. 36) in combination with SAMtools version 0.1.7 (ref. 37) for indel detection. Scripts developed in house at the Cologne Center for Genomics were applied to detect protein changes, affected donor and acceptor splice sites, and overlaps with known variants. On average, 89.6% of the targeted regions were covered by 10×.

Exome sequencing of individuals from family F2. DNA was fragmented by sonication (Covaris). The fragments were end repaired, and adaptors were ligated. For exome sequencing, the DNA was enriched using SeqCap EZ Human Exome Library v.2.0 from NimbleGen (Roche NimbleGen). Each DNA sample was sequenced on an Illumina HiSeq 2000 instrument as paired-end 100-bp reads. For data analysis, the Varbank pipeline version 2.2 and interface was used. Primary data were filtered according to signal purity by Illumina Real-Time Analysis (RTA) software version 1.8. Subsequently, the reads were mapped to human genome reference build GRCh37 using the BWA³⁶ alignment algorithm. GATK version 1.6 (ref. 38) was used to mark duplicate reads, to perform local realignment around short indels, to recalibrate the base quality scores and to call SNPs and short indels together with SAMtools version 0.1 (ref. 37) and Dindel version 1.01 (ref. 39). Scripts developed in house at the Cologne Center for Genomics were applied to detect protein changes, affected donor and acceptor splice sites, and overlaps with known variants. Acceptor and donor splice-site mutations were analyzed with a Maximum Entropy model⁴⁰ and filtered for changes in effect. In particular, we filtered for high-quality, rare (minor allele frequency (MAF) < 0.01) autosomal variants using allele frequencies from the 1000 Genomes Project database and EVS. We also filtered against an in-house database containing variants from 511 exomes from individuals with epilepsy to exclude pipeline-related artifacts (MAF < 0.02).

Whole-genome sequencing in family F1. In brief, whole-genome sequencing was performed by Complete Genomics using a proprietary paired-end, nanoarray-based sequencing-by-ligation technology⁴¹⁻⁴³. Seven individuals were selected for whole-genome sequencing, the six core family members (V.4, V.5, V.8, IV.5, IV.6 and IV40) and IV.19. After quality control, DNA samples were sent to Complete Genomics for sequencing. All quality control, mapping and variant calling for the sequencing data were performed by Complete Genomics as part of their sequencing service using the Standard Sequencing Service pipeline version 2.0. Sequencing reads were mapped against NCBI Build 37. For gene annotations, NCBI Build 37.2 (RefSeq) was used. For further details, see the **Supplementary Note**.

Pyrosequencing. Segregation analysis was performed with Pyrosequencing System PSQHS96A from Qiagen. Pyrosequencing was performed according to the manufacturer's instructions using the PSQ 96 SNP Reagent kit (Biotage), and sequences were analyzed using Pyrosequencing software (PSQ HS96A1.2). All primer sequences are listed in **Supplementary Table 9**.

Sanger sequencing. Variant validation and mutational screening were performed through Sanger sequencing of the entire coding region of *STX1B*. The sequences for the PCR primers and sequencing primers for validation, screening and cosegregation are listed in **Supplementary Tables 10** and **11**. Sequencing reactions were performed at GATC Biotech, Eurofins MWG Operon and the VIB Genetic Service Facility.

Array comparative genomic hybridization. Array CGH using the 180k array (Agilent Technologies, 180K) was performed on the proband of family F6 using DNA isolated from peripheral blood according to the manufacturer's instructions. Scanning and data analysis were performed with Agilent Genomic Workbench standard edition 6.5 (Agilent Technologies). Regions that were identified as showing significant deviation from the reference by the software and that included more than five single oligonucleotides were regarded as aberrant. Differentiation between pathological aberrations and benign copy number polymorphisms (CNPs) was performed by using the Database of Genomic Variants. Targets were mapped according to the hg19 reference genome.

FISH analyses. Molecular cytogenetic analyses (FISH) were performed on buccal cells from the proband of family F6 and phytohemagglutinin (PHA)-stimulated lymphocytes from the parents according to standard protocols. We used the directly labeled probe RP11-297C4 (Blue Gnome) as a specific probe and a centromeric probe for chromosome 16, CEP16 (Abbot/Vysis), as a control probe. We analyzed 33 buccal cells and 15 metaphases and 100 interphases for the stimulated lymphocytes.

Zebrafish maintenance and breeding. Adult zebrafish (*Danio rerio*) of the AB strain (Zebrafish International Resource Center) were maintained at 28.5 °C on a 14-h light/10-h dark cycle under standard aquaculture conditions, and fertilized eggs were collected via natural spawning. Embryos were raised in embryo medium, containing 1.5 mM HEPES, pH 7.6, 17.4 mM NaCl, 0.21 mM KCl, 0.12 mM MgSO₄ and 0.18 mM Ca(NO₃)₂, in an incubator on a 14-h light/10-h dark cycle at 28.5 °C. For all experiments described, larvae at 1–6 d.p.f. were used. All zebrafish experiments were approved by the Ethics Committee of the University of Leuven (Ethische Commissie van de KU Leuven, approval number 061/2013) and by the Belgian Federal Department of Public Health, Food Safety and Environment (Federale Overheidsdienst Volksgezondheid, Veiligheid van de Voedselketen en Leefmileu, approval number LA1210199).

Antisense morpholino knockdowns. To perform stx1b knockdown in zebrafish embryos, we used morpholino antisense oligonucleotides^{17,44} designed to target the region spanning the 5' UTR and/or translational start site in stx1b mRNA, thus blocking translation and leading to a complete or partial loss of syntaxin-1B protein levels. Morpholinos were synthesized by GeneTools. Two different translation-blocking morpholinos were designed to target bases -11 to +14 (stx1b-MO) and -47 to -23 (stx1b-MO2) of the zebrafish stx1b mRNA (**Supplementary Table 12**). A control morpholino

(randomized 25-nucleotide sequence) was used as a negative control (ctrl-MO). Gene knockdown was achieved through microinjection of embryos from the AB (wild-type) strain at the one- to two-cell stage with 2 nl of morpholino according to the previously described method. We titrated the amount of stx1b-MO to 3 ng per injection and the amount of stx1b-MO2 to 7 ng per injection. The same amount of randomized control morpholino as the corresponding stx1b morpholino was used for injection.

SDS-PAGE and protein blotting. To evaluate the extent of knockdown in zebrafish embryos and larvae, SDS-PAGE and immunoblotting were performed on embryos injected with *stx1b* morpholino between 1 and 6 d.p.f. For each condition, 25 embryos or larvae were homogenized in RIPA buffer (ThermoScientific) supplemented with protease inhibitors (Complete Protease Inhibitor Cocktail Tablets, Roche), and samples were fractionated by SDS-PAGE (NuPAGE System, Invitrogen) using a 10% Bis-Tris gel in MOPS running buffer. Proteins were transferred to a nitrocellulose membrane using iBlot (Invitrogen). The membrane was blocked in Odyssey Blocking Buffer (Li-Cor) for 1 h at room temperature and subsequently incubated with primary rabbit antibody to syntaxin-1B (1:2,000 dilution; SYSY, 110 403) overnight at 4 °C and DyLight secondary goat antibody to rabbit IgG (1:10,000 dilution; ThermoScientific, 35571) for 1 h at room temperature. As a loading control, rabbit antibody to GAPDH was used (1:2,000 dilution; Cell Signaling, 2118). Proteins were visualized using the Odyssey 2.1 imaging system (Li-Cor). Quantification of the bands was performed using the Odyssey 2.1 imaging system (Li-Cor).

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Tectal field recordings. Open-field recordings were obtained from zebrafish larval tecta at 5 d.p.f. at (i) room temperature (19.6 °C, s.e.m. = 0.26, n = 15) or (ii) under hyperthermic conditions (31.2 °C, s.e.m. = 0.22, stx1b-MO n = 14; 31.4 °C, s.e.m. = 0.17, ctrl-MO n = 10). Hyperthermia was achieved by transient progressive elevation of the temperature of the microscope stage connected to a temperature controller (TC05, Luigs and Neumann). The temperature of the agarose and buffer surrounding the larvae was monitored using a highly sensitive temperature probe (Testo, 735). Notably, the maximum temperature was controlled so as not to exceed 31.5 °C, as temperatures greater than 32.5 °C resulted in continuous epileptiform discharges. By maintaining a 'sub-threshold' temperature, the sensitivity of larvae with *stx1b* knockdown to hyperthermia relative to controls could be demonstrated. A glass electrode, connected to a high-impedance amplifier, was filled with artificial cerebrospinal fluid (124 mM NaCl, 2 mM KCl, 2 mM MgSO₄, 2 mM CaCl₂, 1.25 mM KH₂PO₄, 26 mM NaHCO₃ and 10 mM glucose). A larva was then embedded in 2% low-melting-point agarose (Invitrogen), and the glass electrode was placed in the optic tectum of the larva. Recordings were performed in current clamp mode with low-pass filtering below 1 kHz, high-pass filtering above 0.1 Hz, a digital gain of 10 and a sampling interval of 10 μs (using a MultiClamp 700B amplifier and Digidata 1440A digitizer, both from Axon). Single recordings were performed for 10 min. Spontaneous events were taken into account when the amplitude exceeded three times the background noise. The analysis of spikes was carried out using Clampfit 10.2 software (Molecular Devices). Zebrafish were taken from the same egg clutch for each set of experimental and corresponding control animals. The distribution of zebrafish for use in different treatment groups was performed in a random fashion. Zebrafish studies and data analysis were not performed in a blinded fashion. A minimum of 10 zebrafish were used per control group, and between 14 and 38 zebrafish were

Spectral analysis. Spectral representations were generated using MATLAB R2007a. Signals were first high-pass filtered with a finite impulse response (cutoff frequency = 20 Hz, order = 512). For time-frequency maps, we used the short-time Fourier transform (function spectrogram). Plots for spectral densities were generated by averaging the periodograms of all events for a given subject. These periodograms were calculated over 150 ms using Welch's method (function p.welch).

used for the experimental conditions. Sample sizes were not predetermined.

Rescue experiments. Wild-type human *STX1B* cDNA under the control of the zebrafish brain-specific *her4* promoter was cloned into the Tol2 expression vector (a gift from G. Weidinger; University of Ulm) using PacI and NotI sites (WT *STX1B*-Tol2). To monitor *STX1B* expression in zebrafish larvae, the sequence for the fluorescent reporter mCherry was cloned in frame, 3' to the *STX1B* sequence and separated by the sequence for the self-cleaving peptide P2A. The mutation in affected individuals encoding p.Val216Glu was cloned into the *STX1B* sequence in the Tol2 expression vector via site-directed mutagenesis (primer sequences are listed in **Supplementary Table 13**).

Transposase (*TRP*) mRNA was capped and transcribed using the T7 Ultra mMessage mMachine kit (Ambion). The rescue experiment was performed by cytoplasmic microinjection of 10 pg of WT *STX1B*-Tol2 or V216E *STX1B*-Tol2 and 50 pg of *TRP* (1-nl volume) into embryos at the one-cell stage and subsequent injection of 3 ng of *stx1b*-MO (2-nl volume) into the yolk. After injections, the embryos were selected for carboxyfluorescein-MO (FAM-MO) fluorescence. Brain-specific mCherry expression was used to sort larvae with low mosaicism and high expression of Stx1b protein at 5 d.p.f. Open-field recordings were performed on double-fluorescent zebrafish larval tecta at 5 d.p.f. at room temperature.

Statistical analysis. Data are presented as mean values \pm s.e.m. Pairwise statistical significance was determined with Student's unpaired *t* test or the Mann-Whitney test for data that failed the normality test, as appropriate, using GraphPad Prism 6 software.

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Corrigendum: Mutations in *STX1B*, encoding a presynaptic protein, cause fever-associated epilepsy syndromes

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In the version of this article initially published online, the amplitudes of the multispike bursts associated with epileptiform paroxysmal events were incorrectly reported as being equal to baseline or exceeding it by threefold. The amplitudes should have been reported as exceeding baseline by at least threefold. The error has been corrected for the print, PDF and HTML versions of this article.

