UNC13B variants associated with partial epilepsy with favourable outcome

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The unc-13 homolog B (UNC13B) gene encodes a presynaptic protein, mammalian uncoordinated 13-2 (Munc13-2), which is highly expressed in the brain—predominantly in the cerebral cortex—and plays an essential role in synaptic vesicle priming and fusion, potentially affecting neuronal excitability. However, the functional significance of the UNC13B mutation in human disease is not known.

In this study, we screened for novel genetic variants in a cohort of 446 unrelated cases (families) with partial epilepsy without acquired causes by trio-based whole-exome sequencing. UNC13B variants were identified in 12 individuals affected by partial epilepsy and/or febrile seizures from eight unrelated families. The eight probands all had focal seizures and focal discharges in EEG recordings, including two patients who experienced frequent daily seizures and one who showed abnormalities in the hippocampus by brain MRI; however, all of the patients showed a favourable outcome without intellectual or developmental abnormalities. The identified UNC13B variants included one nonsense variant, two variants at or around a splice site, one compound heterozygous missense variant and four missense variants that cosegregated in the families. The frequency of UNC13B variants identified in the present study was significantly higher than that in a control cohort of Han Chinese and controls of the East Asian and all populations in the Genome Aggregation Database (gnomAD). Computational modelling, including hydrogen bond and docking analyses, suggested that the variants lead to functional impairment. In Drosophila, seizure rate and duration were increased by Unc13b knockdown compared to wild-type flies, but these effects were less pronounced than in sodium voltage-gated channel alpha subunit 1 (Scn1a) knockdown Drosophila. Electrophysiological recordings showed that excitatory neurons in Unc13b-deficient flies exhibited increased excitability.

These results indicate that UNC13B is potentially associated with epilepsy. The frequent daily seizures and hippocampal abnormalities but ultimately favourable outcome under anti-epileptic therapy in our patients indicate that partial epilepsy caused by UNC13B variant is a clinically manageable condition.
**Introduction**

Partial (focal) epilepsy is a group of common epilepsies with variable aetiologies and outcomes. In some patients, focal epilepsy has an acquired cause such as trauma, infection, immune abnormalities, or neoplasms,1,2; however, in most cases, the aetiology is unknown. Idiopathic focal epilepsy (IFE), also known as localization-related idiopathic epilepsy (G40.0 in ICD-10 2016, World Health Organization), is a common subtype of focal epilepsy with generally good prognosis; it typically manifests in childhood as benign epilepsy with centrotemporal spikes (BECTS; MIM: 117100), which affects around 0.2% of the population.3 Around 4.9% of the patients with BECTS harbour a variant of the glutamate ionotropic receptor N-methyl-D-aspartate type subunit 2A (GRIN2A) gene.4 Other possible causative genes of IFE include DEP domain-containing 5, GATOR1 subcomplex subunit (DEPDC5), elongator acetyltransferase complex subunit 4 (ELP4), leucine-rich glioma inactivated 1 (LGI1), and sashu repeat-containing protein X-linked 2 (SRPX2), with variants of these genes identified in rare cases.5–8 However, the genetic causes of IFE are usually undetermined.9 In this study, we screened for novel genetic variants in a cohort of 446 unrelated cases (families) with partial epilepsy without acquired causes by trio-based whole-exome sequencing (WES). Variants of the unc-13 homolog B (UNC13B) gene were identified in eight unrelated families, and we established a Drosophila knockdown model to investigate the role of UNC13B variant in epilepsy.

**Materials and methods**

**Patients**

A total of 446 unrelated cases (trios) were recruited, including 313 consecutive cases at the Epilepsy Centre of The Second Affiliated Hospital of Guangzhou Medical University from 2012 to 2019; and 133 cases from Fujian Medical University Union Hospital, 900 Hospital of the Joint Logistics Team, People’s Hospital of Xinjiang Uyghur Autonomous Region, and The First Affiliated Hospital of Shanxi Medical University in 2019. Detailed clinical information, including seizure onset age, seizure type and frequency, course of seizure, response to anti-epileptic treatment, family history and findings from general and neurological examinations, was collected. Brain MRI scans were performed to identify abnormalities in brain structure. Video EEGs, including hyperventilation, intermittent photic stimulation and sleep recording, were performed and the results were reviewed by two qualified investigators. Epileptic seizures and epilepsy syndromes were diagnosed and classified according to Commission on Classification and Terminology of the International League Against Epilepsy criteria (1989, 2001, 2010 and 2017). All enrolled patients were diagnosed with epilepsy that was characterized by focal seizures or f ocally originating secondary generalized tonic-clonic seizures. In all cases, EEG recordings showed focal discharges with features of idiopathic epilepsy including shifting, bilateral or multiple focal discharges with normal background. Participants with acquired causes or typical generalized seizures, such as absence, atonic and generalized myoclonic seizures, were excluded. All participants were Han Chinese with four Han Chinese grandparents, and were born to non-consanguineous Chinese parents. In 98 cases, there was a family history of epilepsy or febrile seizures. All enrolled patients were followed up for at least 1 year at epilepsy centres. As a control group, we recruited 150 patients with idiopathic generalized epilepsy. Additionally, WES was performed in 296 healthy Chinese volunteers who served as a normal control group as in our previous report.10 We also analysed a Han Chinese control population (n = 10 640) from a recent large-scale low-depth whole-genome sequencing study on major depressive disorder (we previously obtained the original data through the two provided links),11 and compared the frequencies of the identified variants with those in the control participants of the East Asian and all populations in the Genome Aggregation Database (gnomAD). This study was approved by the ethics committee of The Second Affiliated Hospital of Guangzhou Medical University, and written, informed consent was obtained from all patients and their parents.

**Whole-exome sequencing and genetic analysis**

Blood samples were obtained from the probands, their parents and available family members to determine the origin of the identified genetic variants. Genomic DNA was extracted from the blood samples using the FlexiGene DNA kit (Qiagen). Trio-based WES was performed on a HiSeq 2000 system (Illumina) as previously reported.10,12,13 The sequencing data were generated by massive parallel sequencing with > 125 times average depth and > 98% coverage in the capture region of the chip to obtain high-quality reads that were mapped to the Genome Reference Consortium Human Genome build 37 (GRCh37) by Burrows–Wheeler alignment. Single-nucleotide point variants and indels were called with the Genome Analysis Toolkit. To identify candidate causative variants in each trio, we adopted a case-by-case analytical approach. We first prioritized the rare variants with a minor allele frequency < 0.005 in the 1000 Genomes Project, Exome Aggregation Consortium and gnomAD. We retained potentially pathogenic variants containing frameshift, nonsense, canonical splice site, initiation codon and missense mutations predicted as being damaging using in silico tools (http://varcards.biols.ac.cn/). Finally, we screened for possibly disease-causing mutations/variants in each case under five different models: (i) epilepsy-associated gene; (ii) de novo variant dominant; (iii) autosomal recessive inheritance, including homozygous and compound heterozygous variants; (iv) X-linked; and (v) cosegregation analysis. To identify novel epilepsy-associated genes, we set aside the known epilepsy-associated genes,14 and selected the genes with null variants, de novo variants, bi-allelic variants, hemizygous variants and variants with...
segregations for further studies to define the gene-disease association. The candidate variants were validated by Sanger sequencing. All UNC13B variants identified in this study were annotated to reference transcript NM_006377.3.

Computational modelling and docking

The structure of mammalian uncoordinated 13-2 (Munc13-2), the protein encoded by UNC13B, was modelled using SWISS-MODEL (https://swissmodel.expasy.org/) to predict the effect of missense variants on protein structure. The model of the Thr103Met variant was based on the 2cj5.pdb template and those of the Arg661Cys, Gly794Asp and Gly882Trp variants were based on the 5ue8.1.pdb template in the Protein Data Bank (PDB) (https://www.rcsb.org/). Hydrogen bonding was analysed and visualized using PyMOL v.2.3 software (https://pymol.org/2/). The interaction of the calmodulin-binding domain of neuronal nitric oxide synthase with wild-type or mutant Munc13-2 was analysed. The docking results were visualized using PDBePISA (https://www.ebi.ac.uk/pdbe/pisa/).

Fly stocks

Flies were fed standard cornmeal and maintained in incubator at 25°C (except where otherwise noted) and 60–70% humidity on a 12:12 h light/dark cycle. UAS-UNC13B-RNAi (THU2483/FBst0029548), UAS-Scrna1-RNAi (para-RNAi, positive control) and UAS-Chd3-RNAi (negative control) flies were donated by Tsing Hua Fly Center (Tsinghua University, Beijing, China). The green fluorescent protein (GFP) reporter line UAS-EGFP and a double balance line were purchased from Bloomington Fly Stock Center. The Gal4 driver line tub-Gal4 was a gift from Professor Liu Ji-Yong (Guangzhou Medical University, Guangzhou, China), and the UAS-mCD8::GFP line was a gift from Professor KE Ya (The Chinese University of Hong Kong, Hong Kong). Canton-S was used as the wild-type line in this study.

Seizure behaviour test

The tub-Gal4 line was crossed with Unc13b-RNAi to establish global Unc13b knockdown flies (tub-Gal4> Unc13b-RNAi). The bang-sensitive (BS) test was performed on flies 3–5 days after eclosion and seizure-like behaviour was assessed. Flies were anaesthetized with CO2 and transferred to new clean food vials 1 day before testing. About two to five flies were placed in one vial and mechanically stimulated with a vortex mixer (VWR) at maximum speed for 10 s. The percentage and duration of BS paralysis in flies were recorded.

Electrophysiology and morphological analysis

Attached recording was performed in Unc13b knockdown flies using an established electrophysiological method. Fly brains were dissected as previously described and transferred to a recording chamber with fly external solution and immobilized with a C-sharp holder. The standard external solution contained (in mM) 101 NaCl, 1 CaCl2, 4 MgCl2, 3 KCl, 5 glucose, 1.25 NaH2PO4 and 20.7 NaHCO3 (pH7.2 and 250 mOsm). All dissection procedures were performed under a dissecting microscope. The anterior side of the brain was positioned facing upwards to enable observation of the soma of projection neurons on the brain surface. A patch pipette filled with external solution was used for attaching recorded with a 700B amplifier. Data were acquired with a Digidata 1440B digital–analogue converter (Molecular Devices) and pClamp v.10.5 software (Molecular Devices).

To examine morphologic alterations in the brain, UAS-mCD8::GFP was used to generate tub-Gal4>UAS-mCD8::GFP/UAS-Unc13b-RNAi and tub-Gal4>UAS-mCD8::GFP lines of Unc13b knockdown and control flies, respectively, that were labelled with GFP. The brain was dissected and fixed with 4% paraformaldehyde in PBS with 0.1% Triton™ X-100 for 1 h at 25°C, then washed and permeabilized three times with 0.3% Triton™ X-100 PBS. Images were captured using a confocal microscope (SP8; Zeiss) and analysed with ImageJ software (National Institutes of Health, Bethesda, MD, USA).

Statistical analysis

All quantitative data are presented as mean ± standard error of the mean (SEM). The Student's t-test was used to compare two independent or paired samples. One-way ANOVA was used to compare multiple samples, and Tukey’s post hoc test was used to evaluate differences between two groups. The Kruskal–Wallis test was used to assess non-parametric data, and a two-tailed Fisher's exact test was used to compare allele frequencies between groups. Statistical analyses were performed with GraphPad Prism 7.00 and SPSS 20. The cut-off value for statistical significance was 0.05.

Data availability

Raw data were generated at Institute of Neuroscience, The Second Affiliated Hospital of Guangzhou Medical University. Derived data supporting the findings of this study are available from the corresponding author on request.

Results

Identification of UNC13B variants

Variants in UNC13B were identified in eight unrelated patients with partial epilepsy, including one nonsense variant (c.155G>A/p.Trp45X), two variants at or around a splice site (c.4008+1G>T and c.4330+7G>A) and six missense variants (c.308C>T/p.Arg661Cys, c.662G>A/p.Trp45X/p.Trp45X, c.1190C>T/p.Ser397Phe, c.1981C>T/p.Arg661Cys, c.2381G>A/p.Gly794Asp and c.2644G>T/p.Gly882Trp). Two of the missense variants, c.308C>T/p.Arg661Cys and c.1190C>T/p.Ser397Phe, constituted a pair of compound heterozygous variants. The variants c.135G>A/p.Trp45X and c.4330+7G>A were de novo. The c.4008+1G>T variant was from an unaffected mother. Except for the compound heterozygous variants, the other four heterozygous missense variants were identified in families with more than one affected individual (Fig. 1).

The c.155G>A/p.Trp45X variant was potentially deleterious, yielding a truncated transcript that gave rise to a non-functional Munc13-2 protein or haploinsufficiency. The c.4008+1G>T variant disrupted the original splice donor site and was predicted to introduce a cryptic splice donor site in exon 34 that resulted in the skipping of the exon or deletion of its last nucleotide with consequent translational frameshift. Although the functional outcome is unknown, c.4330+7G>A was a de novo variant that was not listed in any public databases (including 1000 Genomes Project, Exome Sequencing Project and gnomAD). Three other variants (c.135G>A/p.Trp45X, c.2381G>A/p.Gly794Asp and c.2644G>T/p.Gly882Trp) were also not found in gnomAD. The other five variants were present at an extremely low frequency in gnomAD (controls of all populations and East Asian populations; see Supplementary Tables 1 and 2). These variants were not observed in our 296 normal control participants except for c.1190C>T/p.Ser397Phe, which was present in one individual. The c.1190C>T/p.Ser397Phe variant was one of the pair of
compound heterozygous variants in UNC13B (the other being c.308C>T/p.Thr103Met).

We performed a comparative analysis on the frequencies of the variant UNC13B alleles in the present cohort and control populations in gnomAD. Nine mutant alleles in a total of 892 (446 cases) were detected in our cohort, which were present at frequencies of 0.00199 (18/9042) in the controls of the East Asian population and 0.00027 (29/109378) in the controls of all populations in gnomAD. The differences in frequencies of the identified variants between our cohort and the two control populations in gnomAD were statistically significant (9/892 versus 18/9042, \(P = 4.08 \times 10^{-4}\); 9/892 versus 29/109378, \(P = 2.05 \times 10^{-11}\), respectively). Except for c.2644G>T/p.Gly882Trp, these variants were absent in the cohort of 10 640 Han Chinese in the study on major depressive disorder \(^{11}\) (9/892 versus 1/21280, \(P = 2.79 \times 10^{-12}\)) (Supplementary Table 2).

None of the 12 affected individuals had pathogenic or likely pathogenic variants in the 977 genes known to be associated with epileptic phenotypes.\(^{14}\) In control patients with idiopathic

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**Figure 1 Genetic data of cases with UNC13B variants.** (A) Pedigrees and DNA sequencing chromatogram of the eight cases with UNC13B variants and their corresponding phenotypes. (B) Amino acid sequence alignment of the six missense variants with protein substitutions show that Arg221, Arg661 and Gly794 are highly conserved across species. Thr103 and Gly882 are highly conserved in vertebrates but less conserved in lower animals, while Ser397 shows a low degree of conservation. (C) Schematic illustration of the Munc13-2 protein and the location of the UNC13B variants or protein substitutions identified in this study.
generalized epilepsy, we did not find any deleterious, de novo or compound heterozygous UNC13B variants or cosegregating variants.

The missense variants were predicted to be damaging by more than one of the commonly used in silico prediction tools (Supplementary Table 1). Protein sequence alignment indicated that Arg221Gln, Arg661Cys and Gly794Asp were located at residues that are highly conserved across species; Thr103 and Gly882 are highly conserved in vertebrates but less so in lower animals (Fig. 1B). The Ser397Phe was located at a less conserved site but was predicted to be conserved by in silico tools (Supplementary Table 1).

Munc13-2 is a large protein with seven evolutionarily conserved domains including an N-terminal C2 domain (C2A), Ca\(^{2+}\)/phospholipid-binding C2 domain (C2B), C-terminal non-Ca\(^{2+}\)-binding C2 domain (C2C), calmodulin-binding domain (CaM), phorbol ester/diacylglycerol-binding C1 domain (C1) and two Munc13 homology domains (MHD1 and MHD2) (Fig. 1C).\(^{19}\) Ser397Phe and Arg661Cys were located in the CaM and C2B domains, respectively, which are regulated by Ca\(^{2+}\) and mediate neurotransmission and neural plasticity. Alterations in hydrogen bonding caused by Thr103Met, Arg661Cys, Gly794Asp and Gly882Trp were analysed using available templates in SWISS-MODEL and PyMOL (Fig. 2). Thr103 formed two hydrogen bonds with Glu102 that were destroyed by Thr103Met (Fig. 2A). Gly661 formed five hydrogen bonds—three each with Tyr492, Ser659 and Lys663 and two with Glu493. Right: Arg661Cys destroys three of the five hydrogen bonds without affecting those with Ser659 and Lys663. (C) Left: Gly794 (blue) forms one hydrogen bond (yellow) with Ala790. Right: Gly794Asp does not affect hydrogen bonding in Munc13-2. (D) Gly882 (cyan) does not form hydrogen bonds with any other residue, and Gly882Trp does not affect hydrogen bonding in Munc13-2.

Clinical features

UNC13B variants were identified in eight cases (families) with 12 affected individuals (Fig. 1 and Table 1). The eight probands all had focal seizures or focally originating tonic-clonic seizures; all had focal discharges in EEG recordings. Two probands were diagnosed as BECTS and one as benign occipital epilepsy. The other five
| Case ID | Variant (NM_006377.3) | Sex | Age, years | Seizure onset | Seizure course | Seizure timing | Seizure-free duration, years | Effective AED | EEG | Brain MRI | Development | Diagnosis |
|---------|----------------------|-----|------------|--------------|---------------|----------------|-----------------------------|----------------|-----|-----------|-------------|-----------|
| 1       | c.135G>A (p.Trp45X)  | Female | 17         | 7 y          | sGTCS and CPS, 1/mo for 1 y | Nocturnal       | 9 y                         | OXC            | Left occipital spikes and slow spike waves | Normal       | Normal     | BOE        |
| 2       | c.4008+1G>T         | Male | 10         | 5 y          | GTCS, 1–2/mo for 3 y | Mostly nocturnal | 2 y                         | VPA, LEV | Right central-temporal sharp and sharp-slow waves | NA           | Normal     | BECTS      |
| 3       | c.4330+7G>A         | Male | 17         | 12 y         | sGTCS, 1–3/mo for 8 mo | Nocturnal       | 4 y                         | OXC            | Left, right and bilateral central-temporal spikes | Normal       | Normal     | BECTS      |
| 4-1     | c.662G>A (p.Arg221Gln) | Female | 2            | 3 mo         | CPS, 10–15/d for 1 mo | Mostly on awakening | 2 y                        | OXC            | Ictal: right frontal-originating CPS; interictal: no discharge | Normal       | Normal     | PE         |
| 4-2     |                | Female | 34         | 1 y          | FS, 2–3 for 1 y | Mostly diurnal | 32 y                        | –              | NA                   | Normal       | Normal     | FS         |
| 5-1     | c.1981C>T (p.Arg661Cys) | Female | 4            | 8 mo         | sGTCS and CPS, 1–2/wk for 4 mo | –              | 3 y                         | LEV            | Frontal and midline sharp waves and sharp-slow waves | Normal       | Normal     | PE         |
| 5-2     |                | Female | 32         | 3 y          | GTCS in childhood, occasionally in adulthood | –              | 4 y                         | –              | NA                   | Normal       | Normal     | UE         |
| 6-1     | c.2381G>A (p.Gly794Asp) | Female | 19         | 7 mo         | FS once at 7 mo, GTCS and CPS 2–4/y from age 14–18 y | Game-precipitated, nocturnal | 1 y                        | LTG, VPA | Left central-temporal small spikes | Normal       | Normal     | FS, PE     |
| 6-2     |                | Male | 40         | 1 yr         | FS twice | Diurnal and Nocturnal | 39 y                        | –              | NA                   | Normal       | Normal     | FS         |
| 7-1     | c.2644G>T (p.Gly882Trp) | Male | 7            | 2 yr         | FS and afebrile seizures 4–5/y for 2 y | –              | 3 y                         | LEV            | Right frontal and central-temporal spikes and sharp waves, and sharp/slow spike waves | Normal       | Normal     | FS, PE     |
| 7-2     | c.308C>T (p.Thr103Met) | Female | 30         | 2 yr         | FS 1–2/y for 2 y | –              | 26 y                        | –              | NA                   | Normal       | Abnormal   | FS, PE     |
| 8       | c.1190C>T (p.Ser397Phe) | Female | 29         | 22 yr        | sGTCS and CPS, 2–3/mo and up to 5/day for 6 y | –              | 1 y                         | LTG            | Ictal: 1 sGTCS and 4 CPS of indeterminate origin; interictal: left and right temporal spikes and sharp waves | Normal       | Normal     | PE         |

AED = anti-epileptic drug; BECTS = benign childhood epilepsy with centrotemporal spikes; BOE = benign occipital epilepsy; CPS = complex partial seizure; FS = febrile seizure; GTCS = generalized tonic-clonic seizure; LEV = levetiracetam; LTG = lamotrigine; mo = months; NA = not available; OXC = oxcarbazepine; PE = partial epilepsy; sGTCS = secondary generalized tonic-clonic seizure; UE = unclassified epilepsy; VPA = valproate; wk = week; y = years.
proband were diagnosed as partial epilepsy, including two with antecedent febrile seizures. In the eight families, no any other members except the parents have a history of epilepsy or febrile seizures. Intercital or ictal epileptic discharges were detected in all of the probands. The interictal discharges in seven patients showed focal abnormalities with features of idiopathic epilepsies including shifting, bilateral, multiple focal discharges with normal backgrounds or trends of generalization especially during sleep (Fig. 4); no photosensitivity were recorded in these patients. Frequent daily seizures occurred in two patients (Cases 4-1 and 8; Table 1), but no any prolonged seizures or status epilepticus were observed in the affected individuals. All patients were seizure-free after anti-epileptic treatment. The patient with a compound heterozygous variant had secondary generalized tonic-clonic seizures or complex partial seizures at a frequency of up to five times per day (five seizures recorded during one night). The condition lasted for 6 years, which was longer than in other cases. The patient’s brain MRI showed structural asymmetry between the right and left hippocampi; the right hippocampus was smaller than the left one, with a slightly higher signal. The boundary between the left hippocampus and surrounding tissues was indistinct.

Unc13b knockdown in flies

To clarify the relationship between UNC13B variant and seizure sensitivity, we examined BS seizure-like behaviour in tub-Gal4 > Unc13b-RNAi Unc13b knockdown flies. Interestingly, Unc13b knockdown was pre-adult lethal. There were no adults among the tub-Gal4 > Unc13b-RNAi flies treated at 25°C in the pre-adult stage, indicating that Unc13b plays a critical role in the early life of flies. Temporally controlled knockdown was performed to evaluate the effect of Unc13b deficiency on epileptogenesis. The tub-Gal4 > Unc13b-RNAi flies were maintained at 18°C until eclosion, as RNAi efficiency declined at the pre-adult stage. Adult flies were collected and maintained at 29°C for 48 h to increase gene knockdown efficiency before behavioural testing. The tub-Gal4 > Unc13b-RNAi flies exhibited the typical seizure-like behaviour observed in other seizure mutants (bss, tko and para mutants). The three phases of seizure activity in the BS test were observed—namely, seizure, paralysis and recovery (Fig. 5A and Supplementary Video 1). About 24.8% of tub-Gal4 > Unc13b-RNAi flies showed obvious seizure-like behaviour, which was higher than the rate in Unc13b-RNAi control flies (24.83 ± 5.06% (n = 6) versus 4.36 ± 1.29% (n = 7); P = 0.008; Fig. 5B). The tub-Gal4 > Scn1a-RNAi positive control flies had a higher rate of seizures than tub-Gal4 > Unc13b-RNAi flies (48.88 ± 2.60% (n = 5) versus 24.83 ± 5.06% (n = 6); P = 0.006). Most tub-Gal4 > Unc13b-RNAi flies recovered within 1–6 s whereas most controls recovered within 0–1 s. In contrast, the recovery time of tub-Gal4 > Scn1a-RNAi flies was 3–12 s, which was longer than for tub-Gal4 > Unc13b-RNAi flies (Fig. 5C).

Figure 4 Representative EEG recordings and MRI from patients with UNC13B variants. (A) Interictal EEG in Case 1 showed left occipital slow spike waves. (B) Interictal EEG in Case 3 showed left central-temporal slow spike waves. (C and D) Interictal EEG in Case 8 showed spikes or sharp waves in the left or right temporal lobe, or of slow spike waves in the right frontal lobe. (E and F) Coronal and axial T2-FLAIR MRI of Case 8 revealed structural asymmetry in the hippocampus; the right hippocampus was smaller than the left one, with a slightly higher signal. The boundary between the left hippocampus and surrounding tissues was indistinct.
We examined the effect of Unc13b deficiency on the electrophysiological activity of projection neurons, which are important excitatory neurons in the central nervous system of Drosophila, by attached recording. Extracellular activity (action currents) was detected by loose patching; the membrane resistance was 200–250 MΩ. Typical traces of extracellular action currents of wild-type flies are shown in Fig. 6A. The projection neurons of Unc13b knockdown flies (tub-Gal4>Unc13b-RNAi) showed a regular burst firing pattern (Fig. 6B). Meanwhile, tub-Gal4>Unc13b-RNAi flies had significantly higher frequency of extracellular action currents in projection neurons than wild-type flies [8.14 ± 0.94 Hz (n = 6) versus 0.76 ± 0.22 Hz (n = 5); P = 0.0003] (Fig. 6C). There was no significant difference in action current amplitude between tub-Gal4>Unc13b-RNAi and wild-type flies [86.65 ± 7.24 pA (n = 6) versus 76.80 ± 5.35 pA (n = 5); P = 0.33] (Fig. 6D).

We examined possible morphological changes in Unc13b knockdown flies based on GFP expression. The confocal images showed that Unc13b knockdown did not affect the main brain structures (Fig. 7A–F), except for slight loss of the calyx structure that is a part of mushroom body (Fig. 7G and H). As possible abnormalities in the hippocampus were observed in one of the patients (Case 8), we examined the morphology of neurons in the mushroom body, which is considered to be analogous to the mammalian hippocampus, but found that it did not differ between Unc13b knockdown and wild-type flies except for slight blurring of the membrane and irregular cell body shape in the mutants (Fig. 7I and J).

Discussion

The UNC13B gene is located at chromosomal locus 9p13.3 and spans approximately 243 kb of genomic DNA. Eleven UNC13B transcript variants have been described. Isoform 1 (NM_006377.3) composed of 39 exons encodes the 1591-amino acid presynaptic protein Munc13-2, which contains a CaM domain, C1 domain, two
Munc13-homology domains and three C2 domains. Munc13 along with Munc18 functions in vesicle priming and regulates a readily releasable pool of fusion-competent synaptic vesicles by protecting the soluble N-ethylmaleimide-sensitive factor attachment protein receptor (SNARE) complex, which is essential for synaptic vesicle fusion and may modulate neuroexcitability (Supplementary Fig. 1). The syntaxin-binding protein 1 (STXB1) gene encoding Munc18-1 is associated with epileptic encephalopathy (EIEE-4, MIM: 612164). However, the association between UNC13B gene variants and human disease has not been previously reported. Homozygous UNC13B knockout mice exhibit abnormal synapse morphology and sporadic seizures (www.informatics.jax.org/marker/MGI:1342278), and specific knockdown of UNC13B in the medial prefrontal cortex resulted in neural hyperexcitability in the basolateral amygdala. In the present study, we identified UNC13B variants in 12 individuals affected by partial epilepsies and/or febrile seizures from eight unrelated families. The variants included two de novo variants, one compound heterozygous variant and four missense variants that co-segregated in the families. The frequency of UNC13B variants identified in the present study was significantly higher than that in the control cohort of Han Chinese and controls of the East Asian and all populations in gnomAD. In experiments with Drosophila, seizure-like behaviour and increased firing of neurons were observed in Unc13b knockdown flies. These findings provide evidence that UNC13B is potentially associated with epilepsy.

Variants identified in the present study included a truncating variant, splice-site variant and compound heterozygous variant, suggesting that they are associated with a loss of function. Among the six missense variants with protein substitution, Thr103Met abolished hydrogen bonding with Glu102. Arg221Gln was the most highly conserved residue in the protein sequence alignments. Ser397Phe and Arg661Cys were located in the CaM and C2B domains, respectively. Previous studies have shown that mutations in these domains may affect Ca\(^{2+}\) binding and Ca\(^{2+}\)-dependent neurotransmitter release. Gly794Asp and Gly882Trp were located in an area close to the C2B-MHD1 linker, which is associated with C2B inhibition; moreover, Gly794Asp may affect calmodulin docking. In the present study, Unc13b knockdown flies showed seizure-like behaviour and increased firing of excitatory neurons. This along with previous data from UNC13B knockdown and knockout mice suggests that UNC13B loss of function plays a role in cortical excitability and epileptogenesis. However, other pathogenic mechanisms such as gain of function and toxicity cannot be excluded. In fact, UNC13B gain of function is also predicted to promote transmitter release, similar to gain-of-function mutations in the synaptic protein Munc18-1 (encoded by STXB1) that lead to synaptic vesicle exhaustion and epilepsy. Indeed, both loss- and gain-of-function mutations in several genes [e.g. SCN1A, CACNA1A encoding the Ca\(^{2+}\) voltage-dependent calcium channel; and hyperpolarization-activated cyclic nucleotide gated potassium channel 1 (HCN1)] were shown to give rise to similar epilepsy phenotypes. Further studies are needed to clarify the functional consequences of each variant and their contribution to epileptogenesis.

All patients in the present work with UNC13B variants had focal seizures and focal discharges that were usually multifocal. We did not find UNC13B variants in patients with idiopathic generalized epilepsy. UNC13B is predominantly expressed in the cortex, which provides an anatomic basis for the pathogenesis of focal seizures and multifocal EEG discharges. Patients with UNC13B variants had favourable outcome without intellectual or developmental abnormalities and all achieved seizure freedom, including the two patients who experienced daily seizures during their illness. Three individuals had only febrile seizures. In experiments with Drosophila, the frequency of seizures was lower and their duration was shorter in Unc13b knockdown flies compared to Scn1a-deficient flies. SCN1A is the most common epilepsy gene with more than 1727 identified mutations, mostly in patients with severe Dravet syndrome (www.caae.org.cn/gzneurosci/scn1adatabase/). Our findings in flies are consistent with the relatively mild clinical phenotypes and favourable outcomes of patients with UNC13B.

Figure 7 Brain morphology in Unc13b knockdown flies. (A, C, E and G) Serial images of the brain of a wild-type fly from anterior to posterior showing the structure of antennal lobes (A), ellipsoid body (C), fan-shaped body (B), and protocerebral bridge (filled arrowhead) and mushroom body calyx (open arrowhead) (G). (B, D, F and H) Serial images of the brain of a Unc13b knockdown fly from anterior to posterior showing the structure of antennal lobes (B), ellipsoid body (D), fan-shaped body (F), and protocerebral bridge (white arrowhead) and mushroom body calyx (open arrowhead) (H). Scale bar = 100 µm. (I) Cell body of neurons (open arrowhead) in the mushroom body of a wild-type fly. (J) Cell body of neurons (open arrowhead) in the mushroom body of a Unc13b knockdown fly. Scale bar = 10 µm.
variants. Moreover, it was previously reported that normal Munc13 function is required for synaptic secretory activity rather than synaptogenesis in mice.30

The patient with a compound heterozygous variant in UNC13B presented frequent daily seizures, slight structural abnormalities in the hippocampus, had a longer course of illness and initially responded poorly to treatment, suggesting that biallelic variants lead to a more severe phenotype. On the other hand, three heterozygous UNC13B variants (c.4008+1G > T, c.662G > A/p.Arg221Gln and c.1981C > T/p.Arg661Cys) presented a low frequency in control population of gnomAD (Supplementary Table 1). Generally, variants in a gene may differ in damage effect, which varies from mild to severe, depending on factors such as the molecular subregional effect as shown in our recent study.38 It is possible that the UNC13B variants mentioned above were of less damaging effect and were associated with susceptibility or mild epileptic phenotype with incomplete penetrance; or UNC13B is a susceptibility gene of epilepsy. Additional genetic studies are required to determine the pathogenic potential of UNC13B and establish the extremes of severity of the UNC13B mutant phenotype. De novo UNC13B variants were previously reported in patients with bipolar disorder and autism spectrum disorder39-41; therefore, the association between UNC13B variants and neurologic disorders warrants further investigation.

In conclusion, we identified UNC13B variants in individuals affected by partial epilepsies and/or febrile seizures all of whom had favourable outcome with anti-epileptic treatment without intellectual or development abnormalities, including the patients who experienced daily seizures. Unc13b knockout flies resulted in seizure-like behaviour and increased neuronal firing; however, the phenotype was less severe than that of Scn1a knockout flies, consistent with our clinical observations of a milder epilepsy phenotype associated with UNC13B variants. Thus, UNC13B is potentially associated with partial epilepsy. Screening for UNC13B variants can identify individuals who require clinical attention for possibly frequent daily seizures but can be managed with anti-epileptic therapy.

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Competing interests

The authors report no competing interests.

Supplementary material

Supplementary material is available at Brain online.

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