Identification of a novel synaptic protein, TMTC3, involved in periventricular nodular heterotopia with intellectual disability and epilepsy

Sali M.K. Farhan1,2, Kevin C.J. Nixon3, Michelle Everest1,3, Tara N. Edwards3, Shirley Long3, Dmitri Segal3, Maria J. Knip3, Heleen H. Arts1,4,5, Rana Chakrabarti4,6, Jian Wang1, John F. Robinson1, Donald Lee7, Seyed M. Mirsattari3,8, C. Anthony Rupar2,4,6,9, Victoria M. Siu2,4,6, FORGE Canada Consortium, Michael O. Poulter1,3, Robert A. Hegele1,2 and Jamie M. Kramer3,4,10,*

1Molecular Medicine Research Group, Robarts Research Institute, London, ON, Canada, N6A 5B7, 2Department of Biochemistry, 3Department of Physiology and Pharmacology, Schulich School of Medicine and Dentistry, Western University, London, ON, Canada, N6A 5C1, 4Division of Genetics and Development, Children’s Health Research Institute, London, ON, Canada, N6A 5W9, 5Department of Human Genetics, Radboud Institute for Molecular Life Sciences, Radboud University Medical Centre Nijmegen, The Netherlands, 6Department of Pediatrics, 7Department of Medical Imaging, 8Departments of Clinical Neurological Sciences, Medical Biophysics, Medical Imaging and Psychology, 9Department of Pathology and Laboratory Medicine, Schulich School of Medicine and Dentistry, Western University, London, ON, Canada, N6A 5C1 and 10Department of Biology, Faculty of Science, Western University, London, ON, Canada, N6A 5B7

*To whom correspondence should be addressed at: Department of Physiology and Pharmacology, Schulich School of Medicine & Dentistry, Western University, Medical Sciences Building, Room 266, London, ON, Canada, N6A 5C1. Tel: 519-661-3740; Fax: 519-661-3827; Email: James.Kramer@schulich.uwo.ca

Abstract

Defects in neuronal migration cause brain malformations, which are associated with intellectual disability (ID) and epilepsy. Using exome sequencing, we identified compound heterozygous variants (p.Arg71His and p. Leu729ThrfsTer6) in TMTC3, encoding transmembrane and tetratricopeptide repeat containing 3, in four siblings with nocturnal seizures and ID. Three of the four siblings have periventricular nodular heterotopia (PVNHa), a common brain malformation caused by failure of neurons to migrate from the ventricular zone to the cortex. Expression analysis using patient-derived cells confirmed reduced TMTC3 transcript levels and loss of the TMTC3 protein compared to parental and control cells. As TMTC3 function is currently unexplored in the brain, we gathered support for a neurobiological role for TMTC3 by generating flies with post-mitotic neuron-specific knockdown of the highly conserved Drosophila melanogaster TMTC3 ortholog, CG4050/tmtc3. Neuron-specific knockdown of tmtc3 in flies resulted in increased susceptibility to induced seizures. Importantly, this phenotype was rescued by neuron-specific...
expression of human TMTC3, suggesting a role for TMTC3 in seizure biology. In addition, we observed co-localization of TMTC3 in the rat brain with vesicular GABA transporter (VGAT), a presynaptic marker for inhibitory synapses. TMTC3 is localized at VGAT positive pre-synaptic terminals and boutons in the rat hypothalamus and piriform cortex, suggesting a role for TMTC3 in the regulation of GABAergic inhibitory synapses. TMTC3 did not co-localize with Vglut2, a presynaptic marker for excitatory neurons. Our data identified TMTC3 as a synaptic protein that is involved in PVNH with ID and epilepsy, in addition to its previously described association with cobblestone lissencephaly.

**Introduction**

Many neurodevelopmental disorders associated with epilepsy and intellectual disability (ID) are caused by defects in neuronal migration, which result in brain malformations such as lissencephaly, pachygyria, polymicrogyria, and periventricular nodular heterotopias (PVNHs) (1). PVNH is one of the most common brain malformations, and is caused by the failure of neurons to migrate from the ventricular zone to the cortex during early brain development (2). PVNH has been linked to mutations in FLNA (OMIM 300049) (3), ARFGEF2 (OMIM 608097) (4), ERMARD (OMIM 615544) (5), and NEDD4L (OMIM 617201) (6). Functional investigation of these causative genes suggests that PVNH may result from disrupted cytoskeletal dynamics and vesicular trafficking (7). PVNH-causing mutations in NEDD4L have been shown to cause deregulation of Akt and mTor signaling (6). Despite these advances, the mechanisms underlying PVNH are still not well understood, and most cases have no genetic diagnosis.

Recently, the transmembrane and tetratricopeptide repeat containing 3 (TMTC3) gene was implicated in a neuronal migration defect leading to cobblestone lissencephaly (COB), a cortical malformation caused by over migration of neurons beyond the basement membrane. Here, we show that loss of TMTC3 also causes PVNH, in a family with four affected siblings presenting with nocturnal seizures and ID. Furthermore, we demonstrate a neuron-specific role for TMTC3 in mechanically induced seizures in Drosophila, and provide evidence for synaptic localization of TMTC3 in the mammalian brain.

**Results**

**Clinical description of patients with nocturnal seizures and ID**

Four siblings, three females and one male, the only children born to a healthy non-consanguineous Pakistani couple (Fig. 1A), have a similar history of mild to moderate ID and recurrent nocturnal seizures, with onset between one and four years of age (Table 1). The seizures start with upper respiratory airway obstruction, followed by tonic stiffening and coarse tremor of the limbs. Episodes typically last less than 1–2 min and can occur up to 4 times per night, up to 4 to 5 days a week. There is no history of diurnal or photic-induced seizures, except for few rare seizures during the day in II-2. Seizure frequency has decreased with age and on various combinations of carbamazepine, lamotrigine, valproic acid, and clobazam. Currently, at ages ranging between 21 and 30 years, all have been seizure-free for at least a year.

All four siblings were born at term and apparently had normal gross motor development with sitting and walking achieved by 1 year. Three of the four began talking at 1 year, but II-3 did not talk until 4 years. There were cognitive delays from early childhood, with no regression in skills. The two older siblings can read. II-3 and II-4 have behavioral problems consisting of aggression, agitation, irritability, and self-harm. All 4 siblings are bilingual in English and Urdu, and can express themselves to a greater or lesser degree and follow simple instructions. They are all capable of activities of daily living.

Subject II-2, whose seizures were the most difficult to control, has the highest cognitive functioning and has mild ID. The other three siblings have more limited vocabulary and skills, and are functioning in the moderate range of ID. In subject II-4, behavioral problems with aggression towards family and peers are significant and thus she cannot be left alone for any length of time. All have normal strength and can climb, run, and lift objects with no evidence of muscle weakness. None of the sibs has any ophthalmologic concerns.

Common physical features include abnormalities in dentition including enamel hypoplasia, anterior overbite and medial deviation of the mandibular dentition, and flexion contractures of the proximal interphalangeal joints of the 5th fingers (Table 1). Muscle strength and head circumferences are normal. Clinical images of all siblings are shown in the Supplementary Material, Figure S1. Individual II-3, age 23 years, has mild facial dysmorphism with mid-facial hypoplasia, low nasal bridge, several hyperpigmented macules on the tongue, flattening of the helix and bilateral epicanthal folds. She has extensive lymphedema of both lower extremities and her left upper extremity, which began following a fungal infection 10 years ago. She is also the only sib with borderline growth restriction.

Both parents have university degrees. Family history is negative for any other individuals with seizure disorder or intellectual disability.

**Electroencephalogram (EEG) findings:** Subject II-2 had an EEG at age 27 years which showed a moderate degree of generalized slowing in the theta range and an epileptic sharp wave in the left temporal region (Fig. 2A and B). EEG for the other three siblings was not possible, due to behavioral issues. These siblings had EEGs which were reportedly normal in childhood, but details were not available. Sleep EEG’s and video-EEG telemetry could not be carried out due to practical considerations.

**Diagnostic imaging:** MRI of the head in subject II-1, II-2, and II-3 showed bilateral periventricular heterotopias in the temporal lobes (Fig. 2C–E). Subject II-2 also showed a probable developmental venous anomaly in the superior temporal gyrus white matter and subject II-3 showed a stable downward displacement of the cerebellar tonsils, consistent with Arnold-Chiari type I malformation (Table 1). MRI scans at 1.5 T MR scanner in subject II-4 were normal with no definite evidence of neuronal migration defects.

**Compound heterozygous TMTC3 variants in patients with nocturnal seizures and ID**

Based on the family pedigree (Fig. 1A), dominant as well as recessive disease models were applied to determine the genetic
Figure 1. Compound heterozygous TMTC3 variants in patients with nocturnal seizures with developmental delay. (A) Pedigree and electropherograms of the compound heterozygous TMTC3 variants in patients with nocturnal seizures with intellectual disability. Different colored circles in pedigrees represent different variants. (B) Protein structure and associated domains of TMTC3 with respective variants identified. RefSeq numbers: NM_181783.3 and NP_861448.2. Transmembrane regions are shown in blue and the tetratricopeptide repeats (TPR) domains are shown in green. The location of the TMTC3 variants is shown with the black arrows.

Table 1. Clinical features of patients with nocturnal seizures with ID

| Affected individuals | II-1       | II-2       | II-3       | II-4       |
|---------------------|------------|------------|------------|------------|
| Clinical Features   |            |            |            |            |
| Gestation           | 41 weeks   | 41 weeks   | 41 weeks   | 40 weeks   |
| Delivery complications | Induced   | None       | Breech, caesarean section | Repeat caesarean section |
| Sex                 | F          | M          | F          | F          |
| Karyotype           | 46, XX     | 46, XY     | 46, XX     | 46, XX     |
| Birth weight (g)    | 4,082 (+2 SD) | 3,401 (0 SD) | 3,855 (+1 SD) | 3,402 (0 SD) |
| Age at last assessment (years) | 30       | 28         | 23         | 21         |
| Head circumference (cm) | 53.5 (-1SD) | 55.8 (+1 SD) | 53.7 (-1 SD) | 53.3 (-1 SD) |
| Height (cm)         | 164 (+1 SD) | 168 (-1 SD) | 150 (-2 SD) | 159.5 (0 SD) |
| Weight (kg)         | 54.4 (-0.5 SD) | 66.5 (+0.2 SD) | 46.2 (-2 SD) | 64.8 (+1 SD) |
| Associated diagnoses|            |            |            |            |
| Age to walk         | 9 months   | < 1 year   | < 1 year   | < 1 year   |
| Age to talk         | 1 year     | < 1 year   | 4 years    | < 1 year   |
| Able to read        | +          | +          | -          | -          |
| Age at onset of nocturnal seizures (years) | 2       | 5          | 3          | 3          |
| Enamel hypoplasia   | +          | -          | +          | +          |
| Palate              | N          | N          | High arched | N          |
| Anterior overbite   | +          | -          | +          | +          |
| Medial deviation of mandibular dentition | +          | -          | -          | +          |
| Tongue hyperpigmentation | +, single location | -          | +, extensive | -          |
| Flexion contracture of 5th PIP | +          | -          | +          | +          |
| Hair follicle hyperpigmentation | -          | +          | +          | -          |
| Lymphedema          | -          | -          | +          | -          |
| Hyperactive deep tendon reflexes | +          | +          | +          | +          |
| Hallux valgus       | +          | +          | +          | +          |
| MRI findings        |            |            |            |            |
| Cobblestone lissencephaly | -          | -          | -          | -          |
| Bilateral periventricular heterotopias | +          | +          | +          | -          |
| Chiari I malformation | -          | -          | +          | -          |
| Venous anomaly      | -          | -          | +          | -          |

Abbreviations: N, normal; +, present; –, absent; MRI, magnetic resonance imaging.
cause of the disease. Through whole exome sequencing, we observed two novel compound heterozygous variants (c.432 G > C, p.Arg71His; and c.2404insA, p.Leu728fsTer6) in TMTC3 in the affected individuals (Fig. 1B).

TMTC3 encodes the transmembrane and tetratricopeptide repeat containing 3 protein. These variants have not been reported in the Exome Aggregation Consortium (ExAC) database. Overall, there are no homozygous TMTC3 protein truncating variants found in ExAC, which further supports candidate pathogenicity of these variants.

TMTC3 contains 914 amino acids and is characterized by 9 transmembrane and 9 tetratricopeptide repeat (TPR) domains (Fig. 1B). Although the p.Arg71His variant is not located within any of these functional domains, multiple in silico analyses predicted the variant to be detrimental to protein function. This amino acid is conserved in mammals, zebrafish, and Drosophila, suggesting an important role in the function of the protein. The p.Leu728fsTer6 variant is predicted to cause truncation of the protein leading to complete loss of the final three TPR domains (Fig. 1B). TPR domains are critical for protein interactions and are known to be functional only in groups of 3 or 4. These domains represent the main functional component of TMTC3, and therefore their loss is likely to have a drastic impact on the protein. Furthermore, both TMTC3 variants segregated with disease status in the family (Fig. 1A). All four children are heterozygous for both variants, whereas the father is heterozygous only for p.Leu728fsTer6 and the mother is heterozygous only for p.Arg71His.

**Loss of TMTC3 in patients with nocturnal seizures and ID**

To determine the effect of the TMTC3 variants on protein and transcript expression, we evaluated TMTC3 expression levels via immunoblotting and RT-PCR, respectively, using patient-derived fibroblast cells. We observed complete loss of TMTC3 protein in fibroblast cells extracted from two affected individuals relative to both parents and a healthy TMTC3 wild type control.
Neuronal knockdown of Drosophila tmtc3 causes increased susceptibility to mechanically induced seizures

Having identified TMTC3 loss of function mutations in four sibling presenting with epilepsy, we sought to investigate the role of TMTC3 in seizure biology. The TMTC3 protein is highly conserved (60% amino acid similarity and 46% identity) and displays a one-to-one orthology with the Drosophila protein encoded by CG4050, here referred to as tmtc3. According to large scale expression data available on FlyBase, Drosophila tmtc3 is widely expressed, including a relatively high expression level in the adult brain and larval central nervous system (8). Over the course of development, however, tmtc3 has its highest expression in the early embryo (9). Because of the broad expression pattern, and the high expression in early development, we sought to analyze adult flies with a neuron specific TMTC3 knockdown rather than germline mutants, which may have phenotypes related to non-neuronal tissue or early developmental defects. To this end, we tested two Gal4-responsive RNAi transgenes encoding inverted repeats (IR) homologous to Drosophila tmtc3, UAS-tmtc3-IR1 and UAS-tmtc3-IR2. When RNAi expression was induced using the ubiquitous actin promoter to drive Gal4 expression there was a 70% reduction in tmtc3 mRNA with UAS-tmtc3-IR1 and only a 20% reduction with UAS-tmtc3-IR2 (Fig. 4A), therefore our analysis focuses on UAS-tmtc3-IR1.

We investigated the function of tmtc3 in flies using an established seizure paradigm known as bang sensitivity (10,11). In this assay, seizure-like behaviors are induced by mechanical disturbance (vortexing). The response is measured as the amount of time that individual flies are immobilized on their back and unable to right themselves onto their feet. To test for a role in mechanically induced seizures, we used flies with a neuron-specific RNAi knockdown of tmtc3 (genotype: elav-Gal4/+; UAS-dcr2/UAS-tmtc3-IR1) and compared these to genetic background controls possessing the elav-Gal4 driver with no RNAi, or the tmtc3 RNAi transgene with no Gal4 driver (genotypes: elav-Gal4/+; UAS-dcr2/++; UAS-tmtc3-IR1/UAS-dcr2). Notably, the proportion of flies experiencing immobilization in response to vortexing was increased by more than 2-fold upon neuron specific knockdown of tmtc3 compared to both controls (Fig. 4B; see Supplementary videos showing: a normal response with no immobilization in Supplementary Video S1; and a seizure-like response demonstrating immobilization on the back and uncontrollable movement in Supplementary Video S2). To confirm the specificity of this RNAi induced phenotype, we performed rescue experiments using two independent human TMTC3 transgenes (rescue genotypes: elav-Gal4/UAS-hTMTC3-1; UAS-dcr2/UAS-tmtc3-IR1 and elav-Gal4/UAS-hTMTC3-2; UAS-dcr2/UAS-tmtc3-IR1). Expression of both human transgenes did cause a significant reduction in the proportion of flies immobilized in response to vortexing, when compared to knocked down flies (Fig. 4B). Finally, we tested an additional genotype that expresses a UAS-GFP transgene in place of the human UAS-hTMTC3 transgenes (genotypes: elav-Gal4/UAS-GFP, UAS-dcr2/UAS-tmtc3-IR1). The purpose of this control is to ensure that the observed rescue by the human TMTC3 transgene is not due to the addition of an extra UAS site, which may titrate the available Gal4 protein such that the RNAi expression becomes too low to efficiently induce a knockdown. The presence of an additional UAS site did not significantly reduce the proportion of immobilized flies compared to UAS-tmtc3-IR1 alone (P = 0.21). The proportion of flies immobilized in the presence of UAS-GFP was clearly greater than that of the controls and the human...
transgenic rescue genotypes (Fig. 4B), and this difference was statistically significant or approaching significant (tmtc3-IR1 \( P = 0.004 \); elavGal4 \( P = 0.06 \); hTMTC3-1 \( P = 0.08 \); hTMTC3-2 \( P = 0.02 \)). Therefore, it appears that tmtc3 knockdown in neurons increases susceptibility to mechanically induced seizure in Drosophila, and this phenotype can be recovered by transgenic rescue in a humanized fly.

In addition to seizure susceptibility proportions at the population level, we also examined the duration of immobilization in response to vortexing for each individual fly. When considering only flies that were immobilized, the median recovery time is similar between all genotypes (Fig. 4C), suggesting that tmtc3 knockdown does not affect seizure severity, but rather increases the probability of immobilization in response to vortexing.

To further elucidate the underlying mechanisms of the observed immobilization phenotype, we investigated whether the knockdown of Drosophila tmtc3 may cause changes in neuronal morphology. We identified no morphological differences upon examination of several different neuronal structures in tmtc3 knockdown flies; including gross mushroom body morphology (Supplementary Material, Fig. S2A and B), dendrite arborization in type 4 multidendrite neurons (Supplementary Material, Fig. S2C–E), and synaptic morphology at the neuromuscular junction (Supplementary Material, Fig. S2F–H). These structures represent model systems for the analysis of axon, dendrite, and synaptic morphogenesis, respectively. This suggests that Drosophila tmtc3 may not have a widespread role in the regulation of neuronal morphogenesis, however, this analysis is limited to post mitotic knockdown and it is possible that germline null mutations may have a different effect.

TMTC3 is localized at presynaptic terminals in rat brains

TMTC3 is a predicted membrane protein containing 9 transmembrane domains, and has been shown to partially colocalize with an endoplasmic reticulum marker in cultured odontoblasts (12). Considering that TMTC3 has a functional role in Drosophila neurons and appears to play a conserved role in seizure susceptibility in both flies and humans, we sought to investigate the localization of TMTC3 in the mammalian brain, using rat brain sections. First, we used a commercially available anti-TMTC3 antibody (sc-398137, Santa Cruz) directed against the human protein, which has 90% amino acid identity to rat Tmtc3. This antibody showed a restricted expression of TMTC3 with the only significant signal appearing in the hypothalamus.
and piriform cortex (Fig. 5A and B). Immunoreactivity was observed to be punctate and not localized in cell bodies (see white arrows in A and B). This pattern of expression was completely blocked using a blocking peptide against sc-398173, suggesting that the signal is highly specific.

High magnification (60X) images confirm that TMTC3 immunofluorescence is indeed highly punctate, concentrated around cell bodies, but largely absent within cell bodies. We hypothesized that this expression could be in nerve terminals within these regions. To test this, we performed co-localization...
analysis with vesicular GABA transporter (VGAT), a presynaptic marker for inhibitory synapses, and vesicular glutamate transporter 2 (Vglut2), a presynaptic marker for excitatory synapses. TMTC3 did not appreciably co-localize with Vglut2 (Fig. 5C–E). To examine TMTC3 co-localization with VGAT we used a different TMTC3 antibody obtained from another supplier (ab81473; see Methods), as both TMTC3 (sc-398173) and our VGAT antibodies (Synaptic Systems, 131011) were raised in the same species (mouse). As the peptide information for ab81473 is specific for TMTC3 (compare Fig. 5D and G). Using this antibody, we found that TMTC3 was colocalized with a subpopulation of VGAT positive puncta in the hypothalamus (Fig. 5F–H) and piriform cortex (Fig. 5I).

These data show that TMTC3 is localized in a subpopulation of GABAergic inhibitory synaptic terminals and boutons, and absent from excitatory glutamatergic presynapses.

**Discussion**

PVNH is one of the most common forms of cortical brain malformation and for most cases there is no genetic diagnosis. Here, we identify TMTC3 mutations as a novel cause of PVNH, accompanied by nocturnal epileptic seizures and ID (Figs 1–3, Table 1). The identified TMTC3 variants have not been reported in any publically accessible database of genetic variation and our whole exome analysis did not reveal any other plausible candidate genetic variants in the four affected siblings. The ExAC database does not report a single example of homozygous loss of function or damaging missense mutations in TMTC3, indicating that disruption of the TMTC3 protein is likely to be detrimental to cell function. The implication of TMTC3 in PVNH is consistent with a recent report by Jerber et al., which identified a role for TMTC3 in COB, which also results from defects in neuronal migration (13). COB represents over-migration of neuronal cells, whereas PVNH occurs when neurons fail to migrate from the ventricles. Jerber et al., studied 25 families with COB, and six families (nine patients) carried recessive variants in TMTC3, none of whom carried the same recessive variants as the patients described herein. Frameshift or nonsense variants were absent from excitatory glutamatergic presynapses. However, the second TMTC3 antibody obtained from another supplier (ab81473; proprietary, we were unable to obtain a blocking peptide to directly demonstrate specificity. However, the second TMTC3 antibody showed an identical pattern of express as the original one, suggesting that ab81473 is specific for TMTC3 (compare Fig. 5D and G). Using this antibody, we found that TMTC3 was colocalized with a subpopulation of VGAT positive puncta in the hypothalamus (Fig. 5F–H) and piriform cortex (Fig. 5I).

These data show that TMTC3 is localized in a subpopulation of GABAergic inhibitory synaptic terminals and boutons, and absent from excitatory glutamatergic presynapses.

**Discussion**

PVNH is one of the most common forms of cortical brain malformation and for most cases there is no genetic diagnosis. Here, we identify TMTC3 mutations as a novel cause of PVNH, accompanied by nocturnal epileptic seizures and ID (Figs 1–3, Table 1). The identified TMTC3 variants have not been reported in any publically accessible database of genetic variation and our whole exome analysis did not reveal any other plausible candidate genetic variants in the four affected siblings. The ExAC database does not report a single example of homozygous loss of function or damaging missense mutations in TMTC3, indicating that disruption of the TMTC3 protein is likely to be detrimental to cell function. The implication of TMTC3 in PVNH is consistent with a recent report by Jerber et al., which identified a role for TMTC3 in COB, which also results from defects in neuronal migration (13). COB represents over-migration of neuronal cells, whereas PVNH occurs when neurons fail to migrate from the ventricles. Jerber et al., studied 25 families with COB, and six families (nine patients) carried recessive variants in TMTC3, none of whom carried the same recessive variants as the patients described herein. Frameshift or nonsense variants were found in 8 of 14 alleles, and 4 out of 6 families. All nine patients had ID, delayed gross and fine motor skills, delayed language development, and defects in neuronal migration. Eight of nine patients had the hallmark clinical feature, COB, while the remaining individual had subcortical and periventricular hypomyelination, which has been described to precede patchy dysmyelination, and overmigration of neuronal cells in the leptomeningeal space. The majority of patients (six of nine) had diurnal seizures commencing between 4 and 8 months. These observations, in combination with our study, collectively support a role for TMTC3 in a spectrum of cortical malformation disorders causing ID and epilepsy. Our study highlights the clinical variability that can be associated with TMTC3 mutations, by identification of individuals with clear loss of TMTC3 function, who present with a milder phenotype of nocturnal seizures and PVNH.

Functionally, we demonstrate a neuron-specific role for TMTC3 in Drosophila seizure susceptibility (Fig. 4). The “bang sensitivity” test employed here is a classic assay for mechanically induced seizure, characterized by immobilization in response to a strong mechanical stimulus (14). Loss of Drosophila tm3c specifically in post-mitotic neurons doubles the rate of immobilization in response to vortexing (Fig. 4B), suggesting that loss of tm3c renders neurons susceptible to seizure. This phenotype is rescued by expression of a human TMTC3 transgene specifically in neurons. Therefore, although the seizure paradigm employed here is not directly comparable to the human condition, we have illustrated that the human protein is functionally relevant in the context of mechanically induced seizures in Drosophila. To date, this type of humanized rescue experiment is not standard in Drosophila studies investigating human disease genes. However, recent studies have shown that this can be an effective means of assessing human gene function, and potentially disease causing mutations in flies (15–20).

The suspected mechanisms underlying cortical malformation disorders associated with epilepsy are primarily related to defects in cytoskeletal organization and cell signaling pathways, such as the Akt-mTOR pathway. Recently, Nedd4L was associated with PVNH and dysregulation of mTor signaling (21). Other components of the mTor signaling pathway, including the GATOR1 complex and TSC1/2, have been implicated in epilepsy with focal cortical dysplasia and diverse brain lesions suggestive of global defects in neuronal migration (22,23). The potential link between TMTC3 and these biological processes remains unclear.

TMTC3 is a membrane protein that contains several TPR-domains, which mediate protein-protein interactions in many different biological processes (24). Here, we show that TMTC3 is localized at inhibitory GABAergic synapses in the hypothalamus and piriform cortex of the rat brain, but is not present at excitatory glutamatergic synapses (Fig. 5). Interestingly, the TMTC3 paralogs, TMTC1 and TMTC2, are predicted to act as adaptors that nucleate the formation of large protein complexes involved in the regulation of calcium transport across the ER membrane (25). Based on the similar domain structure seen in TMTC3, it is possible that this protein plays a similar role at the synapse. This is supported by protein interaction data for TMTC3 from several proteomic studies (26–29), which are annotated through the BioGRID database (30). Of the 21 TMTC3 interactors listed in BioGRID, six are involved in gated ion channel activity (CHRN, HTR3C, HTR3A, ZACN, GABRE, and SCN3B) and five are localized at the synapse (CHRN, HTR3A, NRG1, GABRE, and DAB1; annotations from PANTHER) (31). Interestingly, neuronal migration is known to depend on synaptic activity and the regulation of calcium gradients (32,33). In mice, migration of neurons in the subventricular zone is dependent on tight regulation of GABA release and uptake (34). TMTC3 might regulate neuronal migration through modulation of synaptic activity or through calcium transport at GABAergic synaptic terminals, however, further studies are needed to investigate this possibility. This study identifies TMTC3 as a novel synaptic protein involved in PVNH, epilepsy and ID, and lays the foundation for future investigations towards understanding the specific role of TMTC3 in the regulation of synaptic function and neuronal migration.

**Materials and Methods**

**Patients**

Four children of a healthy non-consanguineous Pakistani couple were diagnosed with nocturnal seizures of unknown cause with ID. Blood and tissue (skin-derived fibroblasts) samples were collected from all four affected children and both parents following appropriate and informed consent in accordance with
the Research Ethics Board at Western University, London, Ontario, Canada.

DNA isolation
DNA was isolated from blood or cultured cells collected from every family member using the Gentra Puregene Blood kit (Qiagen) per the manufacturer’s instructions.

Next generation sequencing
Whole exome sequencing was performed using genomic DNA from two affected individuals (II-2 and II-3) on the Illumina HiSeq 2000 with 2x100 paired end chemistry in accordance with library protocols used at The Centre for Applied Genomics (TCAG) at The Hospital for Sick Children, Toronto, Ontario as previously described (35,36). Genome Analysis Toolkit (GATK) was used to generate the coverage of the whole exome in the form of variant calling format (VCF) files as previously described (35,37,38).

Variant discovery
Initially, we applied a dominant model analysis on the patients’ exomes with no mutations observed in known epilepsy genes as we were considering the possibility of incomplete penetrance or mosaicism. We also did not observe any promising dominant variants in other genes. We then applied a recessive model of inheritance (homozygous and compound heterozygous) on all variants. We scanned for variants in genes known to cause seizures and/or ID based on literature and OMIM, as well as all protein-coding genes covered by whole exome sequencing. Given the rarity of the disease, we screened for rare variants (minor allele frequency [MAF, <1%]) according to NCBI dbSNP, 1000 Genomes (39), NHLBI ESP Exome Server (40), and Exome Aggregation Consortium (ExAC) (41) datasets to identify any variants. To determine the predicted biological effect of non-synonymous variants on protein function, candidate variants (p.Arg71His and p.Leu728fsTer6) were assessed using the following predictive in silico tools: Polyphen Phenotyping version 2 (PolyPhen-2) (42), Sorting Intolerant From Tolerant (SIFT) (43), and MutationTaster2 (44). To determine amino acid conservation, human protein sequences were aligned to other proteins using the tools SIFT (43), and MutationTaster2 (44). To determine amino acid conservation, human protein sequences were aligned to other proteins using the tools SIFT (43), and MutationTaster2 (44).

Variant validation
Candidate variants were confirmed by Sanger sequencing, as previously described (35). Forward and reverse primers specific to amplify TMTC3 (RefSeq number NM_181783.3) g.88153313 on chromosome 12 (p.Arg71His, c.212 G > A) were: 5'-AAGCCGACTTCCGAAGTGCT-3' and 5'- TT CGTGCACATTGCTTGG-3'. Primers were designed using ClustalW2.

Tissue culture
Skin fibroblasts from patients, both parents, and a healthy (TMTC3 wild type) control were cultured in Petri plates with Dulbecco’s modified Eagle’s medium (GIBCO, Carlsbad, CA, USA) as previously described (35).

Immunoblotting
Protein expression was analyzed by immunoblotting and quantified by densitometry as previously described (35). Anti-TMTC3 (1: 100, Santa Cruz Biotechnology, Dallas, Texas) was used as a primary antibody. For a positive control, a-tubulin (1: 10,000, Sigma-Aldrich, Saint Louis, Missouri) was used. Anti-mouse (1: 10,000, Jackson Immunoresearch Laboratories, Inc) was used as a secondary antibody.

Reverse transcriptase PCR
RNA was isolated from fibroblast cells using the RNeasy Mini Kit per the manufacturer’s instructions (Qiagen) as previously described (35). The data are representative of six independent biological replicates.

Drosophila stocks
Fly lines expressing transgenic RNAi encoding inverted repeats for the CG4050 gene (33248 and 110199, here referred to as UAS-tmtc3-IR1 and UAS-tmtc3-IR2, respectively) and the appropriate genetic background control lines (60,000 and 60,100, respectively) were obtained from the Vienna Drosophila Resource Center (Vienna, Austria). elav-Gal4; UAS-dcr2 was a gift from A. Schenck. UAS-dcr2 (24644 and 24650), R14H06-Gal4 (48667) UAS-mcd8:: GFP (5130) and 477-Gal4, UAS-mcd8:: GFP (8746) were obtained from the Bloomington Drosophila Stock Center (Indiana, USA). For creation of transgenic flies with inducible expression of human TMTC3 (UAS-hTMTC3-1 and UAS-hTMTC3-2), TMTC3 cDNA (Internal ID 53914 of the hORFeome V5.1 of the CCSB Human Orfeome Collection and Entrez Gene ID 160418) was inserted into the pT7W vector at the SPARC BioCentre (SickKids Hospital, Toronto) using standard Gateway cloning. Transgenic flies were created using standard P-element based transgenesis at Best Gene Inc. (California, USA).

RT-qPCR
Quantitative Real-Time PCR (RT-qPCR) was performed to evaluate the potency of UAS-tmtc3-IR1 and UAS-tmtc3-IR2. RNA lines and controls were crossed the ubiquitous Actin-Gal4 driver line and third instar larvae were selected for analysis. RNA was extracted using the RNeasy Liperid Tissue Mini Kit (Qiagen). cDNA was made using the iScript cDNA synthesis kit (BioRad). RT-qPCR was performed using the BioRad CFX 384 and the SYBRgreen master mix (BioRad) with primers directed against CG4050, and the reference genes, jCOP, eIF2β, and RpII140. CG4050 expression was compared in control and RNAi genotypes using a Student’s t-test.

Bang sensitivity assays
Bang sensitivity assays were performed using 5–9-day old male flies, raised at 29°C. The following genotypes were analyzed: UAS-dcr2/ +; UAS-tmtc3-IR1/+ (n = 436), elavGal4/ +; UAS-dcr2/ + (n = 69), elavGal4/ +; UAS-dcr2/UAS-tmtc3-IR1 (n = 75), elavGal4/ UAS-hTMTC3-1; UAS-dcr2/UAS-tmtc3-IR1 (n = 54), elavGal4/UAS- hTMTC3-2; UAS-dcr2/UAS-tmtc3-IR1 (n = 51), and elavGal4/UAS-GFP; UAS-dcr2/UAS-tmtc3-IR1 (n = 127). Male flies were collected...
in groups of ten to twelve and aged for several days. For testing, 5–9-day-old flies were transferred to empty food vials without the use of CO2. After a 5-min acclimatization period, the vials were vortexed at the highest setting for 20 s. Immediately after vortexing, the behavioral response was video recorded. After an additional 10-min rest period, flies were vortexed again in a second trial. Videos were observed for seizure-like responses, characterized by complete immobilization on the back and/or uncontrollable movement for 3 or more seconds (see Video S1 and Video S2). The time of immobilization was recorded for each individual fly as was the proportion of flies immobilized per test vial. The proportion of immobilized flies was averaged between the two trials for each tube and these values were visualized as a box plot (Fig. 4B). Statistical differences between genotypes were compared using the Wilcoxon rank test.

Immunohistochemistry, image acquisition, and analysis

Rat brains: sections were washed with 1x PBS with 0.2% Triton X-100 three times for 5 min and blocked in 10% goat serum and 10% donkey serum in 1x PBS with 0.025% Triton X-100 and 1% bovine serum albumin (BSA) for 1 h. The primary antibodies were diluted in 1x PBS with 1% BSA and 0.025% Triton X-100. The sections were incubated with primary antibodies for 24 h at 4°C; mouse anti-VGAT antibody (1: 200, Synaptic Systems, 131011) with rabbit anti-TMTC3 (1: 500, Abcam ab81473); or rabbit anti-Vgut1 (1: 2000, Synaptic Systems, 135402) with mouse anti-TMTC3 (1: 200 dilution, Santa Cruz, sc-98137). For blocking with of the sc-398137 antibody, a commercially available peptide was used (Santa Cruz, sc-98137 P). The primary antibody was incubated with the blocking peptide at 1 μg/ml for 30 min before incubation with sections for 24 h at 4°C. Sections were then washed twice with 1x PBS with 0.2% Triton X-100 for 5 min. They were incubated with secondary antibodies for 1 h at room temperature; Alexa Fluor 647 donkey anti-rabbit IgG (A31573) and Alexa Fluor 488 donkey anti-mouse IgG antibody (Molecular Probes, A-21202) diluted 1: 1000 in PBS with 1% BSA and 0.025% Triton X-100.

Confocal images were taken on an Olympus IX 60 inverted microscope outfitted with a Perkin Elmer spinning disk confocal attachment with either a 60 × (Numerical aperture = 1.4) immersion oil, or 20 × (N.A. = 0.5). The microscope was equipped with a Hamamatsu Orca ER CCD camera (1300 × 1030 pixels; pixel size 6.5 μm) and images were acquired using Volocity software. Each image shown represents a stack of 10 images spaced 0.2 μm apart in the z-plane, for each wavelength. Image stacks were deconvoluted using Autoquant (Media Cybernetics, Bethesda, MD, U.S.A.) software (using the manufacturer supplied PSF adapted for spinning disk microscopy).

Analysis of the stacks was performed using IMARIS (Bitplane, Cybernetics, Bethesda, MD, U.S.A.) software (using the manufacturer supplied PSF adapted for spinning disk microscopy). Secondary antibodies anti-rabbit-DyLight488 (1: 400, goat, ThermoFisher) and anti-mouse-DyLight594 (1: 400, goat, ThermoFisher). Dorsal type IV multidendritic neurons were imaged using a Zeiss LSM 510 DUO Vario confocal microscope. The entire neuron was captured by combining several fields of view using the tile scan function. Image stacks were imported to Neuron Studio and traced (46). Centrifugal edge labeling was performed and analyzed using the LMeasure program to quantify various features of the neurons (47). A student’s t-test was used to determine if any significant differences were present between the number of bifurcations, number of branches, number of tips, and overall length of the control and UAS-tmtc3-IR1 Type IV multidendritic neurons.

Fly neuromuscular junction (NMJ): the genotypes elavGal4/++; UAS-dcr2/-(control) and elavGal4/++; UASdcr2/UAS-tmtc3-IR1, were processed as described above, with the following changes. Primary antibodies used were: anti-HRP (1: 500, rabbit, Jackson ImmunoResearch) and anti-bi-rp (nc82, 1: 15, mouse, DSHB). Secondary antibodies used were anti-rabbit-DyLight488 (1: 400, goat, ThermoFisher) and anti-mouse-DyLight594 (1: 400, goat, ThermoFisher). To determine whether the knockdown of the tmtc gene had any effect on the morphology of the Drosophila NMJ, images of the NMJ were loaded into the open-source image analysis software, Fiji (48). Using the algorithm, ‘Drosophila_NMJ_Morphometrics’ (49) features of the NMJ were quantified for analysis. A Mann-Whitney test for non-parametric data was used to determine if any significant differences were present between the number of branches, active zones, and the overall length and area of the control and UAS-tmtc3-IR1 NMJ.

Fly mushroom bodies: The mushroom body specific R14H06-Ga4 driver line (50) was used to express tmtc3 RNAi constructs and GFP. Adult male fly brains were dissected from the genotypes UAS-dcr2/mcd8:: GFP; R14H06-Ga4/UAS-tmtc3-IR1, and UAS-dcr2/mcd8::GFP; R14H06-Ga4/+, and fixed for 1 h in 4% paraformaldehyde. GFP labeled mushroom bodies were imaged using a Zeiss LSM 510 DUO Vario confocal microscope.

Supplementary Material

Supplementary Material is available at HMG online.

Acknowledgements

We thank and acknowledge the consent and cooperation of the family members. Many thanks to the FORGE Canada Steering Committee: Kym Boycott (leader; University of Ottawa), Jan Friedman (co-lead; University of British Columbia), Jacques Michaud (co-lead; Université de Montréal), Francois Bernier (University of Calgary), Michael Bruardo (University of Toronto), Bridget Fernandez (Memorial University), Bartha Knoppers (McGill University), Mark Samuels (Université de Montréal), Steve Scherer (University of Toronto). We would like to thank Janet Marcadier (Clinical Coordinator) and Chandree Beaulieu (Project Manager) for their contribution to the infrastructure of the FORGE Canada Consortium. Many thanks to Jacek Majewski (McGill University) for providing valuable feedback during analyses. Thanks to the Bloomberg Drosophila Stock Center.

Figure 5. Image stacks were collapsed to a 2-D image where colocalized volumes (voxels) are shown in white.
Conflict of Interest statement. None declared.

Funding
This work was supported by the Canadian Rare Disease Models and Mechanisms Network, the Canadian Institute for Health Research (CIHR), and Finding of Rare Disease Genes (FORGE) Canada. FORGE Canada was funded by Genome Canada, CIHR, the Ontario Genomics Institute (OGI-049), Genome Quebec, Genome British Columbia, and the McLaughlin Centre. KCJN is supported by a Queen Elizabeth II Ontario Graduate Scholarship. SMKF is supported by the Canadian Institutes of Health Research Frederick Banting and Charles Best Canada Graduate Scholarship. Funding to pay the Open Access publication charges for this article was provided by the Canada Research Chairs program.

References
1. Liu, J.S. (2011) Molecular genetics of neuronal migration disorders. Curr. Neurol. Neurosci. Rep., 11, 171–178.
2. Liu, W., Yan, B., An, D., Xiao, J., Hu, F. and Zhou, D. (2017) Sporadic periventricular nodular heterotopia: Classification, phenotype and correlation with Filamin A mutations. Epilepsy Res., 133, 33–40.
3. Fox, J.W., Lamperti, E.D., Esqoglu, Y.Z., Hong, S.E., Feng, Y., Graham, D.A., Scheffer, I.E., Dobyns, W.B., Hirsch, B.A., Radtke, R.A. et al. (1998) Mutations in filamin 1 prevent migration of cerebral cortical neurons in human periventricular heterotopia. Neuron, 21, 1315–1325.
4. Sheen, V.L., Ganesh, V.S., Topcu, M., Sebire, G., Bodell, A., Hill, R.S., Grant, P.E., Shugart, Y.Y., Imitola, J., Khoury, S.J. et al. (2004) Mutations in ARFGEF2 implicate vesicle trafficking in neural progenitor proliferation and migration in the human cerebral cortex. Nat. Genet., 36, 69–76.
5. Conti, V., Carabalonas, A., Pallesi-Pocachard, E., Parrini, E., Leventer, R.J., Buhler, E., McGillivray, G., Michel, F.J., Striano, P., Mei, D. et al. (2013) Periventricular heterotopia in 6q terminal deletion syndrome: role of the CSorf70 gene. Brain, 136, 3378–3394.
6. Broix, L., Jagline, H., Ivanova, E., Schmucker, S., Drouot, N., Clayton-Smith, J., Pagnamenta, A.T., Metcalfe, K.A., Isidor, B., Louvier, U.W. et al. (2016) Mutations in the HECT domain of NEDD4L lead to AKT-mTOR pathway deregulation and cause periventricular nodular heterotopia. Nat. Genet., 48, 1394–1398.
7. Ferland, R.J., Batiz, L.F., Neale, J., Lian, G., Bundock, E., Lu, J., Kallay Zetchi, C., Seeck, M. et al. (2013) Periventricular heterotopia in 6q terminal deletion syndrome: role of the C6orf70 gene. PLoS Genet, 12, e1006327.
8. Ehaideb, S.N., Iyengar, A., Ueda, A., Iacobucci, G.J., Cranston, M., Bassuk, A.G., Gubb, D., Axelrod, J.D., Gunawardena, S., Wu, C.F. et al. (2014) prickle modulates microtubule polarity and axonal transport to ameliorate seizures in flies. Proc. Natl Acad. Sci. USA, 111, 11187–11192.
9. Paemka, L., Mahajan, V.B., Ehaideb, S.N., Skeie, J.M., Tan, M.C., Wu, S., Cox, A.J., Sowers, L.P., Gecz, J., Jolly, L. et al. (2015) Seizures are regulated by ubiquitin-specific peptidase 9 X-linked (USP9X), a de-ubiquitinase. PLoS Genet, 11, e1005022.
10. Schutte, R.J., Schutte, S.S., Aliga, J., Barragan, E.V., Gilligan, J., Staber, C., Savva, Y.A., Smith, M.A., Reenan, R. and O’Dowd, D.K. (2014) Knock-in model of Dravet syndrome reveals a constitutive and conditional reduction in sodium current. J. Neurophysiol., 112, 902–913.
11. DeMario, F.J. Jr. (2004) Brain abnormalities in tuberous sclerosis complex. J. Child Neurol., 19, 650–657.
12. Baulac, S., Ishida, S., Marsan, E., Miquel, C., Biraben, A., Nguyen, D.K., Nordli, D., Cossette, P., Mei, D. et al. (2015) Familial focal epilepsy with focal cortical dysplasia. Prog. Neurobiol., 128–137.
13. Paemka, L., Mahajan, V.B., Ehaideb, S.N., Skeie, J.M., Tan, M.C., Wu, S., Cox, A.J., Sowers, L.P., Gecz, J., Jolly, L. et al. (2015) Seizures are regulated by ubiquitin-specific peptidase 9 X-linked (USP9X), a de-ubiquitinase. PLoS Genet, 11, e1005022.
14. Schutte, R.J., Schutte, S.S., Aliga, J., Barragan, E.V., Gilligan, J., Staber, C., Savva, Y.A., Smith, M.A., Reenan, R. and O’Dowd, D.K. (2014) Knock-in model of Dravet syndrome reveals a constitutive and conditional reduction in sodium current. J. Neurophysiol., 112, 902–913.
15. DeMario, F.J. Jr. (2004) Brain abnormalities in tuberous sclerosis complex. J. Child Neurol., 19, 650–657.
16. Baulac, S., Ishida, S., Marsan, E., Miquel, C., Biraben, A., Nguyen, D.K., Nordli, D., Cossette, P., Mei, D., Lambrecq, V. et al. (2015) Familial focal epilepsy with focal cortical dysplasia. Prog. Neurobiol., 128–137.
17. Paemka, L., Mahajan, V.B., Ehaideb, S.N., Skeie, J.M., Tan, M.C., Wu, S., Cox, A.J., Sowers, L.P., Gecz, J., Jolly, L. et al. (2015) Seizures are regulated by ubiquitin-specific peptidase 9 X-linked (USP9X), a de-ubiquitinase. PLoS Genet, 11, e1005022.
18. Schutte, R.J., Schutte, S.S., Aliga, J., Barragan, E.V., Gilligan, J., Staber, C., Savva, Y.A., Smith, M.A., Reenan, R. and O’Dowd, D.K. (2014) Knock-in model of Dravet syndrome reveals a constitutive and conditional reduction in sodium current. J. Neurophysiol., 112, 902–913.
19. DeMario, F.J. Jr. (2004) Brain abnormalities in tuberous sclerosis complex. J. Child Neurol., 19, 650–657.
20. Baulac, S., Ishida, S., Marsan, E., Miquel, C., Biraben, A., Nguyen, D.K., Nordli, D., Cossette, P., Mei, D., Lambrecq, V. et al. (2015) Familial focal epilepsy with focal cortical dysplasia. Prog. Neurobiol., 128–137.
adapter proteins involved in calcium homeostasis. J. Biol. Chem., 289, 16085–16099.

26. Gupta, G.D., Coad, E., Goncalves, J., Mojarrad, B.A., Liu, Y., Wu, Q., Gheiratmand, L., Comartin, D., Tkach, J.M., Cheung, S.W. et al. (2015) A dynamic protein interaction landscape of the human centrosome-cilium interface. Cell, 163, 1484–1499.

27. Hein, M.Y., Hubner, N.C., Poser, I., Cox, J., Nagaraj, N., Toyoda, Y., Gak, I.A., Weisswange, I., Mansfeld, J., Buchholz, F. et al. (2015) A human interactome in three quantitative dimensions organized by stoichiometries and abundances. Cell, 163, 712–723.

28. Huttlin, E.L., Ting, L., Bruckner, R.J., Gebreab, F., Gygi, M.P., Szpyt, J., Tam, S., Zarraga, G., Colby, G., Baltier, K. et al. (2015) The BioFlex Network: a systematic exploration of the human interactome. Cell, 162, 425–440.

29. Rolland, T., Taşan, M., Charloteaux, B., Pevzner, S.J., Zhong, Q., Sahni, N., Yi, S., Lemmens, I., Fontanillo, C., Mosca, R. et al. (2014) A proteome-scale map of the human interactome network. Cell, 159, 1212–1226.

30. Chatr-Aryamontri, A., Breitkreutz, B.J., Oughtred, R., Boucher, L., Heinicke, S., Chen, D., Stark, C., Breitkreutz, A., Kolas, N., O’Donnell, L. et al. (2015) The BioGRID interaction database: 2015 update. Nucleic Acids Res., 43, D470–D478.

31. Mi, H., Lazareva-Ulitsky, B., Loo, R., Kejariwal, A., Vandergriff, J., Rabin, S., Guo, N., Muruganujan, A., Doremieux, O., Campbell, M.J. et al. (2005) The PANTHER database of protein families, subfamilies, functions and pathways. Nucleic Acids Res., 33, D284–D288.

32. Spitzer, N.C. (2006) Electrical activity in early neuronal development. Nature, 444, 707–712.

33. Komuro, H. and Rakic, P. (1996) Intracellular Ca2+ fluctuations modulate the rate of neuronal migration. Neuron, 17, 275–285.

34. Bolteus, A.J. and Bordey, A. (2004) GABA release and uptake regulate neuronal precursor migration in the postnatal subventricular zone. J. Neurosci., 24, 7623–7631.

35. Farhan, S.M., Wang, J., Robinson, J.F., Prasad, A.N., Rupar, C.A., Siu, V.M., Consortium, F.C. and Hegele, R.A. (2015) Old gene, new phenotype: mutations in heparan sulfate synthase enzyme, EXT2 leads to seizure and developmental disorder, no exostoses. J. Med. Genet., 52, 666–675.

36. Farhan, S.M. and Hegele, R.A. (2014) Exome sequencing: new insights into lipoprotein disorders. Curr. Cardiol. Rep., 16, 507.

37. DePristó, M.A., Banks, E., Poplin, R., Garimella, K.V., Maguire, J.R., Hartl, C., Philippakis, A.A., del Angel, G., Rivas, M.A., Hanna, M. et al. (2011) A framework for variation discovery and genotyping using next-generation DNA sequencing data. Nat. Genet., 43, 491–498.

38. McKenna, A., Hanna, M., Banks, E., Sivachenko, A., Cibulskis, K., Kernytsky, A., Garimella, K., Altshuler, D., Gabriel, S., Daly, M. et al. (2010) The Genome Analysis Toolkit: a MapReduce framework for analyzing next-generation DNA sequencing data. Genome Res., 20, 1297–1303.

39. Auton, A., Abecasis, G.R., Altshuler, D.M., Durbin, R.M., Abecasis, G.R., Bentley, D.R., Chakravarti, A., Clark, A.G., Donnelly, P., Eichler, E.E. et al. (2015) A global reference for human genetic variation. Nature, 526, 68–74.

40. Fu, W., O’Connor, T.D., Jun, G., Kang, H.M., Abecasis, G., Leal, S.M., Gabriel, S., Rieder, M.J., Altshuler, D., Shendure, J. et al. (2013) Analysis of 6,515 exomes reveals the recent origin of most human protein-coding variants. Nature, 493, 216–220.

41. Lek, M., Karczewski, K.J., Minikel, E.V., Samocha, K.E., Banks, E., Fennell, T., O’Donnell-Luria, A.H., Ware, J.S., Hill, A.J., Cummings, B.B. et al. (2016) Analysis of protein-coding genetic variation in 60,706 humans. Nature, 536, 285–291.

42. Adzhubei, I., Jordan, D.M. and Sunyaev, S.R. (2013) Predicting functional effect of human missense mutations using PolyPhen-2. Curr. Protoc. Hum. Genet., Chapter 7, Unit 7.20.

43. Kumar, P., Henikoff, S. and Ng, P.C. (2009) Predicting the effects of coding non-synonymous variants on protein functional using the SIFT algorithm. Nat. Protoc., 4, 1073–1081.

44. Schwarz, J.M., Cooper, D.N., Schuelke, M. and Seelow, D. (2014) MutationTaster2: mutation prediction for the deep-sequencing age. Nat. Methods, 11, 361–362.

45. Hutchinson, B., Fritschy, J.M. and Poulter, M.O. (2011) A framework for variation discovery and genotyping using next-generation DNA sequencing data. Nat. Genet., 43, 2475–2487.

46. Wearne, S.L., Rodriguez, A., Ehlenberger, D.B., Rocher, A.B., Henderson, S.C. and Hof, P.R. (2005) New techniques for imaging, digitization and analysis of three-dimensional neural morphology on multiple scales. Neuroscience, 136, 661–680.

47. Scorcioni, R., Polavarman, S. and Ascoli, G.A. (2008) L-Measure: a web-accessible tool for the analysis, comparison and search of digital reconstructions of neuronal morphologies. Nat. Protoc., 3, 866–876.

48. Schindelin, J., Arganda-Carreras, I., Frise, E., Kaynig, V., Longair, M., Pietzsch, T., Preibisch, S., Rueden, C., Saalfeld, S., Schmid, B. et al. (2012) Fiji: an open-source platform for biological-image analysis. Nat. Methods, 9, 676–682.

49. Nijhof, B., Castells-Nobau, A., Wolf, L., Scheffer-de Gooyert, J.M., Monedero, I., Torroja, L., Coromina, L., van der Laak, J.A.W.M., Schenck, A. and Graham, L.J. (2016) A new Fiji-based algorithm that systematically quantifies nine synaptic parameters provides insights into drosophila NMJ morphology. PLoS Comput. Biol., 12, e1004823.

50. Jenett, A., Rubin, G.M., Ngo, T.-T.B., Shepherd, D., Murphy, C., Dionne, H., Pfeiffer, B.D., Cavallaro, A., Hall, D., Jeter, J. et al. (2012) A GAL4-driver line resource for Drosophila neurobiology. Cell Rep., 2, 991–1001.