A recurrent de novo mutation in KCNC1 causes progressive myoclonus epilepsy


Progressive myoclonic epilepsies (PMEs) are a group of rare, inherited disorders manifesting with action myoclonus, tonic-clonic seizures and ataxia. We sequenced the exomes of 84 unrelated individuals with PME of unknown cause and molecularly solved 26 cases (31%). Remarkably, a recurrent de novo mutation, c.959G>A (p.Arg320His), in KCNC1 was identified as a new major cause for PME. Eleven unrelated exome-sequenced (13%) and two affected individuals in a secondary cohort (7%) had this mutation. KCNC1 encodes K_i3.1, a subunit of the K_i3 voltage-gated potassium ion channels, which are major determinants of high-frequency neuronal firing. Functional analysis of the Arg320His mutant channel showed a dominant-negative loss-of-function effect. Ten cases had pathogenic mutations in known PME-associated genes (NEU1, NHLRC1, AFG3L2, EPM2A, CLN6 and SERPINI1). Identification of mutations in PRNP, SAC5 and TBC1D24 expand their phenotypic spectra to PME. These findings provide insights into the molecular genetic basis of PME and show the role of de novo mutations in this disease entity.

PMEs are among the most devastating forms of epilepsy. They are clinically and genetically heterogeneous, characterized by core features of action myoclonus, tonic-clonic seizures and progressive neurological decline.1 Most molecularly characterized PMEs are inherited in an autosomal recessive manner, with rare cases showing autosomal dominant or mitochondrial inheritance.2,3 Unverricht-Lundborg disease (ULD; MIM 254800) is the most common form of PME in most patient series, and an important clinical feature is the preservation of cognition.4–3. ULD is caused by mutations in CSTM, and recently discovered mutations in genes including SCARB2 (refs. 5,6) and GOSR2 (ref. 7) also contribute to cases of PME with preserved cognition. Other PMEs might have additional features, particularly dementia. PME-associated genes encode a variety of proteins, many of which are associated with endosomal and lysosomal function,8,9 but the associated disease mechanisms are generally poorly understood.

The precise clinical diagnosis of specific forms of PME is challenging because of genetic heterogeneity, phenotypic similarities and an overlap of symptoms with other epileptic and neurodegenerative diseases. In many cases, there are no distinguishing clinical features or biomarkers. Consequently, a substantial proportion of PME cases remain without a molecular diagnosis.

Here we aimed to identify the causative genes for unsolved PME cases by employing exome sequencing in unrelated individuals assembled from multiple centers in Europe, North America, Asia and Australia over a 25-year period. The extent of previous molecular studies varied, but all cases were negative for mutations in the CSTB gene and approximately half were negative for SCARB2 and GOSR2 mutations. The cohort was thus enriched for finding mutations in new genes and potential atypical phenotypes associated with known disease-related genes.

RESULTS
We performed exome sequencing on 84 unrelated PME cases, of which 70 were sporadic and 14 were from families with pedigrees suggestive of either dominant or recessive inheritance. We therefore analyzed the data seeking pathogenic autosomal recessive or dominant/de novo, sex-linked and mitochondrial DNA (mtDNA) variants (Fig. 1 and Online Methods). On average, 4.15 Gb of sequence was produced within the exome bait regions, with an average coverage of 81 reads.
The c.959G>A mutation causes a substitution of histidine for arginine at codon 320 of the K\textsubscript{CNC1} voltage-sensing domain of the channel (\textit{Fig. 1a}). The highest number of new heterozygous variants occurred in K\textsubscript{CNC1} (11 cases and, in addition, identified pathogenic or probably pathogenic mutations in known disease-related genes in 13 cases). Analysis under the dominant/de novo model (\textit{Fig. 1a}) led to the discovery of a new PME-associated gene, K\textsubscript{CNC1}, with, remarkably, the same recurrent de novo mutation in 11 cases and, in addition, identified pathogenic mutations in known disease-related genes in 3 cases. We did not identify any obvious pathogenic mutations in mtDNA. In total, we identified pathogenic or probably pathogenic mutations in 26 of 84 cases (31.0%).

Identification of a recurrent mutation in \textit{KCNC1}

To identify new pathogenic mutations under the dominant/de novo model, we analyzed the data for potentially deleterious heterozygous variants that were absent in three variant databases. Analysis under the recessive family history (\textit{Fig. 1b}) identified pathogenic or probably pathogenic mutations (see the Online Methods for classification criteria) in known disease-causing genes in 12 cases. Analysis under the dominant/de novo model (\textit{Fig. 1a,b}) led to the discovery of a new PME-associated gene, K\textsubscript{CNC1}, with, remarkably, the same recurrent de novo mutation in 11 cases and, in addition, identified pathogenic mutations in known disease-related genes in 3 cases. We did not identify any obvious pathogenic mutations in mtDNA. In total, we identified pathogenic or probably pathogenic mutations in 26 of 84 cases (31.0%).

were unaffected, and segregation analysis for eight cases where both parental DNA samples were available showed that in each case the mutation occurred \textit{de novo}. Genotyping a set of microsatellite markers in five trios with sufficient DNA available confirmed that the pedigrees were correct. One of the index cases (PME84-1) with the c.959G>A mutation had an affected sibling and two affected children, who were also each heterozygous for the mutation (\textit{Fig. 2a}). The parents of PME84-1 and her unaffected brother were both negative for the mutation. The presence of two mutation-positive and clinically affected children from mutation-negative, unaffected parents suggests the occurrence of mosaicism in one of the parents. A restriction fragment length assay designed to detect both the normal and mutant alleles was carried out on peripheral blood DNA from both parents but showed no indication of mosaicism (data not shown).

We screened for the K\textsubscript{CNC1} c.959G>A mutation in a secondary cohort of 28 PME cases and identified 2 (7.1%) additional unrelated cases heterozygous for the mutation. Sanger sequencing of the available parental samples of one of these individuals confirmed the \textit{de novo} occurrence of the mutation (\textit{Fig. 2a}). In total, we identified 16 cases (13 unrelated) with the c.959G>A mutation. We did not identify any other mutations in K\textsubscript{CNC1} in the exome data. As exon 1 was not sufficiently covered, we carried out Sanger sequencing of it in the 73 exome-sequenced c.959G>A-negative cases, but no potentially deleterious variants were found.

The c.959G>A mutation in \textit{KCNC1} affects a highly evolutionarily conserved arginine residue in segment S4, constituting the main voltage-sensing domain of the channel (\textit{Fig. 2b,c}). The mutation was predicted to be deleterious by all four \textit{in silico} methods used (\textit{Supplementary Table 3}).
To assess the frequency of PME due to KCNC1 c.959G>A mutation, we used a newly published mutational model\(^\text{10}\), which takes into account both the local sequence context of a mutation site and regional factors such as divergence between humans and macaques, to estimate the rate of this specific mutation. A rate of $1.75 \times 10^{-7}$ mutations per person was obtained, indicating that the mutation should occur in 1 out of every 5,700,000 conceptions. Additionally, we examined three other potential mutations encoding changes of the conserved voltage-sensing arginine residues to histidine (red plus sign and arrow). (c) The ClustalX comparison of amino acid sequences for the voltage-sensing S4 segment shows full conservation of Arg320 (arrow) across different species. The four positively charged arginine residues (Arg311, Arg314, Arg317, Arg320) occurring every third position are highlighted in blue. The $K_{\text{CNC3}}$ human KCNC1 KCNC3, fruitfly Shaw, zebrafish KCNCl, chicken KCNCl, mouse KCNCl, rat KCNCl, human KCNC1 (KCNCl), mouse KCNC3 (KCNCl), rat KCNCl, human KCNC1, chicken KCNCl, mouse KCNC3, fruitfly Shaw, zebrafish KCNCl, are in red boxes. Asterisks, colons and periods indicate fully conserved, strongly similar and weakly similar residues, respectively.

**Figure 3** Functional analysis of the p.Arg320His substitution in $K_{\text{CNC3}}$. (a) Representative traces of whole-cell currents recorded in $X$. laevis oocytes, injected with the same amount of cRNA encoding wild-type (WT) or Arg320His $K_{\text{CNC3}}$, during 0.5-s voltage steps (−60 mV to +60 mV). (b) Relative current amplitudes of the wild-type channel ($n = 49$) and Arg320His mutant ($n = 50$) at the end of the voltage step to +60 mV and normalized to the mean current amplitude of the wild-type channel recorded on the same day (Mann-Whitney U test, ***P < 0.001). (c) Protein blot analysis of $X$. laevis oocytes injected with cRNA for either the wild-type channel or the Arg320His mutant using a mouse antibody to the DDK tag. Water-injected oocytes were used as a negative control, and actin served as a loading control. The presence of two bands on the blot is likely to be due to N-glycosylation of the $K_{\text{CNC3}}$ protein (expected protein size of ~56 kDa), which occurs in vivo and in heterologous expression systems\(^\text{50,51}\). (d) Representative whole-cell currents from oocytes injected with a constant amount of cRNA for wild-type channel and either water or cRNA encoding Arg320His mutant in a 1:1 ratio, recorded as in a. (e) Relative current amplitudes remaining upon addition of Arg320His mutant to the wild-type channel ($n = 27$) were determined at the end of a 0.5-s pulse to +60 mV and normalized to the mean current amplitude for the wild-type channel coinjected with water ($n = 32$) recorded on the same day (Student's t test, ***P < 0.001). (f) The current-voltage relationships of cells expressing wild-type channel alone or the wild-type and Arg320His channels (Student's t test, $P < 0.001$) for −60 mV to +50 mV. Lines represent fits of a Boltzmann function. The potential of half-maximal activation ($V_0$) was 19.6 ± 0.5 mV ($n = 19$) and 8.0 ± 1.3 mV ($n = 16$) (Student's t test, $P < 0.001$) and the slope factor $k$ was 12.9 ± 0.2 and 10.2 ± 0.3 (Student's t test, $P < 0.001$) for wild-type channel alone and the wild-type and Arg320His channels together, respectively. The data in b, e and f are presented as means ± s.e.m.
Table 1 Clinical features of cases with the c.959G>A mutation (p.Arg320His) in KCNC1

<table>
<thead>
<tr>
<th>Case ID</th>
<th>Ancestry</th>
<th>Sex</th>
<th>Onset age (years)</th>
<th>Initial symptom</th>
<th>Seizures</th>
<th>Learning disability</th>
<th>Cognitive decline</th>
<th>Age (years); outcome</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Myoclonus</td>
<td>Tonic-clonic</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Exome-sequenced cases</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PME8-1</td>
<td>Italian</td>
<td>M</td>
<td>12</td>
<td>Myoclonus</td>
<td>+++</td>
<td>+</td>
<td>No</td>
<td>Yes</td>
</tr>
<tr>
<td>PME10-1</td>
<td>Italian</td>
<td>M</td>
<td>6</td>
<td>Myoclonus</td>
<td>+++</td>
<td>+</td>
<td>No</td>
<td>Possible</td>
</tr>
<tr>
<td>PME17-1</td>
<td>French</td>
<td>M</td>
<td>&lt;5</td>
<td>Ataxia</td>
<td>+++</td>
<td>+</td>
<td>No</td>
<td>No</td>
</tr>
<tr>
<td>PME18-1</td>
<td>Norwegian</td>
<td>F</td>
<td>10</td>
<td>Myoclonus</td>
<td>+++</td>
<td>+</td>
<td>No</td>
<td>Yes</td>
</tr>
<tr>
<td>PME29-1</td>
<td>US (Italian)</td>
<td>M</td>
<td>9</td>
<td>Tremor/myoclonus</td>
<td>++</td>
<td>+</td>
<td>Yes</td>
<td>No</td>
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<tr>
<td>PME36-1</td>
<td>Italian</td>
<td>F</td>
<td>7</td>
<td>Myoclonus</td>
<td>+++</td>
<td>+</td>
<td>No</td>
<td>No</td>
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<tr>
<td>PME44-1</td>
<td>French Canadian</td>
<td>F</td>
<td>12</td>
<td>Myoclonus</td>
<td>++</td>
<td>+</td>
<td>No</td>
<td>Yes</td>
</tr>
<tr>
<td>PME63-1</td>
<td>Moldavian</td>
<td>F</td>
<td>9</td>
<td>Myoclonus</td>
<td>+++</td>
<td>+</td>
<td>No</td>
<td>No</td>
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<tr>
<td>PME65-1</td>
<td>Portuguese</td>
<td>F</td>
<td>9</td>
<td>Myoclonus</td>
<td>+++</td>
<td>+</td>
<td>No</td>
<td>Yes</td>
</tr>
<tr>
<td>PME84-1</td>
<td>Israeli (Sephardic)</td>
<td>F</td>
<td>10</td>
<td>Tremor</td>
<td>+</td>
<td>+</td>
<td>Yes</td>
<td>No</td>
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<tr>
<td>Sanger-sequenced cases</td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PME84-sister</td>
<td>Israeli (Sephardic)</td>
<td>F</td>
<td>13</td>
<td>Myoclonus</td>
<td>++</td>
<td>+</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>PME84-son</td>
<td>Israeli (Sephardic)</td>
<td>M</td>
<td>12</td>
<td>Ataxia</td>
<td>+</td>
<td>+</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>PME84-daughter</td>
<td>Israeli (Sephardic)</td>
<td>F</td>
<td>14</td>
<td>Tonic-clonic seizure</td>
<td>-</td>
<td>+</td>
<td>Yes</td>
<td>No</td>
</tr>
<tr>
<td>Secondary 1</td>
<td>Danish</td>
<td>M</td>
<td>10</td>
<td>Myoclonus</td>
<td>++</td>
<td>+</td>
<td>No</td>
<td>Yes</td>
</tr>
<tr>
<td>Secondary 2</td>
<td>Israeli</td>
<td>M</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
</tr>
</tbody>
</table>

+, mild; ++, moderate; ++++, severe; −, not observed. NA, data not available.

*Earlier absence seizures. *Ascertainment in Italy. **Severe myoclonus and language barrier prevented good assessment. ***Simple febrile seizures at 6–12 months.

The clinical phenotype of cases with the KCNC1 mutation

We obtained detailed clinical data for 15 of the 16 individuals positive for the KCNC1 mutation (Table 1). The clinical phenotypes were similar and, at disease onset, resembled classical ULD. On a background of usually normal development, the first symptom in the majority of individuals was myoclonus (sometimes reported as tremor) at ages of 6–14 years. Ataxia developed early in one case (PME17-1), but it was otherwise overshadowed by myoclonus as the major motor impediment. There were infrequent tonic-clonic seizures in all cases. During adolescence, myoclonus generally became very severe, limiting ambulation; a walking aid or wheelchair was needed by mid to late teens. Learning disability before seizure onset was noted in some cases, in particular, the nuclear family of PME84-1. There was mild cognitive decline in seven subjects in early adolescence, but this was difficult to quantify owing to the severe motor disability. Early death was not observed. Electroencephalogram recordings showed generalized epileptiform discharges, with photosensitivity in some cases. Magnetic resonance imaging (MRI) scans had no specific features and were regarded as normal or showed cerebellar atrophy. The clinical picture in the family with four affected members (PME84) was milder, with the two older sisters ambulant in the fourth decade of life. Similarly, case 1 from the secondary cohort had a less severe clinical course than the majority of cases.

Mutations in known disease genes

Analysis for mutations in genes known to be associated with PME, epilepsy and neurodegenerative disease identified either pathogenic or probably pathogenic mutations in 15 of the 84 exome-sequenced cases (Table 2; see Supplementary Table 4 for clinical details as well as summaries of the genetic findings, Supplementary Fig. 3 for pedigrees with segregation data and Supplementary Figs. 4 and 5 for conservation of the newly identified mutation sites). These genes fell into three groups.

First, ten cases had a recessively inherited or de novo heterozygous mutation in established PME-related genes, seven with atypical clinical presentations. The ten comprised three cases of Lafora disease (NHLRC1 (ENST00000340650.3) and EPM2A (ENST00000367519.3)), three cases of sialidosis (NEU1; ENST00000375631.4) and one case of neuronal ceroid lipofuscinosis (CLN6; ENST00000249806.5). Two Italian cases, not known to be related, had the same new homozygous missense AFG3L2 mutation (c.1875G>A; p.Met625Ile; ENST000000269143.3) affecting the proteolytic domain of the protein (Supplementary Fig. 4e). Exome data showed that they shared a ~1.75-Mb run of identical homozygous polymorphisms flanking the mutation, indicating that the mutation was identical by descent. The tenth case had a de novo previously described11 pathogenic heterozygous missense mutation (c.1175G>A; p.Gly392Glu; ENST000000295777.5) in the SERPINI1 gene encoding neuroserpin. Mutations in both AFG3L2 and SERPINI1 are very rare known causes of PME.

Second, four cases had mutations in known genes where PME has not been reported as a key part of clinical presentation. One case with adult-onset PME and a father who had died of a similar disease was found to have a previously described12 pathogenic heterozygous missense mutation (c.305C>T; p.Pro102Leu; ENST00000379440.4) in the PRNP gene encoding prion protein. The mutation is a
known cause of Gerstmann-Sträussler-Scheinker disease, one of the inherited prion diseases. Two unrelated cases had probably pathogenic, rare compound heterozygous missense mutations in the SACS gene (c.8393C>A, p.Pro2798Gln in both cases; c.1373C>T, p.Thr458Ile and c.2996T>C, p.Ile999Thr in one case each; ENST00000382298.3). Two of the substitutions (p.Thr458Ile and p.Pro2798Gln) have been reported in individuals with spastic ataxia13–15. One case had a new probably pathogenic homozygous missense mutation (c.1079G>T; p.Arg360Leu; ENST00000293970.5) in TBC1D24, where recessive mutations cause variable neurological disorders, including familial infantile myoclonic epilepsy16–20.

Table 2 Pathogenic or probably pathogenic mutations in known PME, epilepsy or neurodegenerative disease genes

<table>
<thead>
<tr>
<th>Case ID, sex</th>
<th>Gene</th>
<th>Zygosity</th>
<th>Coding DNA change</th>
<th>Protein change</th>
<th>CADD, SIFT, PolyPhen, MutationTaster predictions</th>
<th>Allele frequency in 1000G/EVS (%)</th>
<th>Previously reported pathogenic mutation (reference)</th>
<th>Disease associated with the gene (OMIM gene number)</th>
</tr>
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<tr>
<td><strong>Pathogenic recessive mutations</strong></td>
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<td></td>
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<tr>
<td>PME23-1, F</td>
<td>NEU1</td>
<td>Het</td>
<td>c.1208delG</td>
<td>p.Ser403Thrfs*85</td>
<td>32, NA, NA</td>
<td>0/0</td>
<td>Yes (ref. 52)</td>
<td>PME; sialidosis (608272)</td>
</tr>
<tr>
<td>PME51-1, F</td>
<td>NEU1</td>
<td>Het</td>
<td>c.982G&gt;A</td>
<td>p.Gly328Ser</td>
<td>32, D, D</td>
<td>0/0</td>
<td>Yes (ref. 53)</td>
<td></td>
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<tr>
<td>PME87-1, F</td>
<td>NEU1</td>
<td>Het</td>
<td>c.914G&gt;A</td>
<td>p.Arg305His</td>
<td>9.4, B, PD, D</td>
<td>0/0</td>
<td>No, but p.Arg305Cys is reported (ref. 54)</td>
<td></td>
</tr>
<tr>
<td>PME35-1, F</td>
<td>NHLRC1</td>
<td>Hom</td>
<td>c.436G&gt;A</td>
<td>p.Asp146Asn</td>
<td>15.75, B, PD, D</td>
<td>0/0</td>
<td>Yes (ref. 55)</td>
<td>PME; Lafora disease (608072)</td>
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<tr>
<td>PME81-1, M</td>
<td>NHLRC1</td>
<td>Hom</td>
<td>c.830C&gt;A</td>
<td>p.Ala277Glu</td>
<td>12.72, D, D, D</td>
<td>0/0</td>
<td>No</td>
<td>PME; Lafora disease (607566)</td>
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<tr>
<td>PME82-1, M</td>
<td>EPM2A</td>
<td>Hom</td>
<td>c.590A&gt;T</td>
<td>p.Asp197Val</td>
<td>27.4, D, D, D</td>
<td>0/0</td>
<td>No</td>
<td>PME; neuronal ceroid lipofuscinosis (606725)</td>
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<tr>
<td>PME33-1, M</td>
<td>CLN6</td>
<td>Hom</td>
<td>c.509A&gt;G</td>
<td>p.Tyr170Cys</td>
<td>17.11, D, D, D</td>
<td>0/0</td>
<td>No</td>
<td>Spino-cerebellar ataxia 28 and spastic ataxia with PME (604581)</td>
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<tr>
<td>PME62-1, M</td>
<td>AFG3L2</td>
<td>Hom</td>
<td>c.1875G&gt;A</td>
<td>p.Met625Ile</td>
<td>30, B, PD, D</td>
<td>0/0</td>
<td>No</td>
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<td>PME32-1, F</td>
<td>AFG3L2</td>
<td>Hom</td>
<td>c.1875G&gt;A</td>
<td>p.Met625Ile</td>
<td>30, B, PD, D</td>
<td>0/0</td>
<td>No</td>
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<td><strong>Probably pathogenic recessive mutations</strong></td>
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<tr>
<td>PME14-1, F</td>
<td>TBC1D24</td>
<td>Hom</td>
<td>c.1079G&gt;T</td>
<td>p.Arg360Leu</td>
<td>19.83, D, D, D</td>
<td>0/0</td>
<td>No</td>
<td>Multiple neurological diseases (613577); PME not previously described</td>
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<td>PME15-1, F</td>
<td>SACS</td>
<td>Het</td>
<td>c.8393C&gt;A</td>
<td>p.Pro2798Gln</td>
<td>25.7, D, D, D</td>
<td>0.09/0.29</td>
<td>Possible (ref. 13)d</td>
<td>Autosomal recessive spastic ataxia of the Charlevoix-Saguenay (604490); PME not previously described</td>
</tr>
<tr>
<td>PME75-1, F</td>
<td>SACS</td>
<td>Het</td>
<td>c.2996T&gt;C</td>
<td>p.Ile999Thr</td>
<td>17.35, D, B, D</td>
<td>0/0/0.09</td>
<td>No</td>
<td>Possible (ref. 13)d and yes (ref. 14)</td>
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<tr>
<td>PME42-1, F</td>
<td>SERPINI1</td>
<td>Het</td>
<td>c.1175G&gt;A</td>
<td>p.Gly392Glu</td>
<td>22.5, D, D, D</td>
<td>0/0</td>
<td>Yes (ref. 11)</td>
<td>PME; familial encephalopathy with neuroserpin inclusion bodies (604218)</td>
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<td>PME86-1, F</td>
<td>PRNP</td>
<td>Het</td>
<td>c.305C&gt;T</td>
<td>p.Pro102Leu</td>
<td>19.12, D, D, D</td>
<td>0/0</td>
<td>Yes (ref. 12)</td>
<td>Gerstmann-Sträussler-Scheinker disease (176640); PME not previously described</td>
</tr>
<tr>
<td>PME20-1, F</td>
<td>SCN1A</td>
<td>Het</td>
<td>c.677C&gt;T</td>
<td>p.Thr226Met</td>
<td>20.5, D, D, D</td>
<td>0/0</td>
<td>Yes (ref. 21)</td>
<td>Dravet syndrome (182389)</td>
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</table>

*Genomic positions and more detailed annotations of the mutations are presented in Supplementary Table 3. Mutation coding DNA and amino acid positions are based on Ensembl transcripts (see main text). *A CADD score of >15 indicates deleteriousness for the variant (Online Methods). B, benign (not considered deleterious by the method); PD, possibly deleterious (applies to PolyPhen only); D, deleterious; NA, not available; 1000G, 1000 Genomes Project; EVS, Exome Variant Server of the National Heart, Lung, and Blood Institute (NHGRI) Exome Sequencing Project. *See the Supplementary Note for a discussion of the pathogenicity of the previously reported SACS mutations.
Search for additional genes with new mutations
We analyzed the exome data set for potential new PME-associated genes aside from KCNC1, using both recessive and dominant/de novo models. We did not identify other likely new genes using our criteria of observing putative mutations in a gene in at least two unsolved cases under the recessive model or in at least four unsolved cases using the dominant/de novo model (Supplementary Tables 2 and 7, and Supplementary Note). However, genes of interest in single cases that would warrant further exploration include ALG10 and APOA1BP, which harbored homozygous loss-of-function mutations (Supplementary Table 8).

DISCUSSION
Using an exome sequencing approach in a clinically heterogeneous cohort of 84 unrelated individuals with PME without a specific cause, we reached a genetic diagnosis in 31% of the cases. Notably, we identified a recurrent mutation in KCNC1 as a new cause of PME that explained a substantial proportion of cases. KCNC1 encodes Kv3.1, which functions as a highly conserved potassium ion channel subunit of the Kv3 subfamily of voltage-gated tetrameric potassium ion channels. Although KCNC1 mutations have not been associated with human disease until now, mutations inherited in an autosomal dominant manner or occurring de novo in KCNC3 (Kv3.3) cause spinocerebellar ataxia14–27.

Kv3 channel subunits consist of six membrane-spanning segments (S1–S6), have overlapping expression patterns and can form heterotetramers28. S4 constitutes the main voltage sensor where specific positively charged arginine residues contribute to the gating charge29,30. Electrophysiological analysis showed that the PME-causing p.Arg320His substitution, which affects one of the voltage-sensing residues, causes prominent loss of function with a dominant-negative effect on wild-type Kv3.1 channels. We also observed altered gating properties for the mutant protein, but the physiological consequence of this finding is questionable, as the overall contribution of these altered properties to Kv3.1-mediated current is minor. Similar biophysical properties have been reported for an ataxia-causing alteration in Kv3.3, p.Arg423His31, occurring in the position analogous to p.Arg320His in Kv3.1. Mutations affecting positively charged residues in segment S4 of voltage-gated cation channels might contribute to pathogenicity by generating leak currents through the gating pore32. For example, in the Drosophila Shaker Kv1 channel, the change analogous to p.Arg320His in Kv3.1 increases proton permeability32. However, gating pore currents are not detectable for the analogous Kv3.3 mutant33, calling into question the importance of the phenomenon in the context of Kv3.1. Given the ability of Kv3.3 subunits to form heterotetramers, the dominant-negative Arg320His Kv3.1 mutant is likely to disrupt all Kv3-mediated currents of the neurons in which it is expressed. Reflecting functional redundancy, Kcncl and Kcncc knockouts in mice have relatively mild phenotypes, whereas double-mutant mice show myoclonus, tremor and gait ataxia34–36. Thus, the dominant-negative Kv3.1 and Kv3.3 mutations identified in cases seem to have an effect comparable to double knockouts in mice.

The Kv3 subfamily is distinguished from other Kv channels by more positively shifted voltage-dependent activation and faster activation and deactivation rates. These differences make Kv3 channels major determinants of high-frequency firing in several types of central nervous system (CNS) neurons28. Studies using mutant or pharmacologically suppressed Kv3 channels have demonstrated that loss of Kv3 function disrupts the firing properties of fast-spiking neurons28,37,38, affects neurotransmitter release49 and induces cell death40. The expression of Kv3.1 is limited to the CNS, with the exception of a subpopulation of T lymphocytes41,42. It is preferentially expressed in specific subsets of fast-spiking neurons, with prominent expression in inhibitory GABAergic interneurons43,44. Therefore, it is likely that the p.Arg320His substitution in Kv3.1 results in disinhibition due to the impaired firing of fast-spiking GABAergic interneurons. This mechanism is likely to contribute particularly to myoclonus and tonic-clonic seizures. Furthermore, dysfunction and/or degeneration of cerebellar neurons, where Kv3.1 is expressed45, are likely to contribute to motor impairment. Modulation of Kv3 channel function may provide a possibility for pharmacological intervention in patients with KCNC1 mutations. However, although drugs with anticonvulsant effects activating other Kv channels exist43, there is no activator of Kv3 channels currently available.

The initial clinical presentation and evolution of ULD14 and the disorder in individuals with the KCNC1 mutation, designated here as ‘MEAK’ (myoclonus epilepsy and ataxia due to potassium channel mutation), are similar. They have overlapping ages of onset and moderate-to-severe incapacitating myoclonus, infrequent tonic-clonic seizures and mild, if any, cognitive decline. Differences emerge later in disease progression, as the clinical course for MEAK is generally more severe. ULD is caused by mutations in CSTB, encoding cystatin B, which is implicated in oxidative stress and inflammation45,46. Evidence from the mouse model for ULD suggests that altered GABAergic signaling contributes to latent hyperexcitability47,48, implying a possible convergent pathway for ULD and MEAK.

The recurrence of the KCNC1 mutation is likely due to its location in a CpG dinucleotide, corresponding to a class of sites that represent mutation hotspots49. We estimated that the mutation occurs in 1 out of 5,700,000 conceptions, thus potentially affecting hundreds of individuals globally. The observation of four cases positive for the KCNC1 mutation ascertained in a multicenter clinical collaboration in Italy5 supports this estimate, assuming that probably not all existing cases were ascertained and that the mutation might reduce lifespan. We did not observe any other mutations in KCNC1, which is among the top 1% of the most constrained genes46. For example, the estimated mutation rates for the three other potential arginine-to-histidine substitutions affecting voltage-sensing residues are equal to that for p.Arg320His, and these mutations thus should have been observed in our cohort if the phenotypic consequences were the same.

The fact that we only identified the mutation affecting Arg320 suggests that this residue is biophysically special. Indeed, codon-specific consequences of S4-altering mutations have been demonstrated, for example, in Kv3.3 (ref. 31).

The identification of mutations in previously established disease-related genes expands the phenotypic and genotypic spectra of PME. Highlighting the usefulness of exome sequencing as a diagnostic tool in a heterogeneous cohort of affected individuals previously subjected to molecular analyses, we identified pathogenic mutations in known PME-associated genes in ten individuals, of whom the majority had atypical symptoms (Supplementary Table 4 and Supplementary Note). Notably, we identified mutations in three known disease-related genes (PRPN, SACS and TBC1D24), where PME has not been appreciated as part of the clinical spectrum. These cases are discussed in the Supplementary Note. The majority of all solved cases also had variants in other known disease-associated genes (Supplementary Tables 5 and 6). These variants, however, did not fulfill our criteria for pathogenicity. It is possible that they modify the clinical outcome, thus contributing to some atypical disease presentations.

The genetic basis of disease remained unknown in over two-thirds of the cases. Aside from KCNC1, there were no other new PME-related
genes definitively identified (Supplementary Tables 2 and 7, and Supplementary Note); however, of the nine genes with homozygous loss-of-function mutations in single cases, some are interesting candidates (Supplementary Table 8). In light of de novo mutations being established as an important cause of PME, exome sequencing in a family trio setting could be pursued to further dissect this heterogeneous cohort. Also, the role of copy number variants (CNVs) and epistatic mutations in PMEs should be assessed. Our findings, especially the discovery of MEAK, will aid in molecular diagnostics and potential therapeutic interventions in PME, and the exome data generated will facilitate the further identification of disease-relevant genes.


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

Accession codes. Raw aligned sequence reads have been submitted to the European Genome-phenome Archive (EGA) by the Wellcome Trust Sanger Institute under study accessions EGAS00001000048 and EGAS00001000038.

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AUTHOR CONTRIBUTIONS

COMPETING FINANCIAL INTERESTS
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Mutations in Next generation sequencing for molecular diagnosis of...
ONLINE METHODS

Study subjects. The study cohort consisted of 84 unrelated individuals with a clinical presentation of PME, collected from multiple centers in Europe, North America, Asia and Australia over a 25-year period for molecular study, where a specific diagnosis had not hitherto been made. Seventy-three cases were of European origin, seven were of West Asian origin, three were of South Asian origin and one was of Chinese origin. The extent of previous molecular investigations varied; mutations in the CSTR gene had been excluded in all, 43 cases tested negative for mutations in SCARB2 and 39 cases tested negative for mutations in GOSR2. A variable set of other genes was also screened for some cases. The study was approved by the institutional review board at the Helsinki University Central Hospital (Finland) and the human research ethics committees of Austin Health (Melbourne, Australia) and the University of South Australia (Adelaide, Australia). Informed consent for DNA analysis was obtained from study participants in line with local institutional review board requirements at the time of collection. Family members, when available, were recruited for segregation analysis, which was carried out using Sanger sequencing.

Seventy cases were sporadic. Three probands had either an affected parent or affected offspring. Ten probands had at least one affected sibling, and one proband had an affected cousin with known parental consanguinity. Fifteen of the cases were reported to be the result of a consanguineous union. On the basis of the inbreeding coefficients obtained with FEstim56, the number of cases resulting from consanguinity was 18. No cryptic relatedness between cases (PIHAT > 0.05) was detected by PLINK57 identity-by-descent (IBD) analysis.

Sanger sequencing was performed on a secondary cohort of 28 cases with PME or possible PME for the KCNC1 recurrent mutation. These cases were excluded from our original cohort for exome sequencing owing to insufficient DNA or inadequate clinical data. Informed consent from study participants in the secondary cohort was obtained as described above for the exome sequencing cohort.

Exome sequencing. Exome sequencing was carried out at the Wellcome Trust Sanger Institute (Hinxton, UK). Genomic DNA (approximately 1 µg) extracted from peripheral blood for each sample was fragmented to an average size of 150 bp and subjected to DNA library creation using established Illumina paired-end protocols. Adapter-ligated libraries were amplified and indexed via PCR. A portion of each library was used to create an equimolar pool comprising 4–8 indexed libraries. Each pool was hybridized to SureSelect Human All Exon 45Mb V3 RNA baits (Agilent Technologies), and sequence targets were captured and amplified in accordance with the manufacturer’s recommendations. Enriched libraries were subjected to 75-base paired-end sequencing (HiSeq 2000, Illumina) following the manufacturer’s instructions. Each pool of indexed samples was sequenced twice on two different flow cells.

Sequence read alignment and processing. Alignment of the sequenced DNA fragments to the human reference genome was performed using the Burrows-Wheeler Alignment Tool (BWA)58. The reference sequence used was the 100 Genomes Project Phase II reference (hg19), which is based on GRCh37 and the 1000 Genomes Project Phase I data as a reference. Third, we used genotype information from the 3,268 Finnish exomes to identify heterozygous indels present in the same sequencing reads that were manually removed. Second, to detect rare variants segregating together in populations (likely to be inherited from only one parent), Beagle70 was applied to phase genotypes using 100 Genomes Project Phase I data as a reference. Third, we used genotype information from the 3,268 Finnish exomes to detect heterozygous variants likely to be on the same allele. After filtering the variant data using a recessive hypothesis, before taking advantage of the phased information, we visualized the sequencing reads surrounding the variants that passed filtering using the Integrative Genomics Viewer (IGV) to detect likely false positive calls residing mainly within segmental duplication regions where mapping of reads is more challenging. As a technical comparison set for variant visualization, we used 43 in-house exomes that had been processed in the same sequencing, read processing and variant calling pipelines as the exomes in this study. After excluding low-quality variants, we implemented the phasing information in the cleaned data and repeated the recessive filtering.

In the dominant/de novo filtering strategy, we assumed full penetrance of the mutations and, because the great majority of cases had disease onset before adulthood (10/84 cases had onset at 18 years or older, maximum onset age of 26 years), we included heterozygous variants absent from the three variant databases. Additionally, dbsNP Build 138 variants were excluded except those with clinical association in NCBI ClinVar. After applying the filtering criteria, we excluded low-quality variants on the basis of IGV visualization of sequencing reads as described for the filtering of recessive variants.
Analysis of filtered variant data under recessive and dominant/de novo strategies. Exome data were analyzed both for variants in genes previously associated with PME, epilepsy and/or neurodegenerative disorders and variants in new genes. The association of genes with mendelian diseases was annotated on the basis of the OMIM database. Additionally, genes involved in monogenic forms of epilepsy and neurodegenerative disease were retrieved on the basis of the diagnostic panels developed by Lemke and colleagues. Genes involved in PME were also retrieved from the literature.

To identify new recessive or dominant/de novo genetic causes, genes with variants surviving the filtering strategy were ranked on the basis of the number of PME cases with variants in the gene. Genes that are highly polymorphic in human populations and were thus likely to rank high in the data analysis only as a result of their high benign mutation load were given less priority if at least one of the following criteria was fulfilled: (i) the gene had a residual variation intolerance score (RVIS) (estimating the tolerance of genes for functional variation) in the most tolerant tenth percentile (this criterion was used only in the dominant/de novo model) and (ii) the gene was identified as hyperpolymorphic by Fuentes Fajardo and colleagues. Other measures used in the prioritization of candidate genes and variants were known disease association and the function (UniProt database) and the literature (expression pattern (GTEx database) and the literature) of the gene.

Analysis of mitochondrial DNA variants. Because PME might also be caused by mutations in the mitochondrial genome, we analyzed the mtDNA for possible disease-associated mutations. Even though the data for mtDNA are not included in the SureSelect exome capture kit, we obtained an average of 32.7x sequencing coverage per sample in the mitochondrial genome, owing to the abundance of mtDNA in cells. We called mtDNA variants using the GATK UnifiedGenotyper. At sites for the most commonly reported mutations associated with diseases showing myoclonus as part of the clinical presentation, namely myoclonic epilepsy with ragged red fibers (MERRF) and mitochondrial encephalomyopathy, lactic acidosis and stroke-like episodes (MELAS), the average coverage was at least 24.8x. We searched for new mitochondrial mutations by excluding known mtDNA polymorphisms in the MITOMAP database. The same database was also used to obtain positions for known disease-associated mtDNA mutations.

Classification criteria for mutations in known as well as new disease-associated genes. We used a three-tier scale to classify mutations passing variant filtering in genes known to be associated with PME, epilepsy or neurodegenerative disease: (i) pathogenic, (ii) probably pathogenic and (iii) unlikely to be pathogenic. Classification criteria were as follows.

Pathogenic. The mutation was in a known PME-associated gene, i.e., where mutations cause a disease with PME as a key feature of the presentation, or in a gene known to be associated with epilepsy or neurodegenerative disease, had previously been reported as pathogenic and corresponded to a phenotype in the case that was compatible to those of previous cases. In addition, the mutation was required to map to a conserved domain where other pathogenic mutations had previously been reported. Segregation data, when available, had to comply with the expected inheritance pattern in the family, and the mode of inheritance had to concur with that for previously reported cases. If no segregation data were available, the corresponding phenotype had to present substantial overlap with those of previously reported cases, and, in the case of previously reported pathogenic compound heterozygous mutations, the mutations should not have occurred in cis in the original report.

Probably pathogenic. Either the mutation occurred in only one case in a previously established disease-related gene with phenotypic overlap with PME or occurred in a previously established neurological disease gene with little phenotypic overlap with PME and two or more cases had mutations in the gene. In addition, the mutation was required to map to a conserved domain where other pathogenic mutations had been reported previously. Segregation data for the mutation had to comply with the expected inheritance pattern in the family, and the mode of inheritance had to concur with that for previously reported cases.

Unlikely to be pathogenic. This category included mutations that did not fulfill the above criteria.

To consider new disease-associated genes as candidates for PME, we required them to harbor mutations in at least two cases in the recessive variant filtering strategy and in at least four cases in the dominant/de novo variant filtering strategy. The higher threshold in the dominant/de novo strategy was set to limit the number of candidates to a feasible level for follow-up, as exome variant data for the parents were not available for filtering inherited variants in data analysis.

Variant validation and segregation analysis. Candidate mutations in known and new disease-associated genes were confirmed and segregation of the variants was analyzed, when DNA from parents or siblings was available, by bidirectional Sanger sequencing (ABI BigDye 3.1. Applied Biosystems) on an ABI 3730xl DNA Analyzer. Primers (sequences available from the authors upon request) were designed with Primer-BLAST. Sequences were analyzed using Sequencer (Gene Codes Corporation) and visualized using 4Peaks (Nucleoebies). Evaluation of the quality of the sequence reads over the recurrent c.959G>A mutation in KCN1 is described in the Supplemental Note.

Parental testing of the cases with the c.959G>A mutation in KCN1. Parental testing was carried out for five cases (those with a sufficient amount of parental DNA available) with the de novo c.959G>A mutation in KCN1 to exclude false paternity and inadvertent sample substitution. Biparental testing was performed using 12 highly polymorphic microsatellite markers: D3S3680, D4S418, D6S289, D7S2560, D8S281, D13S175, D13S221, D15S117, D19S1150, DXS1113, DXS1036 and DXS7423. PCR was performed using the Qiagen Multiplex PCR kit according to the manufacturer’s instructions. The reverse primer of each pair was labeled with either HEX or FAM. Products were analyzed on an ABI 3131 Genetic Analyzer.

Analysis of parental mosaicism for the c.959G>A mutation in KCN1 in the family of PME84-1. The PCR product corresponding to exon 2 of KCN1 was amplified using the same primers and conditions as for sequencing, and 10 µl of the PCR reaction was digested with 2 U HpyCH4V (New England BioLabs) for 2 h at 37 °C under the conditions recommended by the manufacturer. Fragments were visualized by electrophoresis of 10 µl of the digest on a 2% agarose gel in TBE buffer. Gels were stained with RedSafe (INtRON Biotechnology). PCR and digestion were performed in duplicate.

Evaluation of the expected rate of mutations encoding arginine-to-histidine changes in the S4 segment of K_v3.1. The expected rates for the four possible mutations encoding arginine-to-histidine changes (CGC>CAC) in the voltage-sensing arginine residues of the K_v3.1 S4 segment (p.Arg311His, p.Arg314His, p.Arg317His and p.Arg320His; Fig. 2b,c) were established using a recently developed statistical framework, which takes into account both the local sequence context of a mutation site and regional factors such as divergence between humans and macaques.

Functional analysis of the p.Arg320His substitution in K_v3.1. Mutagenesis and RNA preparation. We used the QuikChange kit (Stratagene) to engineer the missense mutation c.959G>A (p.Arg320His) into human KCN1 cDNA (NM_004972: this construct corresponds to the K_v3.1a isoform (511 amino acids), which has a shorter cytoplasmic C-terminal domain but identical biophysical properties in comparison to the longer K_v3.1b (585 amino acids) isoform) cloned in a pCMV-Entry vector obtained from OriGene Technologies. This clone encodes a C-terminal Myc-DDK tag. Introduction of the mutation was confirmed and the presence of additional mutations was excluded by Sanger sequencing. Primer sequences are available upon request.

RNA was prepared using the T7 mMessage mMachine kit from Ambion. Oocyte preparation and injection. The use of animals and all experimental procedures were approved by local authorities (Regierungspraesidium Tübingen, Tübingen, Germany). Extracted ovaries pieces (X. laevis) obtained from the Institute of Physiology I, Tübingen, were treated with collagenase (1 mg/ml type CLS II collagenase, Biochrom) in OR-2 solution (8.25 mM NaCl, 2.5 mM KCl, 1 mM MgCl_2 and 5 mM HEPES, pH 7.6), washed thoroughly and stored at 16 °C in Barth solution (88 mM NaCl, 2.4 mM NaHCO_3, 1 mM...
KCl, 0.33 mM Ca(NO₃)₂, 0.41 mM CaCl₂, 0.82 mM MgSO₄ and 5 mM Tris-HCl, brought to pH 7.4 with NaOH (Sanko Junyus Co., Ltd.). Equal volumes (50 µl) of cRNA with the concentration adjusted to 2 µg/µl were injected into the same batch of oocytes plated in 96-well plates using Roboinjector (Multi Channel Systems). Recordings were performed in parallel at days 2–3 after injection. Amplitudes of interest for all currents recorded on the same day were normalized to the mean value obtained for wild-type Kv3.1 on that day so that the normalized data from different experiments could be pooled. Sample sizes were not predetermined.

Automated oocyte two-microelectrode voltage clamp. Potassium ion currents in oocytes were recorded at room temperature (20–22 °C) on Roboocyte2 (Multi Channel Systems) using prepulled and prepositioned intracellular glass microelectrodes with a resistance of 0.3–1 MΩ when filled with a solution of 1 M KCl and 1.5 M potassium acetate. The bath solution was ND96 (93.5 mM NaCl, 2 mM KCl, 1.8 mM CaCl₂, 2 mM MgCl₂ and 5 mM HEPES, pH 7.5). Currents were sampled at 5-kHz intervals. For the analysis of channel activation, we kept cells at the holding potential of −90 mV and used 0.5-s depolarizing steps (∆10 mV) from −60 mV to +60 mV, followed by a step to −90 mV for 0.5 s to analyze tail currents.

Data analysis. Voltage clamp recordings were analyzed using Roboocyte2+ (Multi Channel Systems), Clampfit (pClamp 8.2, Axon Instruments), Excel (Microsoft) and Origin (OriginLab Corp.) software. The voltage dependence of channel activation was determined from tail current amplitudes recorded at −90 mV. A Boltzmann function was fit to each current-voltage relationship, I(V) = I_{max}/(1 + exp((V - V_{0.5})/k)) + C, where I_{max} is the maximum tail current amplitude at test potential V, V_{0.5} is the half-maximal activation potential, k is a slope factor reflecting the characteristics of voltage-dependent channel gating and C is a constant. Maximum current amplitudes were compared at the end of a 0.5-s test pulse to +60 mV. All data are shown as mean values ± s.e.m. Statistical analysis was performed with GraphPad software, and significant differences (P < 0.05) were determined using the Student’s t test or Mann-Whitney U test.

Protein blotting. For protein blots, injected Xenopus oocytes were lysed in buffer containing 20 mM Tris, 100 mM NaCl, 1 mM EDTA, 0.5% Triton X-100 and 10% glycerol with Complete protease inhibitors (Roche). After we determined protein concentrations (BCA system, Thermo Fisher Scientific), 20 µg of protein for each sample was separated by SDS-PAGE on 8% polyacrylamide gels. The proteins were transferred onto nitrocellulose membrane (Whatman, GE Healthcare Europe), and protein blotting was performed using a mouse monoclonal antibody to the DDK tag (1:1,500 dilution; OriGene Technologies, TA50011). Chemiluminescence detection was carried out according to the manufacturer's protocol (ECL Western Detection Kit, Amersham Pharmacia Biotech Europe). Actin was used as a loading control.