De novo loss- or gain-of-function mutations in KCNA2 cause epileptic encephalopathy

Epileptic encephalopathies are a phenotypically and genetically heterogeneous group of severe epilepsies accompanied by intellectual disability and other neurodevelopmental features. Using next-generation sequencing, we identified four different de novo mutations in KCNA2, encoding the potassium channel Kv1.2, in six isolated patients with epileptic encephalopathy (one mutation recurred three times independently). Four individuals presented with febrile and multiple afebrile, often focal seizure types, multifocal epileptiform discharges strongly activated by sleep, mild to moderate intellectual disability, delayed speech development and sometimes ataxia. Functional studies of the two mutations associated with this phenotype showed almost complete loss of function with a dominant-negative effect. Two further individuals presented with a different and more severe epileptic encephalopathy phenotype. They carried mutations inducing a drastic gain-of-function effect leading to permanently open channels. These results establish KCNA2 as a new gene involved in human neurodevelopmental disorders through two different mechanisms, predicting either hyperexcitability or electrical silencing of Kv1.2-expressing neurons.

Many of the voltage-gated potassium channels (Kv1–Kv12) are expressed in the central nervous system (CNS), having an important role in neuronal excitability and neurotransmitter release. Mutations in potassium channel–encoding genes cause different neurological diseases, including benign familial neonatal seizures (KCNQ2, encoding Kv7.2; KCNQ3, encoding Kv7.3), neonatal epileptic encephalopathy (KCNQ2), episodic ataxia type 1 (EA1) (KCNA1, encoding Kv1.1.1) and peripheral nerve hyperexcitability (KCNA1, KCNQ2). In addition, antibodies against Kv1.1 or associated proteins such as contactin-associated protein 2 (CASPR2) or leucine-rich, glioma-inactivated 1 (LGII) cause limbic encephalitis or neuromyotonia. Therefore, potassium channel genes represent interesting candidates for neurodevelopmental disorders.

To identify mutations in presumed genetic forms of epilepsy, we designed a targeted resequencing panel comprising 265 known and 220 candidate genes for epilepsy (Supplementary Table 1). Screening a pilot cohort of 33 patients, we identified mutations in known epilepsy-related genes in 16 cases. We evaluated the remaining 17 cases for mutations in candidate genes (Supplementary Note), which led to the detection of a heterozygous de novo mutation in KCNA2, c.1214C>T (encoding p.Pro405Leu), affecting the highly conserved pore domain of the voltage-gated potassium channel Kv1.2 (NM_004974, CCDS827). This mutation was not present in control databases (1000 Genomes Project, Exome Variant Server (EVS), dbSNP138 or the Exome Aggregation Consortium (ExAC) database).

The affected female (patient 1) carrying this mutation had remarkable early development until the onset of epilepsy at 17 months of age. The phenotype included febrile and afebrile alternating hemiconic seizures and status epilepticus, reminiscent of Dravet syndrome. The electroencephalogram (EEG) showed multifocal spikes with marked activation during sleep. After seizure onset, ataxia and delay of psychomotor and language development became apparent. She had postnatal short stature, growth hormone deficiency and hypothyroidism. Seizures and ataxia responded poorly to antiepileptic drugs (topiramate, oxcarbazepine, valproic acid and bromide), including acetazolamide (known to be effective in EA1 caused by mutations in KCNA1; ref. 18). At last follow-up at 8 years of age, she had remained seizure free for the past 6 months without previous change of medication.

We identified further KCNA2 mutations in several parallel studies (Supplementary Fig. 1). First, we performed whole-exome sequencing in 86 parent-offspring trios with epileptic encephalopathy (31 with Dravet syndrome negative for mutations in SCN1A, 39 with myoclonic-atonic epilepsy (MAE) and 16 with electrical status epilepticus in slow-wave sleep (ESES)). Second, we performed panel sequencing (Supplementary Note) in 147 adults with a broad spectrum of epilepsy phenotypes associated with intellectual disability. Third, we performed whole-exome sequencing in an adult cohort of 10 independent trios with severe epilepsy and intellectual disability and whole-exome sequencing in another cohort of 12 independent, isolated index cases with early-onset ataxia and epilepsy.

We identified six additional independent cases with previously unreported heterozygous KCNA2 variants (Table 1, Supplementary Fig. 2 and Supplementary Note). Patient 2 (initially classified as having MAE) carried the de novo mutation c.788T>C (encoding p.Ile263Thr). Patient 3 (intellectual disability with neonatal-onset focal epilepsy and cerebellar hypoplasia) carried the variant c.440G>A (encoding p.Arg147Lys), of unknown inheritance. We considered p.Arg147Lys to be a variant of unknown relevance because (i) it could not be confirmed as de novo, (ii) it was predicted to be benign using seven of nine prediction tools, (iii) a lysine occurs naturally at this position in Drosophila melanogaster and zebrafish, and (iv) the change did not show functional consequences (Supplementary Fig. 3, Supplementary Tables 1 and 2, and Supplementary Note). Patient 4 (initially classified as having Dravet
syndrome with prominent focal seizures) and patient 5 (intellectual disability with febrile seizures, focal seizures and status epilepticus) also carried the de novo mutation encoding p.Pro405Leu (Fig. 1c and Supplementary Fig. 2). Patients 1, 2, 4 and 5 eventually became seizure free between 4 and 15 years of age, whereas intellectual disability and (in patients 1 and 4) mild to moderate ataxia remained unchanged. Recurrence of the variant encoding p.Pro405Leu (in patients 1 and 4) mild to moderate ataxia remained unchanged.

Patient 6 carried the de novo mutation c.894G>T encoding p.Leu298Phe). His phenotype was different and much more severe, presenting with severe intellectual disability with gradual loss of language and motor skills, pharmacoresistant generalized tonic-clonic, atypical absence and myoclonic seizures, facial dysmorphism, generalized epileptic discharges and moderate ataxia (Table 1 and Supplementary Note). Similarly, patient 7 carrying the de novo mutation c.890G>A (encoding p.Arg297Gln) presented with a more severe phenotype consisting of moderate intellectual disability, moderate to severe ataxia and pharmacoresistant seizures.

We subsequently screened a follow-up cohort of 99 patients, comprising 47 individuals with unresolved epileptic encephalopathy, short stature and/or ataxia as well as 52 individuals with intellectual disability and idiopathic severe growth hormone deficiency, without detecting additional sequence alterations by Sanger sequencing. We excluded

### Table 1 Main phenotypic characteristics of patients carrying a disease-causing de novo KCNA2 mutation

| Patient 1 | Patient 2 | Patient 3 | Patient 4 | Patient 5 | Patient 6 | Patient 7 |
|-----------|-----------|-----------|-----------|-----------|-----------|-----------|
| Cohort    | First epilepsy panel (n = 33) | MAE (n = 39) | DS (n = 31) | Adult EE 1 (n = 147) | Adult EE II (n = 10) | Ataxia and epilepsy (n = 12) |
| Mutation  | c.1214C>T, p.Pro405Leu | De novo | De novo | De novo | De novo | De novo |
| Functional consequence | Loss of function | Loss of function | De novo | Loss of function | Gain of function | Gain of function |
| Sex, age | F, 11 months | M, 7 years | M, 5 months | M, 19 years | M, 36 years | M, 26 years |
| Development before seizure onset | Normal | Normal | Normal | Normal | Normal | Normal |
| Age at seizure onset | 17 months | 11 months | 10 months | 8 months | 6 months | 5 months |
| Seizure type at onset | FS, hemiclonic seizures | MC, MA | FS, FDS | Seizure free since age 15 years | GTCS | Febrile SE |
| Other seizure types | FS, MC, FDS, focal motor seizures, secondary GTCS | FS, FDS, focal motor seizures, possible extension spasms | FS, focal motor seizures, secondary GTCS | Seizure free since age 15 years | GTCS bimonthly on polytherapy | GTCS once a year on lamotrigine |
| Seizure outcome | Seizure free since age 7.5 years | Seizure free since age 4 years | Seizure free since age 4 years | Seizure free since age 15 years | Seizure free since age 15 years | Seizure free since age 15 years |
| EEG at onset | Focal sharp waves | Focal sharp waves and spikes | Normal | Sharp waves, bilateral centrotoperiorofrontal spikes | Sharp waves, multifocal epileptiform discharges activated by sleep | At age 6 years, generalized spike waves and polyspike waves |
| Course of EEG | Multifocal sharp waves and sharp slow waves, accentuated over the left frontocentral region with marked increase during sleep | Multifocal sharp waves and polyspikes since age 6 years, normal | Focal sharp waves from age 2 years, sharp waves, spike waves and polyspike waves over both centrotemporal regions, independently or bilaterally synchronous (left more than right) increase during sleep | At age 4 years, multifocal epileptiform discharges activated by sleep | At age 6 years, generalized spike waves and polyspike waves |
| Neurological examination | Mild-moderate ataxia, constant myoclonus | Normal | Mild ataxia, myoclonus at rest in hand and fingers | Normal | Moderate ataxia, occasional myoclonus at rest | Moderate-severe ataxia, hyper-reflexia |
| Development at last follow-up | Mild-moderate ID, delayed speech development | Mild-moderate ID | Learning disability, delayed speech development | Moderate ID, delayed speech development | Severe ID, no speech, requires help with all aspects of daily activities | Moderate ID |
| MRI | Normal | Normal | Normal | Normal | Normal | Normal |
| Additional features | GH deficiency, IGFl-1: −0.7 s.d. (1 year, 2 months), −8.5 s.d. (3 years, 5 months) | Subclinical hypothyroidism | Normal | Severe scoliosis | Facial dysmorphism (broad forehead, bulbous nasal tip, deep-set eyes, synophrys, full lips) | Normal |

DS, Dravet syndrome; F, female; FDS, focal dyscognitive seizures; FS, febrile seizures; GH, growth hormone; GTCS, generalized tonic-clonic seizures; ID, intellectual disability; HC, head circumference; M, male; MA, myoclonic-atonic seizures; MC, myoclonic seizures; MRI, magnetic resonance imaging; NA, not available; SE, status epilepticus.
copy number variants (CNVs) affecting KCNA2 in all 99 follow-up cases as well as in 86 trio cases analyzed by whole-exome sequencing using a multiplex amplicon quantification technique developed in house (Online Methods and Supplementary Fig. 1).

To validate our findings statistically and corroborate KCNA2 as a new disease-predisposing gene for epileptic encephalopathy, we calculated the probability for recurring KCNA2 mutations occurring by chance in our cohorts. Comparing the allele frequency of the 6 KCNA2 nonsynonymous variants in our validation cohort (6/(354 × 2); excluding the first mutation encoding p.Pro405Leu detected in the discovery cohort of 33 patients) with those of missense and nonsense variants reported in the largest available control database (ExAC; 144/122,828) showed a significant enrichment of KCNA2 variants in our patient cohorts using Fisher’s exact test ($P = 2.6 \times 10^{-4}$). Further statistical evidence is provided in the Supplementary Note.

KCNA2 had not until now been associated with a human disease. However, during the review process of this manuscript, a single case report was published describing a 7-year-old boy with a KCNA2 de novo mutation encoding p.Arg297Gln presenting with ataxia and myoclonic epilepsy, similar to our patient 7 (ref. 19). In addition, the Pingu mouse presenting with ataxia and growth retardation carries a Kcnma2 loss-of-function mutation, encoding p.Ile402Thr, in close proximity to the p.Pro405Leu substitution; Kcnma2 knockout mice present with severe seizures and premature death.$^{20,21}$

KCNA2 belongs to the Kv1 family of potassium channels (Kv1.1–Kv1.8), all members of which are expressed in the CNS. These channels consist of four subunits with six transmembrane segments (S1–S6). S4 forms the voltage sensor, and S5 and S6 form the pore region containing a selectivity filter and gating ion flow$^{22}$ (Fig. 1a).

All four KCNA2 sequence alterations detected in patients 1–7 (except the one in patient 3) were localized to highly conserved and functionally important protein regions (Fig. 1b) and were predicted to be pathogenic: p.Pro405Leu disrupts the highly conserved, Kv1-specific PVP motif in S6, which is thought to link the gate to the voltage sensor$^{23,24}$. A PVP→AVP change in Kv1.5 leads to a non-functional

**Figure 1** Mutations affecting the Kv1.2 potassium channel. (a) Structure of the voltage-gated potassium channel Kv1.2 with transmembrane segments S1–S4 forming the voltage sensor domain (light gray) and segments S5 and S6 forming the pore region (dark gray) with its pore-forming loop. Variants are localized to highly conserved regions in the S3 segment (p.Ile263Thr, light blue), the S4 segment constituting the voltage sensor (p.Arg297Gln, red; p.Leu298Phe, orange) and the S6 segment (p.Pro405Leu, dark blue). (b) Ile263, Arg297, Leu298, Pro405 and their respective surrounding amino acids show evolutionary conservation. (<(Xemopus laevis) Rattus norvegicus) Mus musculus Bos taurus Dario rerio Xenopus laevis Drosophila melanogaster DI AllIPYPF RVRVRVVF TIALIPVPVI DI AllIPYPF RVRVRVVF TIALIPVPVI DIVAPIPF RVRVRVVF TIALIPVPVI DIVAPIPF RVRVRVVF TIALIPVPVI DIVAPIPF RVRVRVVF TIALIPVPVI

**Figure 2** Functional effects of the KCNA2 mutations encoding p.Pro405Leu and p.Ile263Thr. (a) Representative current traces of Kv1.2 wild-type (WT) channels recorded in a Xenopus oocyte during voltage steps (from −80 mV to +70 mV). (b) Effect of increasing amounts of injected cRNA for wild-type KCNA2 on current amplitude (0.25, 0.5, 1, 2, 4, 8; n = 10; wild type, n = 44; bottom: Ile263Thr, n = 10; wild type, n = 34). A dominant-negative effect of the Pro405Leu and Ile263Thr mutants on wild-type Kv1.2 was seen when a constant amount of cRNA for the wild-type channel (amount 1 in b) was injected with either water or increasing amounts of cRNA for the mutant channels (top: Pro405Leu: ratio 1:1, n = 47; ratio 1:2, n = 40; ratio 1:4, n = 36; bottom: Ile263Thr: ratio 1:1, n = 34; ratio 1:2, n = 42; ratio 1:4, n = 38). Coexpression of Pro405Leu or Ile263Thr and wild-type channel led to a significant reduction in the current amplitude in comparison to expression of wild-type channel alone (indicated by an asterisk). Groups were statistically different (one-way ANOVA, $P < 0.001$; post-hoc Dunn’s method, $P < 0.05$). Shown are means ± s.e.m. (e) Immunoblot analysis for lysates of CHO cells transiently transfected with cDNA for wild-type or Pro405Leu Kv1.2 (middle). For Pro405Leu mutant channels, there was a shift from 57 kDa to −55.5 kDa (n = 3). Kv1.2 wild-type and Ile263Thr (n = 3) channels showed similarly sized bands (57 kDa). The mock condition consisted of transfection with water.
p.Arg297Gln and p.Leu298Phe directly affect the S4 voltage sensor, and p.Arg297Gln has been described before to induce a negative shift of the K\textsubscript{V1.2} activation curve\textsuperscript{27,28}. We found a sigmoidal relationship for the wild-type channel, current amplitudes significantly decreased for the wild-type channel, current amplitudes significantly decreased in comparison to those obtained with similar amounts of cRNA for the wild-type channel alone (Fig. 2b). Hence, both the Pro405Leu and Ile263Thr mutants exert a clear dominant-negative effect on wild-type K\textsubscript{V1.2} channels. Furthermore, Ile263Thr caused a depolarizing shift in voltage-dependent activation, and we found slight changes in inactivation for Pro405Leu (Supplementary Fig. 5).

In contrast to the Pro405Leu and Ile263Thr mutants, both Arg297Gln and Leu298Phe induced strong gain-of-function effects. Neutralization of the second arginine in the voltage sensor in K\textsubscript{V1.2} through the p.Arg297Gln substitution increased current amplitudes by ninefold and shifted the voltage dependence of steady-state activation by −40 mV in comparison to the wild-type channel (Fig. 3a–c).

The gain of function for the Leu298Phe mutant was even more pronounced, with a 13-fold increase in current amplitude and a shift of −50 mV in the activation curve (Fig. 3a–c). As a consequence of the permanently open mutant channels, the resting membrane potentials of oocytes expressing Arg297Gln or Leu298Phe channels were about 40 mV more negative than the resting potentials of oocytes expressing wild-type channel (Fig. 3d). Both mutants exerted a dominant effect on the wild-type protein, as coexpression with cRNA for either Arg297Gln or Leu298Phe and wild-type K\textsubscript{V1.2} in a 0.5:0.5 ratio resulted in very similar alterations as observed with one of the mutants (1.0) alone (Fig. 3b–d).
To examine protein production and stability, we performed SDS-PAGE analysis of total cell lysates using a monoclonal antibody to Kv1.2. Representative immunoblots showed that all mutations resulted in a protein expression level similar to that observed for the 57-kDa band corresponding to the wild-type channel (Figs. 2e and 3e). We found a slight but reproducible shift in size for the band corresponding to Pro405Leu in both oocytes and mammalian cells (Fig. 2e, top and middle). The steric properties of proline can disrupt secondary structure elements, which could be important for the function of the conserved PVP motif. Introduction of a leucine in this motif (LVP) could induce a structural change, resulting in altered gel migration.

Kv1.2 belongs to the delayed rectifier class of potassium channels enabling efficient neuronal repolarization after an action potential. Loss-of-function mutations predict hyperexcitability neuronal membranes and repetitive neuronal firing due to impaired repolarization. This hypothesis is corroborated by the epileptic phenotype of the Kcna2 knockout mouse. In stark contrast, p.Arg297Gln and p.Leu298Phe substitutions are predicted to result in permanently open channels. p.Leu298Phe substitutions are predicted to result in permanently open channels. J.R.L. (DFG Le1030/11-1), P.D.J. (G.A.136.11.N, FWO/ESF-ECRP), H.S.C. (TUBITAK 110558) and I.H. (DFG HE 5145-3-1) received financial support within the EurePIEPIOMICS RES and EuroEPIOMICS CoGEE networks (http://www.europeomics.org/), a Eurocores project of the European Science Foundation. R.S. received funding from the European Union (E-Rare JTC grants 01G1408B and PFOJ-GA-2012-326681). J.M.S. received further support from the Ministerio de Economía y Competitividad (SAF2010-18586). H. Lerche, S.B., and S. Maljevic received further support from the Federal Ministry for Education and Research (BMBF; program on rare diseases, IonNeuroonet: 01GM1105A). S.Z. received support from the US National Institutes of Health (R01NS072248). S.M.S. received support from the Wellcome Trust (084730), National Institute for Health Research (NIHR) University College London Hospital Biomedical Research Centre and Epilepsy Society. UK. M. Synofzik received support from the Interdisciplinary Center for Clinical Research (IZKF) Tübingen (2191-0-0). A.S. received funding for a postdoctoral fellowship from the Fonds Wetenschappelijk Onderzoek.

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COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

URLs

dbSNP Build 138, http://www.ncbi.nlm.nih.gov/projects/SNP/; 1000 Genomes Project database, http://www.1000genomes.org/; Exome Variant Server (EVS), http://evs.gs.washington.edu/EVS/; Exome Aggregation Consortium (ExAC) database, http://exac.broadinstitute.org/; PolyPhen-2, http://genetics.bwh.harvard.edu/pph2/; MutationTaster, http://www.mutationtaster.org/; Multiplex Amplicon Quantification (MAQ), http://www.multiplicom.com/multiplex-amplicon-quantification-maq; Multiplex Amplicon Quantification (MAQ) Software, http://www.multiplicom.com/maq-s; ANNOVAR, http://www.openinformatics.org/annovar/annovar_filter.html#jsb23; GEM.app browser, https://genomics.med.miami.edu/.

METHODS

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

Accession codes. Data for the panel sequencing cohort are accessible on the GEM.app browser under the “EuroEPIOMICS CH/DK” cohort. Data for the trio exome sequencing cohorts are accessible on the GEM.app browser under the “RES EE trio sequencing” cohort.

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

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ONLINE METHODS

Whole-exome and panel sequencing analysis. High-throughput sequencing was performed as described previously by our group for whole-exome analysis\(^\text{31}\) and panel analysis\(^\text{17}\).

The panel used to screen the pilot cohort of 33 patients (including the index patient) comprised 485 known and putative epilepsy-related genes (Supplementary Table 1). The candidates comprised genes that were suggestive of being involved in epileptogenesis on the basis of several reasons, for example, genes belong to neurotransmitter receptor families or other ion channels, genes discussed by different research groups as putatively involved in epilepsy, genes associated with seizures in animals or associated with human neurodevelopmental phenotypes, etc. The gene panel used to screen the second cohort of 147 patients was an updated version of the initial panel. To improve sequence coverage and adapt the panel for purely diagnostic purposes, we excluded a few metabolic and mitochondrial genes as well as most candidate genes and added all recently published new epileptic encephalopathy–associated genes. This panel finally contained 280 genes including 20 candidates for research settings (Supplementary Table 2).

Study cohorts are described in more detail in the Supplementary Note and Supplementary Figure 1. Informed consent was obtained from the patients or their legal guardians prior to analysis. The study was approved by the ethics committees of the University of Antwerp, Belgium; University of Bern, Switzerland; and University of Kiel, Germany.

Sanger sequencing analysis and copy number variation analysis. We performed bidirectional Sanger sequencing of all three exons of KCNA2 (ENST000000485317, NM_004974) and its intron-exon boundaries using the BigDye Terminator v3.1 Cycle Sequencing kit on an ABI 3730XL DNA Analyzer\(^\text{32}\). The comparison PCR amplification of fluorescently labeled target and reference amplicons followed by fragment analysis on the ABI 3730 DNA Analyzer\(^\text{32}\). This assay comprises multiplex PCR amplification of fluorescently labeled target and reference amplicons followed by fragment analysis on the ABI 3730 DNA Analyzer\(^\text{2,5}\). The comparison PCR reaction consisted of three test amplicons located in the genomic region containing KCNA2 and three reference amplicons located on different chromosomes (the primer mix is available upon request).

Additionally, the genomic region containing KCNA2 was screened for CNVs by use of an in house–developed technique for multiplex amplicon quantification (MAQ). With this MAQ technique, we screened all 99 individuals of the Sanger sequencing cohort as well as all 86 individuals of the whole-exome sequencing cohort (Supplementary Fig. 1). This assay comprises multiplex PCR amplification of fluorescently labeled target and reference amplicons followed by fragment analysis on the ABI 3730 DNA Analyzer\(^\text{2,5}\). The comparison of normalized peak areas for the test individual and the average of seven control individuals yields target amplicon doses indicating the copy number of the target amplicon (using the in house–developed MAQ software). The multiplex PCR reaction consisted of three test amplicons located in the genomic region of KCNA2 and three reference amplicons located on different chromosomes (the primer mix is available upon request).

Pathogenicity predictions. For prediction of the pathogenicity of nonsynonymous variants, we used the ANNOVAR\(^\text{33}\) table_annovar.pl script together with the LJ23 database (dbNSFP)\(^\text{34}\) from June 2013 comprising prediction scores from SIFT, PolyPhen-2 (HDIV and HVAR), LRT, MutationTaster, MutationAssessor, FATHMM, MetaSVM and MetaLR. Scores were used as given on the ANNOVAR webpage. Three additional conservation scores (GERP+, phyloP and SiPhy) were used to determine the conservation of a genomic position (more details are provided in Supplementary Table 2).

Testing the enrichment of pathogenic variants. To test the enrichment of probably damaging nonsynonymous KCNA2 variants in our data, we used the ExAC database as a control data set. It comprises data from 61,486 individuals from various exome sequencing projects, including data for control cohorts, but also data from studies on neurological disorders such as schizophrenia and bipolar disorder. We extracted all 64 nonsynonymous (missense and nonsense) variants for KCNA2 from ExAC (November 2014). Some of these occurred in more than one individual yielding all together 144 alleles with variation in KCNA2 out of a total of 122,828 alleles in the ExAC database. Significant enrichment of nonsynonymous variants was then tested determining the difference in allele counts for our data set and the ExAC data set using Fisher’s exact test.

Probability assessment of de novo mutation events. We first obtained an estimate for the single-nucleotide mutation rate in the KCNA2 gene. This rate equals the product of the average de novo mutation rate in humans of $1.2 \times 10^{-8}$ mutations per nucleotide per generation\(^\text{35}\) and the length of the largest coding sequence of KCNA2 (CCDS database, 827.1) of 1,500 bp, yielding $1.8 \times 10^{-9}$ mutations per generation. The probability of observing a de novo mutation in KCNA2 in k out of n parent–offspring trios then simply follows a binomial distribution with a success probability equaling the gene-based mutation rate, binomial$(k, n, 1.8 \times 10^{-9})$ (Supplementary Note).

Functional investigations. Mutagenesis and RNA preparation. Site-directed mutagenesis was performed to engineer the mutations into human KCNA2 cDNA using QuiKChange (Agilent Technologies; primer sequences are available upon request). Mutant cDNA was fully resequenced before being used in experiments to confirm the introduced mutation and exclude the presence of any additional sequence alterations. cRNA was prepared using the SP6 mMessage kit from Ambion. cDNA for human K\(_{\text{v}1.2}\) in the pcDNA3.1 vector was kindly provided by S. Grissmer (Ulm University).

Electrophysiology. Xenopus oocytes were obtained from the Institute of Physiology I, Tübingen, Germany. Preparation of the oocytes was performed as described previously\(^\text{2,5}\). Experiments were approved by the local Animal Care and Use Committee (Regierungspräsidium Tübingen, Tübingen, Germany). Oocytes were treated with collagenase (1 mg/ml type CLS II collagenase, Biochrom) in OR-2 solution (82.5 mM NaCl, 2.5 mM KCl, 1 mM MgCl\(_2\), 1.8 mM CaCl\(_2\), 0.82 mM MgSO\(_4\) and 5 mM Tris-HCl, pH 7.4 with NaOH) supplemented with 50 µg/ml gentamicin (Biochrom). We injected 50 nl of cRNA encoding wild-type or mutated K\(_{\text{v}1.2}\) subunits (1 µg/µl, which corresponds to the cRNA amount of 1.0) into oocytes using the Roboocyte2 (Multi Channel Systems), and oocytes were stored for 2 d (at 17°C) before the experiment. Current amplitudes for wild-type and mutant channels recorded on the same day were normalized to the mean value with a cRNA amount of 1.0 for wild-type K\(_{\text{v}1.2}\) recorded on that day to pool the normalized data from different experiments together.

Automated two-electrode voltage-clamp. Potassium currents in oocytes were recorded at room temperature (20–22°C) using Roboocyte2 (Multi Channel Systems). For two-electrode voltage-clamp (TEVC) recordings, oocytes were impaled with two glass electrodes with a resistance of 0.4–1 MΩ containing a solution of 1 M KCl and 1.5 M potassium acetate and clamped at a holding potential of −80 mV. Oocytes were perfused with an ND96 bath solution containing 93.5 mM NaCl, 2.5 mM KCl, 1.8 mM CaCl\(_2\), 2 mM MgCl\(_2\) and 5 mM HEPES (pH 7.6). Currents were sampled at 5 kHz.

Voltage-clamp protocols and data analysis. The membrane was depolarized to various test potentials from a holding potential of −80 mV to record potassium currents. The activation curve was derived from the current-voltage relationship that was obtained by measuring the peak current at various step depolarizations from the holding potential of −80 mV (10 mV increment, depolarized to +70 mV). The following Boltzmann function was fitted to the obtained data points

$$g(V) = \frac{g_{\text{max}}}{[1 + \exp((V-V_{\text{rev}})/k_V)]} \text{,}$$

with $g(V) = I(V) - I_{\text{rev}}$ being the conductance, $I$ the recorded current amplitude at test potential $V$, $V_{\text{rev}}$ the potassium reversal potential, $g_{\text{max}}$ the maximal conductance, $V_{1/2}$ the voltage of half-maximal activation and $k_V$ a slope factor. Voltage-dependent inactivation of wild-type and mutated K\(_{\text{v}1.2}\) channels was analyzed using 25-s conditioning pulses at potentials ranging from −60 mV to 0 mV (10-mV increment) from a holding potential of −80 mV; the test pulse was 30 mV. A standard Boltzmann function was fitted to the inactivation curves

$$I(V) = \frac{I_{\text{max}}}{[1 + \exp((V-V_{1/2})/k_I)]} \text{,}$$

with $I$ being the recorded current amplitude at the conditioning potential $V$, $I_{\text{max}}$ the maximal current amplitude, $V_{1/2}$ the voltage of half-maximal inactivation and $k_I$ a slope factor.
Protein blot analysis. For protein blotting, injected Xenopus oocytes were lysed in a buffer containing 20 mM Tris, 100 mM NaCl, 1 mM EDTA, 0.5% Triton X-100 and 10% glycerol with cOmplete protease inhibitors (Roche). In addition, for the mutation encoding p.Pro405Leu, CHO cells were transfected with 10 µg/µl DNA using Mirus TransIT-LT1 reagent. CHO cells were lysed in a buffer containing 2 M Tris (pH 7.5), 3 M NaCl, 0.2 M EDTA, 0.2 M EGTA, 0.25 M sodium pyrophosphate, 0.1 M β-glycerophosphate, 0.1 M sodium orthovanadate, 1 M DTT, 0.1 M 1% Triton X-100 and 25× cOmplete solution (Roche). After measuring protein concentrations (BCA Systems, Thermo Fisher Scientific), 15–20 µg of protein was separated by SDS-PAGE on 8% polyacrylamide gels. Proteins were transferred onto polyvinylidene fluoride (PVDF) membranes (PALL Corporation) and protein blotting was performed using a mouse antibody to K V1.2 (NeuroMab clone K14/16). Water-injected oocytes and untransfected and water-transfected CHO cells were used as controls.

Data and statistical analysis. Sample size was estimated by using GraphPad StatMate software. TEVC recordings were analyzed using Roboocyt e 2+ (Multi Channel Systems) and Clampfit (pClamp, Axon Instruments), Origin 6.1 (Origin-Lab Corporation) and Excel (Microsoft) software. Data were tested for a normal distribution using SigmaPlot12 (Systat software). For statistical evaluation, one-way ANOVA with Dunnett’s post-hoc test (normally distributed data) or one-way ANOVA on ranks with Dunn’s post-hoc test (data not normally distributed) was used to compare multiple groups, with one-way ANOVA testing the overall difference between groups and post-hoc tests examining the difference between specific groups. For unpaired data sets, Student’s t test (normally distributed, unpaired data sets) or Mann-Whitney rank-sum test (data not normally distributed) was used. All data are shown as means ± s.e.m. For all statistical tests, significance with respect to the control is indicated in the figures using the following symbols: *P < 0.05, **P < 0.01, ***P < 0.001.

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