

# Spectrum of Phenotypic, Genetic, and Functional Characteristics in Patients With Epilepsy With *KCNC2* Pathogenic Variants

Niklas Schwarz, MD,\* Simone Seiffert, MSc,\* Manuela Pendziwiat, MSc,\* Annika Verena Rademacher, MD, Tobias Brünger, PhD, Ulrike B.S. Hedrich, MD, Paul B. Augustijn, MD, Hartmut Baier, MD, Allan Bayat, MD, Francesca Bisulli, MD, PhD, Russell J. Buono, MD, Ben Zeev Bruria, MD, Michael G. Doyle, MD, Renzo Guerrini, MD, Gali Heimer, MD, Michele Iacomino, PhD, Hugh Kearney, PhD, Karl Martin Klein, MD, Ioanna Kousiappa, PhD, Wolfram S. Kunz, MD, Holger Lerche, MD, Laura Licchetta, MD, PhD, Ebba Lohmann, MD, Raffaella Minardi, PhD, Marie McDonald, MD, Sarah Montgomery, MS, Lejla Mulahasanovic, MD, Renske Oegema, MD, PhD, Barel Ortal, MD, Savvas S. Papacostas, MD, Francesca Ragona, MD, Tiziana Granata, MD, Phillip S. Reif, MD, Felix Rosenow, MD, Annick Rothschild, MD, Paolo Scudieri, PhD, Pasquale Striano, MD, PhD, Paolo Tinuper, MD, George A. Tanteles, MD, Annalisa Vetro, PhD, Felix Zahnert, MD, Ethan M. Goldberg, MD, Federico Zara, PhD, Dennis Lal, PhD, Patrick May, PhD, Hiltrud Muhle, MD, Ingo Helbig, MD, and Yvonne Weber, MD

## Correspondence

Dr. Weber  
yweber@ukaachen.de

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## Abstract

### Background and Objectives

*KCNC2* encodes Kv3.2, a member of the Shaw-related (Kv3) voltage-gated potassium channel subfamily, which is important for sustained high-frequency firing and optimized energy efficiency of action potentials in the brain. The objective of this study was to analyze the clinical phenotype, genetic background, and biophysical function of disease-associated Kv3.2 variants.

### Methods

Individuals with *KCNC2* variants detected by exome sequencing were selected for clinical, further genetic, and functional analysis. Cases were referred through clinical and research collaborations. Selected de novo variants were examined electrophysiologically in *Xenopus laevis* oocytes.

### Results

We identified novel *KCNC2* variants in 18 patients with various forms of epilepsy, including genetic generalized epilepsy (GGE), developmental and epileptic encephalopathy (DEE)

\*These authors contributed equally to this work.

From the Departments of Neurology and Epileptology (N.S., S.S., U.B.S.H., H.L., Y.W.) and Neurodegenerative Diseases (E.L.), Hertie-Institute for Clinical Brain Research, University of Tübingen; Institute of Clinical Molecular Biology (M.P., I.H.), Christian-Albrechts-University of Kiel; Departments of Neuropediatrics (M.P., A.V.R., H.M., I.H.) and Pediatrics I (A.V.R.), University Medical Center Schleswig-Holstein, Christian-Albrechts-University, Kiel; Cologne Center for Genomics (T.B., D.L.), University of Cologne, Germany; Epilepsy Center SEIN (P.B.A.), Heemstede, the Netherlands; Epilepsiezentrum Bodensee (H.B.), Weissenau, Germany; Department of Genetics and Precision Medicine (A.B.), Danish Epilepsy Centre, Dianalund, Denmark; IRCCS Istituti delle Scienze Neurologiche di Bologna (F.B., L.L., R.M., P.T.); Department of Biomedical and Neuromotor Sciences (F.B., L.L., P.T.), University of Bologna, Italy; Department of Biomedical Sciences (R.J.B.), Cooper Medical School of Rowan University, Camden, NJ; Pediatric Neurology Unit (B.Z.B., G.H.), Edmond and Lily Safra Children's Hospital, Chaim Sheba Medical Center, Ramat Gan; Sackler School of Medicine (B.Z.B., G.H.), Tel Aviv University, Israel; FutureNeuro SFI Research Centre (M.G.D., H.K.) and Department of Neurology, Beaumont Hospital (M.G.D., H.K.), Royal College of Surgeons in Ireland; StAR MD Programme (M.G.D.), Royal College of Surgeons in Ireland in collaboration with Blackrock Clinic, Dublin, Ireland; Pediatric Neurology, Neurogenetics and Neurobiology Unit and Laboratories, and Department of Neuroscience (R.G., A.V.), A. Meyer Children's Hospital, University of Florence; IRCCS Istituto Giannina Gaslini (M.I., P. Striano), Genova, Italy; Epilepsy Center Frankfurt Rhine-Main, Department of Neurology (K.M.K., P.S.R., F. Rosenow), University Hospital Frankfurt; LOEWE Center for Personalized Translational Epilepsy Research (CePTER) (K.M.K., P.S.R., F. Rosenow), Goethe-University Frankfurt, Germany; Departments of Clinical Neurosciences, Medical Genetics, and Community Health Sciences (K.M.K.), Hotchkiss Brain Institute & Alberta Children's Hospital Research Institute, Cumming School of Medicine, University of Calgary, Canada; Department of Neurobiology and Epilepsy Center (I.K., S.S.P.), The Cyprus Institute of Neurology and Genetics, Nicosia; Institute of Experimental Epileptology and Cognition Research and Department of Epileptology (W.S.K.), University of Bonn, Germany; Division of Medical Genetics, Department of Pediatrics (M.M., S.M.), Duke University, Durham, NC; CeGaT GmbH and Praxis für Humangenetik Tübingen (L.M.), Germany; Division of Neurology (E.M.G., I.H.), University Medical Center Utrecht, the Netherlands; The Genomic Unit, Sheba Cancer Research Center (B.O.), Cancer Research Center, Wohl Institute for Translational Medicine (B.O.), and The Institute for Rare Diseases, The Edmond and Lily Safra Children's Hospital (A.R.), Sheba Medical Center, Tel Hashomer, Israel; Medical School (S.S.P.), University of Nicosia, Cyprus; Department of Pediatric Neuroscience (F. Ragona, T.G.), Fondazione IRCCS Istituto Neurologico Carlo Besta, Member of the ERN EpiCARE, Milan, Italy; Sheba Medical Center, Tel-Hashomer; Sackler Faculty of Medicine (A.R.), Tel Aviv University, Israel; Departments of Neurosciences, Rehabilitation, Ophthalmology, Genetics, and Maternal and Child Health (P. Scudieri, P. Striano, F. Zara), University of Genova, Italy; Clinical Genetics and Genomics Department (G.A.T.), The Cyprus Institute of Neurology and Genetics, Nicosia; Department of Neurology (F. Zahnert), Epilepsy Center Hessen, Philipps-University Marburg, Germany; Division of Neurology (E.M.G., I.H.), The Epilepsy NeuroGenetics Initiative (ENGIN) (E.M.G., I.H.), and Department of Biomedical and Health Informatics (DBHI) (I.H.), Children's Hospital of Philadelphia, PA; Epilepsy Center, Neurological Institute (D.L.), and Genomic Medicine Institute, Lerner Research Institute (D.L.), Cleveland Clinic, OH; Stanley Center for Psychiatric Research (D.L.), The Broad Institute of Harvard and MIT, Cambridge, MA; Analytic and Translational Genetics Unit (D.L.), Massachusetts General Hospital, Boston; Luxembourg Centre for Systems Biomedicine (P.M.), University Luxembourg; Department of Neurology (I.H.), Perelman School of Medicine, University of Pennsylvania, PA; and Department of Epileptology and Neurology (Y.W.), University of Aachen, Germany.

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## Glossary

**CSWS** = continuous spikes and waves during sleep; **DEE** = developmental and epileptic encephalopathies; **EOAE** = early-onset absence epilepsy; **FE** = focal epilepsy; **GGE** = genetic generalized epilepsy; **JME** = juvenile myoclonic epilepsy; **MAE** = myoclonic-atonic epilepsy; **MTR** = missense tolerance ratio; **VPA** = valproic acid; **WT** = wild-type.

including early-onset absence epilepsy, focal epilepsy, and myoclonic-atonic epilepsy. Of the 18 variants, 10 were de novo and 8 were classified as modifying variants. Eight drug-responsive patients became seizure-free using valproic acid as monotherapy or in combination, including severe DEE cases. Functional analysis of 4 variants demonstrated gain of function in 3 severely affected DEE cases and loss of function in 1 case with a milder phenotype (GGE) as the underlying pathomechanisms.

## Discussion

These findings implicate *KCNC2* as a novel causative gene for epilepsy and emphasize the critical role of  $K_{V3.2}$  in the regulation of brain excitability.

Epilepsy is one of the most prevalent neurologic diseases, with  $\approx 50$  million people affected worldwide.<sup>1</sup> Particularly in children and young adults, epilepsy represents a substantial disease burden relative to other neurologic conditions.<sup>2</sup> The identification of epilepsy-associated genes in the past decade has dramatically improved the understanding of epileptogenesis. More than 10 epilepsy-associated genes coding for potassium channels have been identified. Based on this knowledge, new precision medicine approaches like the treatment with the potassium channel blocker 4-aminopyridine in *KCNA2*-related developmental and epileptic encephalopathies (DEE)<sup>3</sup> were developed. Based on the original description, potassium channels are divided into Shaker, Shal, Shab, and Shaw subtypes.<sup>4</sup> The Shaw-related potassium channel family ( $K_{V3}$ ) plays a pivotal role in the excitability of the CNS by regulating action potential duration and firing pattern of neurons as well as neurotransmitter release.<sup>5,6</sup> So far, only *KCNC1* and *KCNC3* as members of this potassium channel gene family have been implicated in human neurologic diseases (progressive myoclonus epilepsy and spinocerebellar ataxia).<sup>7,8</sup> *KCNC2* codes for the potassium channel  $K_{V3.2}$  (an additional member of the  $K_{V3}$  sub family) mainly expressed in the brain in the interneurons of cortex, thalamus, hippocampus, and basal ganglia.<sup>5</sup>

We identified 16 different heterozygous variants in *KCNC2* in 18 unrelated individuals with DEE including early-onset absence epilepsy (EOAE) and other, more mild epilepsy syndromes such as genetic generalized epilepsy (GGE), epilepsy with focal epilepsy (FE), and myoclonic-atonic epilepsy (MAE), and provide a detailed phenotypical, genetic, and functional analysis emphasizing the role of *KCNC2* as a novel disease gene in human epilepsies.

## Methods

### Patients

Participating patients and relatives were identified through clinical and research collaborations in our study, including

individuals contributing through the Epi25 consortium.<sup>9</sup> Recruitment was performed by the local centers. The data collection included age at onset, sex, seizure types, epilepsy syndrome, result of neurologic examination, cognitive state and development, EEG, brain MRI results, treatment, and outcome. The epilepsy syndromes were classified using the actual International League Against Epilepsy criteria<sup>10</sup> by trained neurologists, epileptologists, and neuropediatricians. The clinical, neuroimaging, and electrophysiologic data were reviewed in detail by 2 experienced clinicians. The epilepsy syndromes were only subclassified (e.g., juvenile myoclonic epilepsy [JME]) as a subtype of GGE if the clinical aspects met the above mentioned criteria; if not, the term GGE was chosen. Individuals with *KCNC2* variants were selected and variants were identified by whole exome sequencing in single or trio setting. Segregation analysis was performed when possible. No other relevant variants were detected in these cases based on the classification criteria by the American College of Medical Genetics.<sup>11</sup>

### Standard Protocol Approvals, Registrations, and Patient Consents

Written informed consent was obtained from all participants in the study or by their legal representatives. The study was approved by each of the following local ethics committees: Universities of Aachen, Bonn, Frankfurt, and Kiel, Germany; Epilepsy Centre Heemstede, the Netherlands; Epilepsiezentrum Bodensee, Weissenau, Germany; Danish Epilepsy Centre, Dianalund, Denmark; IRCCS Istituti delle Scienze Neurologiche di Bologna, Italy; Cooper Medical School of Rowan University, Camden, NJ; Royal College of Surgeons in Ireland, Dublin; University of Florence, Italy; The Cyprus Institute of Neurology and Genetics, Nicosia; Duke University, Durham, NC; University Medical Center Utrecht, the Netherlands; Sheba Medical Center, Tel Hashomer, Israel; Fondazione IRCCS Istituto Neurologico Carlo Besta, Milan, Italy; Tel-Aviv University, Israel; University of Genova, Italy; Philipps-University Marburg, Germany; and Cleveland Clinic, OH.

## Methods

### Sanger Sequencing Analysis

We performed bidirectional Sanger sequencing of the respective areas of *KCNC2* (NM\_139137) using the BigDye Terminator v3.1 Cycle Sequencing kit on an ABI3730XL DNA Analyzer to confirm the described mutations and define the inheritance model (Applied Biosystems; primer sequences available upon request).

The presented *KCNC2* variants were grouped into 2 different categories. The first category (group 1, pathogenic) includes patients with de novo variants. We also acquired variants absent in large population databases (except D128E, once described in gnomAD) that were either inherited from an unaffected parent or variants where a positive family history could be determined without knowledge available about the inheritance model. We defined these as likely pathogenic or modifying variants (group 2) if a minimum of 3/4 prediction scores indicate them as potentially damaging and the CADD score was high (>20) (eTable 1, [links.lww.com/WNL/B901](https://links.lww.com/WNL/B901)).

### Protein Structure Analysis

No experimentally solved protein structure was available for the human  $K_V3.2$  channel. We generated a model of the protein structure using the RaptorX webserver<sup>12</sup> that covered all 638 residues of the protein (NM\_139137; NP\_631875). Two scores were used for the identification of variant sensitive amino acid residues. First, we used our recently developed and validated score that identifies paralog conserved regions across genes of the same gene family (Para\_zscore).<sup>13</sup> In a follow-up study, we showed that paralogous conserved regions are enriched for patient variants,<sup>14</sup> in particular in neurodevelopmental diseases.<sup>13</sup> Second, we used the missense tolerance ratio (MTR) score, which quantifies the constraint of each residue to missense variants in the general population. It was demonstrated that the most constrained regions are enriched for pathogenic variants in ClinVar and HGMD.<sup>15</sup> Variants and critical regions were visualized in PyMOL.<sup>16</sup>

### Functional Investigations

Four variants were selected for functional analysis according to the associated phenotype, the location, and the predicted effect on the structure of the protein.

### Backbone and RNA Preparation

Vector pcDNA3.1 (+) + insert *KCNC2* wild-type (WT) and the mutant clones (NM\_139137: c.375C > G/p.Cys125Trp/C125W; c.404A > G/p.Glu135Gly/E135G; c.656T > C/p.Phe219Ser/F219S; c.1309A > G/p.Thr437Ala/T437A) were acquired from GenScript USA Inc. WT and mutant cDNA sequences were fully resequenced before being used in experiments to confirm the variant and exclude the presence of any additional sequence alterations. cRNA was prepared using the SP6 mMessage kit from Ambion according to the manufacturer's instructions.

### Electrophysiology

Collagenase-treated *Xenopus laevis* oocytes were acquired from Ecocyte Bioscience (1 mg/mL type CLS II collagenase [Biochrom] in OR-2 solution [in mM] 82.5 NaCl, 2.5 KCl, 1 MgCl<sub>2</sub>, and 5 HEPES, pH 7.5), washed 3 times, and stored at 16°C in Barth solution ([in mM] 88 NaCl, 2.4 NaHCO<sub>3</sub>, 1 KCl, 0.33 Ca[NO<sub>3</sub>]<sub>2</sub>, 0.41 CaCl<sub>2</sub>, 0.82 MgSO<sub>4</sub>, and 5 Tris-HCl, pH 7.4 with NaOH) supplemented with 50 µg/mL gentamicin (Biochrom). To compare current amplitudes of WT and mutant channels, 70 nL of cRNA encoding WT or mutant *KCNC2* cRNA (*c* = 1 µg/µL) were injected on the same day using the same batch of oocytes. For recordings of homozygous conditions of the WT or mutant  $K_V3.2$  channels, 70 nL of cRNA (1.0) were injected. To be able to record the heterozygous condition, 35 nL of the WT and 35 nL of the related mutant *KCNC2* cRNA were injected. Injection was performed with the automated Roboinject system (Multi Channel Systems) and oocytes were incubated for 5 days (at 17°C) before the experiments were performed. Potassium currents in oocytes were recorded at room temperature (20–22°C) using Roboocyte2 (Multi Channel Systems). For 2-electrode voltage-clamp (TEVC) recordings, oocytes were impaled with 2 glass electrodes (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 (in mM) 93.5 NaCl, 2 KCl, 1.8 CaCl<sub>2</sub>, 2 MgCl<sub>2</sub>, and 5 HEPES (pH 7.6). Currents were sampled at 2 kHz.

### Western Blot Analysis

For protein blotting, injected *Xenopus laevis* oocytes were lysed in a buffer containing 20 mM Tris HCl (pH 7.6), 100 mM NaCl, 1% Triton X-100, and 1X complete protease inhibitors (Roche). After measuring the protein concentrations (BCA Systems [Thermo Fisher Scientific]), 50 µg of protein were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis on 8% polyacrylamide gels. Proteins were transferred onto polyvinylidene fluoride membranes (Pall Corporation) and protein blotting was performed using a monoclonal mouse antibody (S410-17) against  $K_V3.2$  (*KCNC2*) (MAS-27683 [Thermo Fisher]) with a concentration of 1:500. Water-injected oocytes were used as negative control.

### Data and Statistical Analysis

Data analysis and graphical illustrations were achieved using Roboocyte2+ (Multi Channel Systems), Excel (Microsoft), and GraphPad Prism Software. Normality was tested using the Shapiro-Wilk test and statistical evaluation for multiple comparisons was conducted using 1-way analysis of variance on ranks with Dunn post hoc test. Statistical testing was performed with SigmaPlot 12.0 (Systat Software, Inc.) and differences between groups were considered significant with *p* < 0.05. Data are reported as mean ± SEM.

**Table 1** Clinical and Genetic Information on the Analyzed Cohort

Patient ID	Protein variant	Inheritance mode, FH	F/M	Syndrome	Age at onset (current age)	Seizure types	Neurologic examination	Intellectual disability	EEG	Brain MRI	Treatments	Outcome (last treatment)
<b>Group 1: Pathogenic variants (de novo), 10/18</b>												
1	C125W	DN	M	DEE (EOAE)	1.5 mo (15 y)	Absence, Myo, GTCS	Facial dysmorphism, hypotonia, ataxia, ASD	Mild-moderate	G (PSW)	N	CBZ, CZP, LEV, TPM, VPA	SF (VPA, CZP)
2	E135G	DN	M	DEE	1 y (7 y)	Myo, atonic	N	Mild	G	N	ESM, LEV, VPA	DR
3	D167Y <sup>15</sup>	DN	F	DEE (WS to LGS)	1 mo (10 y)	IS, atypical absence, Myo, focal unaware, GTCS	Ataxia, speech disturbance, macrocephaly	Mild-moderate	Hypsarrhythmia, later MF	Arachnoidal cyst (T-L), progressive brain atrophy	CLB, LEV, VPA, LTG, PER, CBD, VGB, ST, OXC, TPM, LCM, VNS	DR
4	F219S	DN <sup>a</sup>	F	GGE	18 y (34 y)	GTCS	N	No	G (PSW)	N	NA	DR
5	R351K	DN	M	DEE (CSWS)	7 mo (15 y)	GTCS, Myo, focal unaware	N	Mild	CSWS	N	VPA, LTG, STM, LEV, ST, CLB, TPM	SF (VPA)
6	R351K	DN <sup>b</sup>	M	DEE (CSWS)	8 mo (30 y)	GTCS, Myo, absence, focal unaware	ASD, hyperactivity, impaired sleep, aphasia	Severe	CSWS	N	VPA, CLB, ST, ESM, VGB, LEV, CZP, TPM, LTG, CBZ, DZP, PB	DR
7	F382C	DN	M	MAE	NA (4 y)	Myo, atonic, clonic	N	No	G (PSW)	Venous anomaly	STM	DR
8	T437A	DN	M	DEE (EOAE)	8 mo (8 y)	Absence, tonic	Regression in language development, cannot follow commands	Severe	G	N	KD, CZP, CLB, DZP, LEV, PER, VPA, ZNS	DR
9	T437N	DN	F	DEE	11 mo (15 y)	FS, Myo	N	Moderate	G	N	VPA, CNZ, RUF	DR
10	T437N	DN	M	DEE	3 mo (12 y)	FS, Myo, absence	Hyperactivity	Moderate	G	N	PB, VPA, LEV, CNZ, CLB, ESM, LTG	DR
<b>Group 2: Likely pathogenic (8/18)</b>												
11	T32A	Mc (neg)	F	GGE (JAE)	10 y (21 y)	GTCS, absence	N	No	N	NA	VPA	SF (VPA)
12	D128E	NA (neg)	F	GGE (JAE)	14 y (67 y)	Absence, GTCS	N	No	G (PSW)	Generalized brain atrophy	VPA, TPM, ESM	SF (VPA, ESM)
13	D144E	NA <sup>c</sup>	M	GGE (JAE)	10 y (29 y)	GTCS, absence	N	No	G (3 Hz spike wave discharges)	NA	LEV, VPA	DR (SF 2 y VPA)
14	V330M	Mc (neg)	F	GGE	3 y (11 y)	GTCS	N	Mild	G (PSW)	N	VPA	SF (VPA)
15	S333T	Mc (neg)	F	Dravet-like (DEE)	2 y (20 y)	GTCS, Myo, absence	N	Mild	G	N	VPA	SF (VPA)
16	I465V	NA <sup>d</sup>	M	FE	13 mo (NA)	FS, SE, GTCS, focal aware and unaware	NA	Na	T-B	N	15 AEDs tried, details NA, VNS	DR

Continued



**Table 1** Clinical and Genetic Information on the Analyzed Cohort (continued)

Patient ID	Protein variant	Inheritance mode, FH	F/M	Syndrome	Age at onset (current age)	Seizure types	Neurologic examination	Intellectual disability	EEG	Brain MRI	Treatments	Outcome (last treatment)
17	N530H	NA (neg)	F	GGE (JME)	8 y (45 y)	GTCS, Myo	Depression and anxiety	No	G (3–4 Hz spike wave discharges)	NA	VPA, TPM, CLB, CBZ, PB	DR
18	S636F	Mc <sup>e</sup>	M	FE	12 y (42 y)	Focal aware, GTCS	N	No	G	N	VPA	SF (VPA)

Abbreviations: ASD = autism spectrum disorder; BRV = brivaracetam; CAE = childhood absence epilepsy; CBZ = carbamazepine; CLB = clobazam; CSWS = continuous spikes and waves during sleep; CZP = clonazepam; DEE = developmental and epileptic encephalopathies; dn = de novo; DR = drug-resistant; DZP = diazepam; EAOE = early-onset absence epilepsy; ESM = ethosuximide; FE = focal epilepsy; FH = family history; G = generalized; GBP = gabapentin; GGE = genetic generalized epilepsy; GTCS = generalized tonic-clonic seizure; IS = infantile spasms; JAE = juvenile absence epilepsy; JME = juvenile myoclonic epilepsy; KD = ketogenic diet; LCM = lacosamide; LEV = levetiracetam; LGS = Lennox-Gastaut syndrome; LTG = lamotrigine; MAE = myoclonic-atic tonic epilepsy; MF = multifocal; Myo = myoclonic seizures; N = normal; NA = not available; neg = negative; OXC = oxcarbazepine; P = posterior; PB = phenobarbital; PER = perampal; PHT = phenytoin; PSW = polyspike wave discharges; RUF = rufinamide; SE = status epilepticus; SF = seizure-free; ST = steroids; STM = sulthiame; T-B = temporal bilateral; T-L = temporal left; TLE = temporal lobe epilepsy; TPM = topiramate; VGB = vigabatrin; VNS = vagus nerve stimulation; VPA = valproic acid; WS = West syndrome; ZNS = zonisamide.

Affected relatives of patients were not available for genetic testing unless noted otherwise. Variants in *italic* were functionally measured. Subclassifications of syndromes (for example, JME for GGE) are only given if classical aspects are present. More detailed EEG information is only given if applicable.

<sup>a</sup> Brother with GGE (KCNC2 negative).

<sup>b</sup> Brother with rolandic epilepsy.

<sup>c</sup> Maternal uncle with epilepsy.

<sup>d</sup> Father and first cousin had febrile convulsions.

<sup>e</sup> GTCS in sister (KCNC2 negative, variant of uncertain significance in *CLCN2*).

## Data Availability

Anonymized data not published within this article will be made available by request from any qualified investigator.

## Results

### Patients

We identified 18 patients carrying 16 different *KCNC2* variants (Table 1), 10 male and 8 female, and separated the patients into 2 categories regarding the potential pathogenicity of their respective *KCNC2* variants. Within these groups, we could describe the following phenotypes.

### Pathogenic Variants (de novo, Group 1): 10/18 Patients

#### Myoclonic-Atonic Epilepsy

One of the 10 patients presented with myoclonic, atonic, and generalized tonic-clonic seizures (exact onset not applicable but before 4 years of age), which were drug-resistant. EEG recordings showed polyspike-wave discharges. The neurologic examination and the mental presentation were normal. On brain MRI, the patient had a developmental venous anomaly, which is a nonspecific finding for epilepsy.

#### Genetic Generalized Epilepsy

One of the 10 patients presented a drug-resistant subtype of GGE featuring generalized tonic-clonic seizures with onset at 18 years of age. EEG recordings showed typical generalized epileptic discharges with polyspike-waves. The neurologic examination and the brain MRI results were normal.

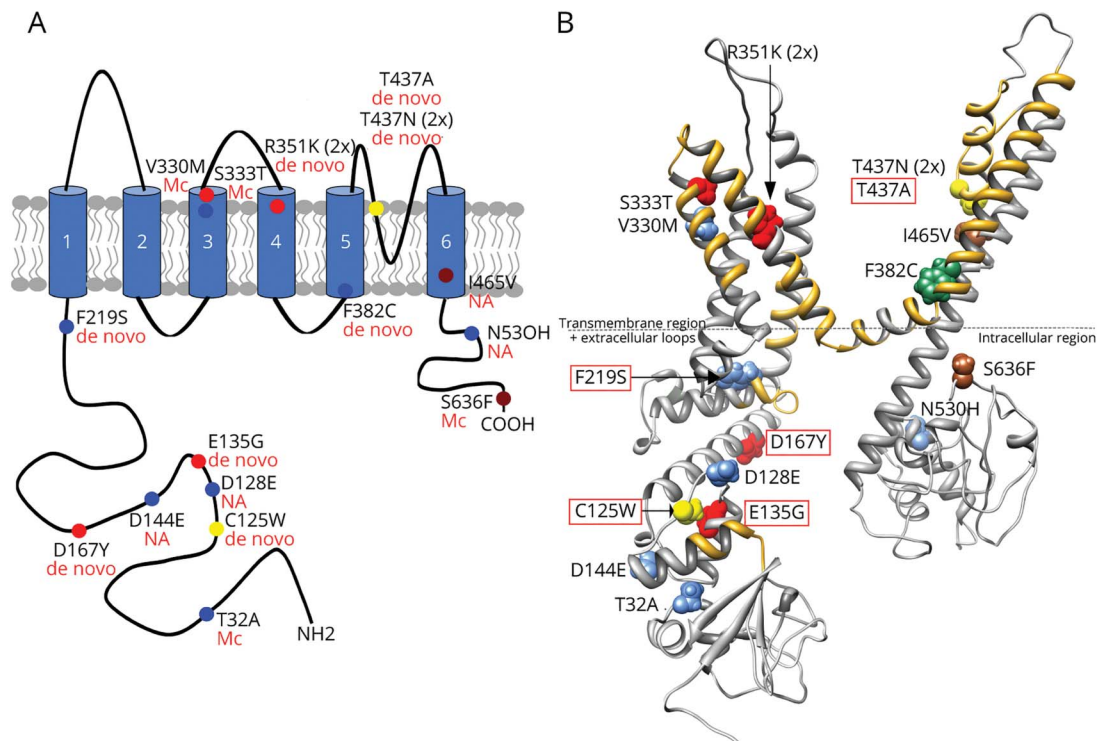
#### Developmental and Epileptic Encephalopathy

Six of the 10 patients with a DEE could be identified carrying a de novo *KCNC2* variant. The age at onset ranged from the first month of life to 1 year. Moderate to severe intellectual disability was present in all patients. Further neuropsychiatric findings were present in 3/6 patients: 1 patient demonstrated an ataxic gait, hypotonia, speech disturbance, and macrocephaly; a second patient had autism spectrum disorder, impaired sleep, hyperactivity, and aphasia; and the third patient presented with isolated hyperactivity. Depending on the syndrome, EEG showed continuous spikes and waves during sleep (CSWS) (2 patients), hypsarrhythmia and later multifocal spikes (1 case), or bilateral/generalized discharges (3 cases). The brain MRI was normal in 5 patients; 1 patient showed an arachnoid cyst combined with brain atrophy. Only 1 patient achieved seizure freedom, using valproic acid (VPA).

#### Early-Onset Absence Epilepsy

Two of the 10 patients had EAOE, with an onset between 1.5 and 8 months. The EAOE syndrome also can be defined as a subtype of DEE but we preferred to describe these patients separately. Both had normal MRI and generalized epileptiform discharges on EEG. They had mild intellectual disability and demonstrated additional abnormalities including facial dysmorphism (more detailed information was

**Figure 1** Structure of KV3.2 Encoded by *KCNC2*



A) Schematic structure of the  $K_v3.2$  subunit. The subunit consists of 6 transmembrane segments (1–6) with long C- and N-terminal regions. The N-terminal plays a crucial role for the tetramerization of the channel. The 4th transmembrane segment works as the selectivity filter for  $K^+$  ions. Variants are color-coded according to the phenotype of the patient: red = developmental and epileptic encephalopathy, yellow = early-onset absence epilepsy, blue = genetic generalized epilepsy, green = myoclonic-atonic epilepsy, and brown = focal epilepsy. (B) The 3D structure of  $K_v3.2$  predicted by RaptorX with *KCNC2* variants and phenotypes included. The golden areas within the structure are highly conserved regions characterized by paralog conservation (Paraz score) and depletion of population variants (missense tolerance ratio [MTR] score). Extracellular loops are shown above the dotted line; the intracellular N- and C-terminal regions are shown below the line. The splice variant and E608K are not shown within the structure. E608K is only expressed in transcript number NM\_139136 and not on NM\_139137, which was used to create the structure. Red-rimmed variants were selected for functional analysis either measured here or previously described by us.<sup>17</sup>

not available), hypotonia, ataxia, and autism spectrum disorder. One of the patients showed a regression in language development. The other patient achieved seizure freedom using a combination of VPA and clobazam.

## Likely Pathogenic Variants (Group 2)—8/18 Patients

### Genetic Generalized Epilepsy

Five of the 8 patients presented with subtypes of GGE featuring absences, myoclonic, and generalized tonic-clonic seizures (onset age 3–14 years). EEG recordings showed 3–4 Hz spike-wave complexes, polyspike waves, or normal interictal results. The neurologic examination was normal in all patients. One patient with GGE had psychiatric symptoms with depression and anxiety, while another showed mild intellectual disability. GGE subclassification was possible in 4/5 patients with GGE of this subgroup of variants—3 with juvenile absence epilepsy and 1 with JME. On the available brain MRI, 1 patient had generalized brain atrophy, which is a nonspecific finding for epilepsy. Four of the 5 patients with GGE were seizure-free (1 lasting >2 years) using VPA in monotherapy or in combination.

### Focal Epilepsies

Two of the 8 patients had FE. Neurologic examination and brain MRI were normal in 1 patient and not available in the other. Neither had intellectual disability. EEG demonstrated bilateral temporal and generalized discharges. One patient achieved seizure freedom using VPA.

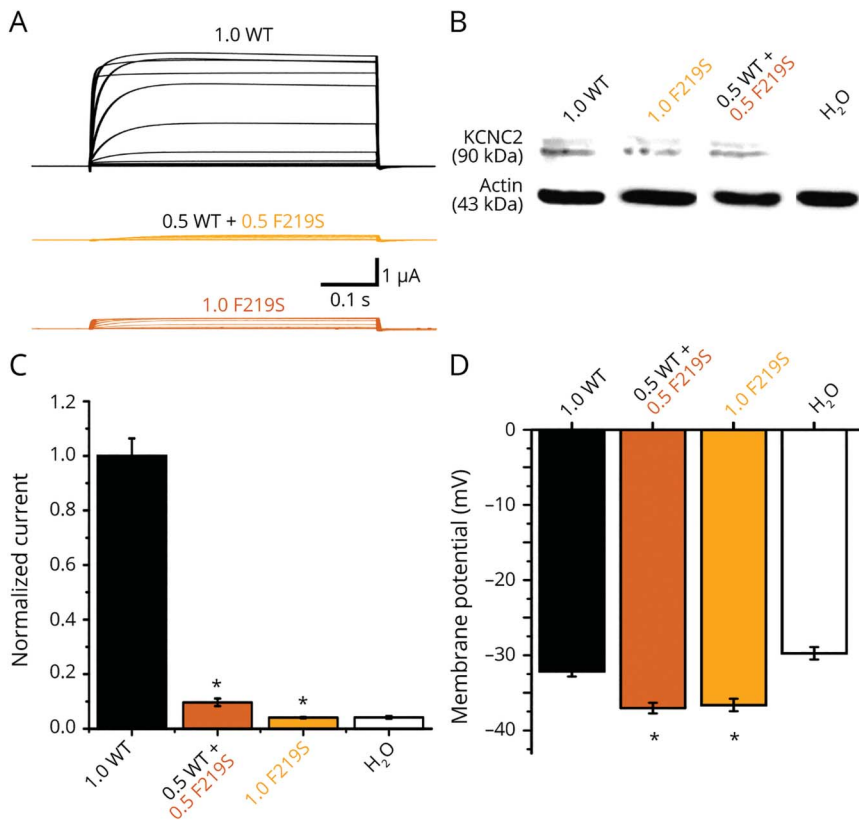
### Developmental and Epileptic Encephalopathy

One of the 8 patients had DEE. The age at onset was 2 years. This patient had mild intellectual disability but no other neuropsychiatric findings. The EEG showed generalized discharges and the brain MRI was normal. The patient received seizure freedom using VPA.

### Genetic Findings

We present 16 unique heterozygous missense variants identified in 18 patients including a first initial case published already by our group.<sup>17</sup> Fifteen of the 16 variants were not found in control cohorts previously. The remaining variant was found once in the control cohort gnomAD (D128E). The presented *KCNC2* variants were grouped into 2 different categories (see also Table 1 and Methods). Pathogenic variants (group 1) were found in 10/18 cases. Modifying variants

**Figure 2** Electrophysiologic Analysis of the p.F219S *KCNC2* Variant



Functional analysis for the F219S variant compared with wild-type (WT). The figures show that the milder phenotype F219S-GGE has a dramatically dominant negative effect in a sense of a loss of function. (A) Representative traces of  $K_v3.2$  currents in *Xenopus laevis* oocytes expressing WT, F219S, or a 1:1 mixture of both in response to the voltage steps from  $-70$  mV to  $+30$  mV. (B) Immunoblot analysis for lysates of *X laevis* oocytes injected with cRNA for  $K_v3.2$  WT, F219S, equal amounts of WT + F219S, or water. All channels showed a band at about 90 kDa. (C) Mean current amplitudes of analyzed oocytes injected with WT ( $n = 101$ ), F219S ( $n = 39$ ), equal amounts of WT + F219S ( $n = 29$ ), or water ( $n = 44$ ). (D) Resting membrane potentials of oocytes injected with WT ( $n = 101$ ), F219S ( $n = 39$ ), equal amounts of WT + F219S ( $n = 29$ ), or water ( $n = 44$ ). Shown are means  $\pm$  SEM. Statistically significant differences between WT channels and the tested groups were verified by analysis of variance on ranks (indicated by asterisks).

(group 2) were detected in 8/18 cases, either inherited from an unaffected parent ( $n = 4$ ) or variants where a positive family history could be determined without knowledge available about the inheritance model ( $n = 4$ ).

Interestingly, 2 variants were identified in our cohort with a similar clinical picture with regard to seizure onset, seizure types, and developmental delay. Both patients (2 cases) with the variant R351K presented with a CSWS phenotype and all 3 patients carrying the T437N/A variant had severe DEE with onset between 3 and 11 months of age, dominant absences and myoclonic seizures, moderate to profound intellectual impairment, and multiple drug-resistant epilepsy.

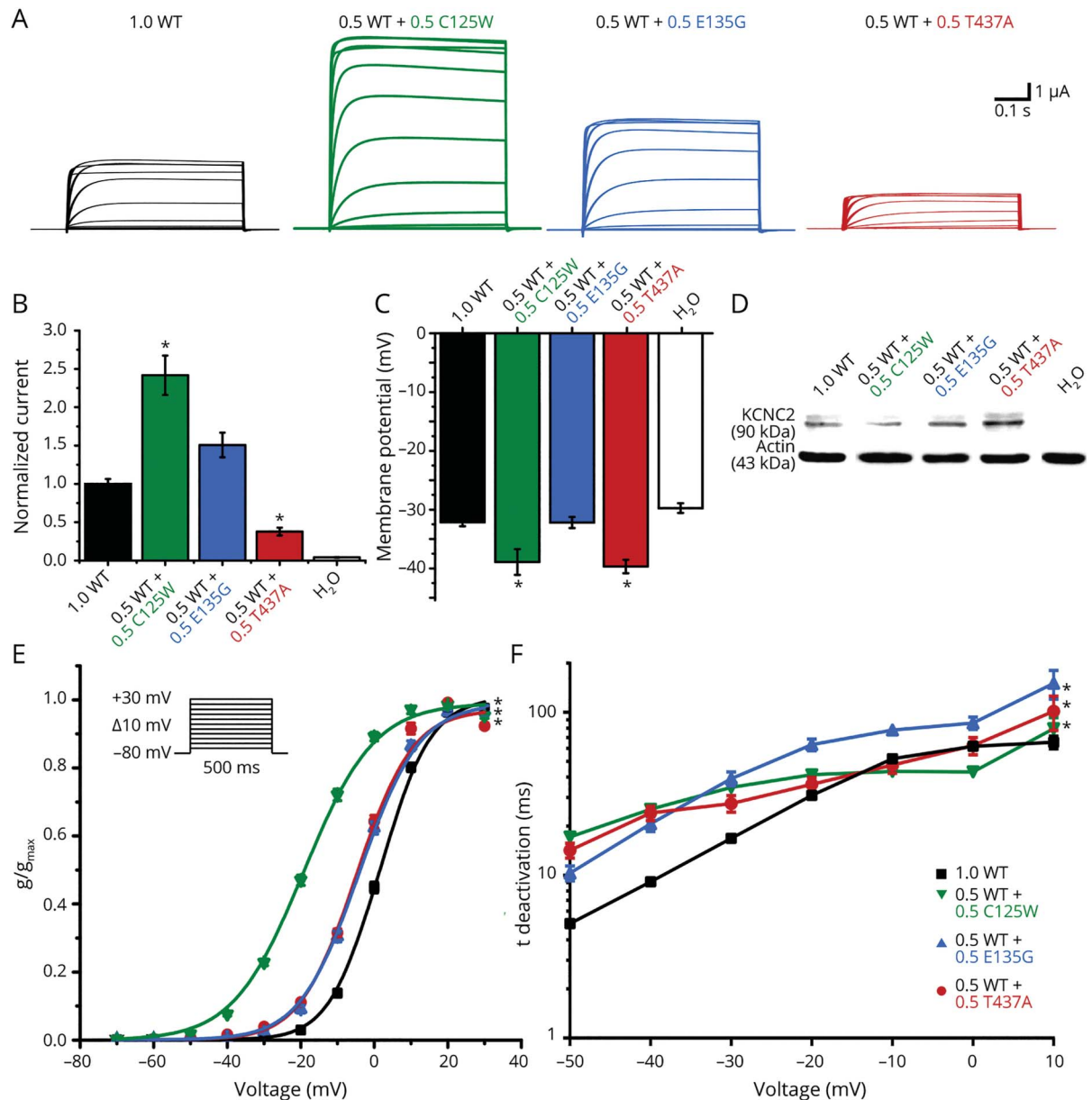
### Protein Structure Analysis

Based on knowledge of potassium channel structure, we located the *KCNC2* variants across the 6 transmembrane segments of the  $K_v3.2$  subunit as well as in the long cytoplasmic N- and C-terminal regions<sup>18</sup> (Figure 1A). The fourth transmembrane domain of the  $K_v3.2$  subunit builds a voltage sensor and the extracellular loop between the fifth and the sixth transmembrane domain serves as a selectivity filter for potassium ions. The identified variants were located in the N- and C-terminal portion of the protein as well as in the last 4 transmembrane domains, but the

localization of each variant did not correlate with a specific phenotype. Because there was no crystal structure available, we modeled the structure of the  $K_v3.2$  subunit and mapped the identified variants onto our model (Figure 1B). By combining the evidence of both scores used, we detected critical regions ( $>5$  consecutively amino acids) characterized by paralog conservation (Para\_zscore  $> 0$ ) and depletion of population variants (MTR  $< 0.459$ ). A total of 9 of 18 patient variants (F219S, V330M, S333T, R351K [2 patients], F382C, T437A/N [3 patients]) are located within these special regions. These regions/variants were all located close to or within the transmembrane region of the protein. Interestingly, one further variant (I465V) was located in the transmembrane region. All others were localized in the cytoplasmic regions. Three variants were de novo cytosolic N-terminal variants (C125W, E135G, D167Y) within or close to the structured N-terminal cytoplasmic region, the so-called T1 domain, which is important for the tetramerization of the protein.<sup>19</sup> The C-terminal region that harbored 2 patient variants is predicted to be mainly unstructured.

We could not detect a strong phenotype-localization correlation in the predicted structure of the  $K_v3.2$  subunit, but most of the identified variants are located in specific relevant and conserved areas of the channel.

**Figure 3** Electrophysiologic Analysis of Selected *KCNC2* Variants



Functional analysis of the variants C125W, E135G, and T437A compared with wild-type (WT). The more severe phenotypes C125W-EOAE (increased current amplitude/activation at more hyperpolarized potentials/slow deactivation), E135G-DEE (normal current/activation at more hyperpolarized potentials/slow deactivation), and T437A-EOAE (reduced current amplitude/activation at more hyperpolarized potentials/slow deactivation) demonstrate gain of function. (A) Representative traces of *K<sub>v</sub>3.2* currents recorded in *Xenopus laevis* oocytes expressing wild-type (WT) or the different variants (C125W, E135G, T437A) in response to voltage steps from -70 mV to +30 mV (with an increment of 10 mV). (B) Mean current amplitudes of oocytes injected with WT (n = 101) and equal amounts of WT + C125W (n = 40), WT + E135G (n = 31), WT + T437A (n = 41), or water (1.0, n = 44). (C) Resting membrane potentials of oocytes injected with WT (n = 101) and equal amounts of WT + C125W (n = 40), WT + E135G (n = 31), WT + F219S (n = 29), WT + T437A (n = 41), or water (n = 44). Shown are means ± SEM. Statistically significant differences between WT channels and the tested groups were verified by analysis of variance on ranks (indicated by asterisks). (D) Immunoblot analysis for lysates of *Xenopus laevis* oocytes injected with cRNA for *K<sub>v</sub>3.2* channel for WT (n = 101), equal amounts of WT + C125W (n = 40), WT + E135G (n = 31), and WT + T437A (n = 42). Lines illustrate Boltzmann function fit to the data points. All activation curves showed a significant shift to more hyperpolarized potentials in comparison with WT channel alone. All data are shown as means ± SEM. (F) Mean voltage-dependent deactivation time constant of *K<sub>v</sub>3.2* channel WT (n = 72), WT + C125W (n = 40), WT + E135G (n = 12), and WT + T437A (n = 20). All deactivation curves showed a significantly slower deactivation in comparison with channels only containing WT subunit. All data are shown as means ± SEM.

### Functional Analysis

We analyzed 4 different variants, which were selected according to the location of the variant within the channel structure as well as the phenotype of the patient (C125W-EOAE, E135G-DEE, F219S-GGE, and T437A-EOAE; see also Figure 1). All of them showed a de novo inheritance

pattern and none of them was found in control cohorts (gnomAD). C125W and E135G are located within the T1 domain, which is important for the tetramerization of the channel (see eFigure 1, [links.lww.com/WNL/B901](https://www.lww.com/WNL/B901)). The Zn<sup>2+</sup> coordinating motif is also located in this region (Hx<sub>5</sub>Cx<sub>20</sub>CC), which is important in bridging the interaction interface between 2



proteins and stabilizing the tetrameric protein structure. The Zn<sup>2+</sup> location within the Shaw and Shaker family is different, suggesting that zinc may play a role in differentiating Shaw from Shaker T1 in assembly.<sup>19</sup> F219S is located shortly before the first transmembrane domain and T437A is located within the P-domain (TxT/SxGY/FG), which acts as the K<sup>+</sup> selectivity filter, and affects the second important amino acid of the P-domain motif.<sup>18</sup>

The functionally analyzed variant F219S-GGE showed a complete loss of function in the homozygous and heterozygous state with current amplitudes comparable to the ones obtained from water-injected control oocytes (Figure 2A). Thus, this variant causes a dominant negative effect on WT channels (Figure 2, A and C). T437A-EOAE led to a significant reduction of the current amplitude, while C125W-EOAE showed a significant increase in the current amplitude. The analysis of E135G-DEE could not demonstrate any differences compared with oocytes injected with the WT subunit (Figure 3, A and B). To make sure that all variants have been expressed in the injected oocytes, we performed a Western blot analysis. This analysis showed a band at about 90 kDa in all protein lysates except for the water-injected control oocytes. As loading control, the housekeeping gene  $\beta$ -actin was used, and all protein lysates showed a band at about 40 kDa. Thus, all variants showed expression in oocytes (Figures 2B and 3D). The analysis of the channel kinetics of the variants C125W-EOAE, E135G-DEE, and T437A-EOAE showed a significant shift of the activation curve to more hyperpolarized potentials (Figure 3E) and in total a slower deactivation compared with oocytes expressing WT channels (Figure 3F). E135G-DEE showed a significantly slower deactivation time constant compared with cells injected with RNA encoding only WT subunits at all recorded voltages except for 0 mV. For C125W-EOAE and T437A-EOAE, the deactivation time constant was significantly reduced between -50 mV and -20 mV or -30 mV, respectively, in comparison with oocytes expressing WT channels (Figure 3F). Thus, analysis of the variants C125W, E135G, and T437A in total demonstrates a gain of function in the channel kinetics. The membrane potential of the variants C125W, F219S, and T437A was significantly shifted to more hyperpolarized potentials (Figures 2D and 3C).

Taken together, the variant T437A-EOAE showed a mixed effect on the channel function, with a gain of function based on changes in the channel kinetics and a loss of function based on a decrease in the normalized current amplitude. For the other 2 variants (C125W-EOAE and E135G-DEE), the gain of function was the predominant effect.

Overall, we could detect 18 patients carrying a *KCNC2* variant, of which 10/18 were defined as pathogenic and 8/18 as modifying, with a variable phenotype including GGE (6/18 cases in total: 1 with pathogenic variants, 5 modifying), DEE (7/18 cases in total: 6 with pathogenic variants, 1 modifying), EOAE (2/18 cases in total: 2 pathogenic), FE (2/18 cases in total: 2 modifying), and MAE (1/18: pathogenic). The 10

pathogenic variants are mainly located in the T1 domain (responsible for tetramer formation), voltage sensor, and P domain (K<sup>+</sup> selectivity filter; see also eFigure 1, links.lww.com/WNL/B901). Three of the 8 modifying variants are located in the T1 domain; others, in nonspecific areas. The functional analysis demonstrated in the more severe phenotypes a clear gain-of-function effect (DEE and EOAE), whereas the milder phenotype F219S-GGE causes a dramatic dominant negative loss-of-function effect.

## Discussion

We describe pathogenic variants in *KCNC2* as a novel genetic etiology for human epilepsies. Phenotypes range from mild generalized epilepsies to severe DEEs related to specific functional changes.

The variants collected ranged from pathogenic to modifying. Therefore, we divided these into 2 different categories. The first category includes only de novo variants that are pathogenic (10/18 variants). The second category includes patients with variants in *KCNC2* that are likely to be disease-causing or to have only a modifying effect because the prediction scores indicated pathogenicity but the variants were inherited by unaffected family members or the inheritance mode was unclear (8/18). The clinical spectrum observed in *KCNC2*-related disorders is very broad concerning the extent of severity. DEE was the main phenotype (39% in total cohort, including EOAE: 50%), but closely followed by GGE (34% in total cohort), FE (11% in total cohort), and 1 case with MAE (5% in total cohort). Nevertheless, the recurrent variants had a recognizable homogenous clinical picture with a CSWS syndrome (R351K) and similar onset, seizure types, mental status, and drug resistance (T437N/A), hinting at potential unique variant-specific genotype-phenotype correlation.

In general, the *KCNC2* cohort showed a reduced drug response, as only 8/18 patients were seizure-free (4/8 patients with GGE). In addition, the GGE-*KCNC2* drug-responsive patients seem to have a poor prognosis as only 66% were drug-responsive compared with approximately 90% in the general GGE population.<sup>20</sup> Nevertheless, it is interesting that all drug-responsive patients became seizure-free using VPA as monotherapy or in combination, including with severe DEE. The patients responding to VPA carry variants that mainly cluster in 2 regions. One is the intracellular N-terminal part including the variants (T32A, D128E, D144E) and the other one the extracellular region of the third and fourth domain including the variants V330M, S333T, and R351K. VPA is an antiepileptic drug with a broad spectrum of mechanisms of action. VPA has been demonstrated to limit high-frequency repetitive firing in cultured neurons.<sup>21</sup> This effect is linked to the modulation of sodium, calcium, and potassium channels, especially with a use-dependent decrease in inward sodium currents. VPA increases the amplitude of the late potassium outward currents, further increasing the threshold for

epileptiform activity,<sup>21</sup> which might explain the special effect of this drug in our cohort. However, the cohort is small and further studies are needed to prove this hypothesis.

*KCNC2* has previously been proposed as a modifying factor<sup>17</sup> and to play a role in other neuropsychiatric diseases such as ataxia,<sup>22,23</sup> schizophrenia,<sup>24</sup> bipolar disorder,<sup>25</sup> and other DEEs.<sup>26</sup> Our patients also displayed additional neurologic features including facial dysmorphism, ataxia, speech disturbance, depression, hyperactivity, and autism spectrum disorder, with additional support to this hypothesis. We could demonstrate a much broader clinical spectrum including GGE, EOAE, FE, and MAE.

The virtual structure of  $K_V3.2$  identified very important regions characterized by paralog conservation (Paraz score) and depletion of population variants (MTR score). We could not find a strong phenotype–structure association but the variants of our patients were concentrated at the C-terminus, N-terminus, and transmembrane segments 3 to 6, indicating high relevance of these regions to channel function.

We selected 4 variants for functional analysis based on the phenotype as well as the location within the channel. The functional results demonstrated a gain of function in the more severe phenotypes DEE and EOAE, whereas in the GGE-associated variant, a dramatic loss-of-function effect was observed. Among all  $K_V$  channels, channels of the  $K_V3$  family in particular play a crucial role in the rapid repolarization of action potentials and therefore dictate action potential duration. The  $K_V3.2$  subunit is predominantly expressed in the brain specifically in parvalbumin-expressing GABAergic interneurons in neocortex as well as somatostatin-expressing GABAergic interneurons in deep cortical layers<sup>5,27</sup> and therefore may be responsible for the modulation of excitation. Furthermore, functional analysis demonstrated that voltage-gated potassium channels are important for the regulation of firing, action potential duration, and neurotransmitter release.<sup>5,6</sup> The  $K_V3.2$  subunit and the  $K_V3$  family in general have some unusual electrophysiologic properties compared with other potassium channels such as the fast rate of deactivation upon repolarization. This rate is significantly faster than that of any other known neuronal voltage-gated  $K^+$  channel. Thus,  $K_V3.2$  plays a crucial role in fast-spiking interneurons.<sup>28</sup> It is therefore reasonable that the development of epileptic seizures in the cases described here may be due to impaired function of inhibitory interneurons. In many generalized epilepsies, inhibitory interneurons play an essential role, for example in Dravet syndrome and genetic epilepsy with febrile seizures plus, which are associated with pathogenic variants in *SCN1A* encoding the main  $Na^+$  channel in inhibitory neurons,<sup>29-31</sup> or in progressive myoclonic epilepsy due to *KCNC1* mutations.<sup>8</sup>

$K_V3.2^{-/-}$  knockout mice presented with specific changes in their cortical EEG patterns and showed increased susceptibility to epileptic seizures.<sup>32</sup> This emphasizes the importance of inhibitory interneurons being able to generate high-

frequency firing so that balanced cortical operations can occur. As described for other variants in potassium channel genes associated with epilepsy,<sup>33</sup> the recorded  $K_V3.2$  variants demonstrate gain- and loss-of-function effects.

*KCNC2* is a novel and important gene for a broad spectrum of epilepsy syndromes with an intriguing phenotype–pathophysiology correlation.

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## Disclosure

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## Appendix Authors

Name	Location	Contribution
<b>Niklas Schwarz, MD</b>	Departments of Neurology and Epileptology, Hertie-Institute for Clinical Brain Research, University of Tübingen, Germany	Drafting/revision of the manuscript for content, including medical writing for content; major role in the acquisition of data; analysis or interpretation of data; other
<b>Simone Seiffert, MSc</b>	Departments of Neurology and Epileptology, Hertie-Institute for Clinical Brain Research, University of Tübingen, Germany	Drafting/revision of the manuscript for content, including medical writing for content; major role in the acquisition of data; analysis or interpretation of data; other
<b>Manuela Pendiwiat, MSc</b>	Institute of Clinical Molecular Biology and Department of Neuropediatrics, University Medical Center Schleswig-Holstein, Christian-Albrechts-University, Kiel, Germany	Drafting/revision of the manuscript for content, including medical writing for content; major role in the acquisition of data; analysis or interpretation of data; other
<b>Annika Verena Rademacher, MD</b>	Department of Neuropediatrics and Pediatrics I, University Medical Center Schleswig-Holstein, Christian-Albrechts-University, Kiel, Germany	Drafting/revision of the manuscript for content, including medical writing for content; analysis or interpretation of data; other

## Appendix (continued)

Name	Location	Contribution
<b>Tobias Brünger, MSc</b>	Cologne Center for Genomics, University of Cologne, Germany	Analysis or interpretation of data; other
<b>Ulrike B.S. Hedrich, MD</b>	Departments of Neurology and Epileptology, Hertie-Institute for Clinical Brain Research, University of Tübingen, Germany	Drafting/revision of the manuscript for content, including medical writing for content; analysis or interpretation of data; other
<b>Paul B. Augustijn, MD</b>	Epilepsy Center SEIN, Heemstede, the Netherlands	Drafting/revision of the manuscript for content, including medical writing for content; other
<b>Hartmut Baier, MD</b>	Epilepsiezentrum Bodensee, Weissenau, Germany	Drafting/revision of the manuscript for content, including medical writing for content; other
<b>Allan Bayat, MD</b>	Department of Genetics and Precision Medicine, Danish Epilepsy Centre, Dianalund, Denmark	Drafting/revision of the manuscript for content, including medical writing for content; other
<b>Francesca Bisulli, MD, PhD</b>	IRCCS Istituti delle Scienze Neurologiche di Bologna; and Department of Biomedical and Neuromotor Sciences, University of Bologna, Italy	Drafting/revision of the manuscript for content, including medical writing for content; other
<b>Russell J. Buono, MD</b>	Department of Biomedical Sciences, Cooper Medical School of Rowan University, Camden, NJ	Drafting/revision of the manuscript for content, including medical writing for content; other
<b>Ben Zeev Bruria, MD</b>	Pediatric Neurology Unit, Edmond and Lily Safra Children's Hospital, Chaim Sheba Medical Center, Ramat Gan; and Sackler School of Medicine, Tel Aviv University, Israel	Drafting/revision of the manuscript for content, including medical writing for content; other
<b>Michael G. Doyle, MD</b>	FutureNeuro SFI Research Centre and Department of Neurology, Beaumont Hospital, Royal College of Surgeons in Ireland; and StAR MD Programme, Royal College of Surgeons in Ireland in collaboration with Blackrock Clinic, Dublin, Ireland	Drafting/revision of the manuscript for content, including medical writing for content; other
<b>Renzo Guerrini, MD</b>	Pediatric Neurology, Neurogenetics and Neurobiology Unit and Laboratories, and Department of Neuroscience, A. Meyer Children's Hospital, University of Florence, Italy	Drafting/revision of the manuscript for content, including medical writing for content; other
<b>Gali Heimer, MD</b>	Pediatric Neurology Unit, Edmond and Lily Safra Children's Hospital, Chaim Sheba Medical Center, Ramat Gan; and Sackler School of Medicine, Tel Aviv University, Israel	Drafting/revision of the manuscript for content, including medical writing for content; other

## Appendix (continued)

Name	Location	Contribution
<b>Michele Iacomino, PhD</b>	IRCCS Istituto Giannina Gaslini, Genova, Italy	Drafting/revision of the manuscript for content, including medical writing for content; other
<b>Hugh Kearney, PhD</b>	FutureNeuro SFI Research Centre and Department of Neurology, Beaumont Hospital, Royal College of Surgeons in Ireland, Dublin	Drafting/revision of the manuscript for content, including medical writing for content; other
<b>Karl Martin Klein, MD</b>	Epilepsy Center Frankfurt Rhine-Main, Department of Neurology, University Hospital Frankfurt; LOEWE Center for Personalized Translational Epilepsy Research (CePTER), Goethe-University Frankfurt, Germany; and Departments of Clinical Neurosciences, Medical Genetics, and Community Health Sciences, Hotchkiss Brain Institute & Alberta Children's Hospital Research Institute, Cumming School of Medicine, University of Calgary, Canada	Drafting/revision of the manuscript for content, including medical writing for content; other
<b>Ioanna Kousiappa, PhD</b>	Department of Neurobiology and Epilepsy Center, The Cyprus Institute of Neurology and Genetics, Nicosia	Drafting/revision of the manuscript for content, including medical writing for content; other
<b>Wolfram S. Kunz, MD</b>	Institute of Experimental Epileptology and Cognition Research and Department of Epileptology, University of Bonn, Germany	Drafting/revision of the manuscript for content, including medical writing for content; other
<b>Holger Lerche, MD</b>	Departments of Neurology and Epileptology, Hertie-Institute for Clinical Brain Research, University of Tübingen, Germany	Drafting/revision of the manuscript for content, including medical writing for content; other
<b>Laura Licchetta, MD, PhD</b>	IRCCS Istituti delle Scienze Neurologiche di Bologna; and Department of Biomedical and Neuromotor Sciences, University of Bologna, Italy	Drafting/revision of the manuscript for content, including medical writing for content; other
<b>Ebba Lohmann, MD</b>	Department of Neurodegenerative Diseases, Hertie-Institute for Clinical Brain Research, University of Tübingen, Germany	Drafting/revision of the manuscript for content, including medical writing for content; other
<b>Raffaella Minardi, PhD</b>	IRCCS Istituti delle Scienze Neurologiche di Bologna, Italy	Drafting/revision of the manuscript for content, including medical writing for content; other
<b>Marie McDonald, MD</b>	Division of Medical Genetics, Department of Pediatrics, Duke University, Durham, NC	Drafting/revision of the manuscript for content, including medical writing for content; other
<b>Sarah Montgomery, MS</b>	Division of Medical Genetics, Department of Pediatrics, Duke University, Durham, NC	Drafting/revision of the manuscript for content, including medical writing for content; other

## Appendix (continued)

Name	Location	Contribution
<b>Leijla Mulahasanovic, MD</b>	CeGaT GmbH and Praxis für Humangenetik Tübingen, Germany	Drafting/revision of the manuscript for content, including medical writing for content; other
<b>Renske Oegema, MD, PhD</b>	Division Biomedical Genetics, University Medical Center Utrecht, the Netherlands	Drafting/revision of the manuscript for content, including medical writing for content; other
<b>Barel Ortal, MD</b>	The Genomic Unit, Sheba Cancer Research Center, and Cancer Research Center, Wohl Institute for Translational Medicine, Sheba Medical Center, Tel Hashomer, Israel	Drafting/revision of the manuscript for content, including medical writing for content; other
<b>Savvas S. Papacostas, MD</b>	Department of Neurobiology and Epilepsy Center, The Cyprus Institute of Neurology and Genetics, Nicosia; and Medical School, University of Nicosia, Cyprus	Drafting/revision of the manuscript for content, including medical writing for content; other
<b>Francesca Ragona, MD</b>	Department of Pediatric Neuroscience, Fondazione IRCCS Istituto Neurologico Carlo Besta, Member of the ERN EpiCARE, Milan, Italy	Drafting/revision of the manuscript for content, including medical writing for content; other
<b>Tiziana Granata, MD</b>	Department of Pediatric Neuroscience, Fondazione IRCCS Istituto Neurologico Carlo Besta, Member of the ERN EpiCARE, Milan, Italy	Drafting/revision of the manuscript for content, including medical writing for content; other
<b>Phillip S. Reif, MD</b>	Epilepsy Center Frankfurt Rhine-Main, Department of Neurology, University Hospital Frankfurt; and LOEWE Center for Personalized Translational Epilepsy Research (CePTER), Goethe-University Frankfurt, Germany	Drafting/revision of the manuscript for content, including medical writing for content; other
<b>Felix Rosenow, MD</b>	Epilepsy Center Frankfurt Rhine-Main, Department of Neurology, University Hospital Frankfurt; and LOEWE Center for Personalized Translational Epilepsy Research (CePTER), Goethe-University Frankfurt, Germany	Drafting/revision of the manuscript for content, including medical writing for content; other
<b>Annick Rothschild, MD</b>	The Institute for Rare Diseases, The Edmond and Lily Safra Children's Hospital, Sheba Medical Center, Tel Hashomer; and Sackler Faculty of Medicine, Tel Aviv University, Israel	Drafting/revision of the manuscript for content, including medical writing for content; other
<b>Paolo Scudieri, PhD</b>	Departments of Neurosciences, Rehabilitation, Ophthalmology, Genetics, and Maternal and Child Health, University of Genova, Italy	Drafting/revision of the manuscript for content, including medical writing for content; other

Continued



## Appendix (continued)

Name	Location	Contribution
<b>Pasquale Striano, MD, PhD</b>	IRCCS Istituto Giannina Gaslini; and Departments of Neurosciences, Rehabilitation, Ophthalmology, Genetics, and Maternal and Child Health, University of Genova, Italy	Drafting/revision of the manuscript for content, including medical writing for content; other
<b>Paolo Tinuper, MD</b>	IRCCS Istituti delle Scienze Neurologiche di Bologna; and Department of Biomedical and Neuromotor Sciences, University of Bologna, Italy	Drafting/revision of the manuscript for content, including medical writing for content; other
<b>George A. Tanteles, MD</b>	Clinical Genetics and Genomics Department, The Cyprus Institute of Neurology and Genetics, Nicosia	Drafting/revision of the manuscript for content, including medical writing for content; other
<b>Annalisa Vetro, PhD</b>	Pediatric Neurology, Neurogenetics and Neurobiology Unit and Laboratories, and Department of Neuroscience, A. Meyer Children's Hospital, University of Florence, Italy	Drafting/revision of the manuscript for content, including medical writing for content; other
<b>Felix Zahnert, MD</b>	Department of Neurology, Epilepsy Center Hessen, Philipps-University Marburg, Germany	Drafting/revision of the manuscript for content, including medical writing for content; other
<b>Ethan M. Goldberg, MD</b>	Division of Neurology and The Epilepsy NeuroGenetics Initiative (ENGIN), Children's Hospital of Philadelphia, PA	Drafting/revision of the manuscript for content, including medical writing for content; other
<b>Federico Zara, PhD</b>	Departments of Neurosciences, Rehabilitation, Ophthalmology, Genetics, and Maternal and Child Health, University of Genova, Italy	Drafting/revision of the manuscript for content, including medical writing for content; other
<b>Dennis Lal, PhD</b>	Cologne Center for Genomics, University of Cologne, Germany; Epilepsy Center, Neurological Institute, and Genomic Medicine Institute, Lerner Research Institute, Cleveland Clinic, OH; Stanley Center for Psychiatric Research, The Broad Institute of Harvard and MIT, Cambridge; and Analytic and Translational Genetics Unit, Massachusetts General Hospital, Boston	Drafting/revision of the manuscript for content, including medical writing for content; other
<b>Patrick May, PhD</b>	Luxembourg Centre for Systems Biomedicine, University Luxembourg	Drafting/revision of the manuscript for content, including medical writing for content; other

## Appendix (continued)

Name	Location	Contribution
<b>Hiltrud Muhle, MD</b>	Department of Neuropediatrics, University Medical Center Schleswig-Holstein, Christian-Albrechts-University, Kiel, Germany	Drafting/revision of the manuscript for content, including medical writing for content; study concept or design; analysis or interpretation of data; other
<b>Ingo Helbig, MD</b>	Institute of Clinical Molecular Biology and Department of Neuropediatrics, University Medical Center Schleswig-Holstein, Christian-Albrechts-University, Kiel, Germany; and Division of Neurology, The Epilepsy NeuroGenetics Initiative (ENGIN), and Department of Biomedical and Health Informatics (DBHI), Children's Hospital of Philadelphia; and Department of Neurology, Perelman School of Medicine, University of Pennsylvania, PA	Drafting/revision of the manuscript for content, including medical writing for content; Study concept or design; analysis or interpretation of data
<b>Yvonne Weber, MD</b>	Departments of Neurology and Epileptology, Hertie-Institute for Clinical Brain Research, University of Tübingen; and Department of Epileptology and Neurology, University of Aachen, Germany	Drafting/revision of the manuscript for content, including medical writing for content; major role in the acquisition of data; study concept or design; analysis or interpretation of data

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