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Clinical spectrum and genotype–phenotype associations of *KCNA2*-related encephalopathies

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Recently, *de novo* mutations in the gene *KCNA2*, causing either a dominant-negative loss-of-function or a gain-of-function of the voltage-gated K^+ channel $K_v1.2$, were described to cause a new molecular entity within the epileptic encephalopathies. Here, we report a cohort of 23 patients (eight previously described) with epileptic encephalopathy carrying either novel or known *KCNA2* mutations, with the aim to detail the clinical phenotype associated with each of them, to characterize the functional effects of the newly identified mutations, and to assess genotype–phenotype associations. We identified five novel and confirmed six known mutations, three of which recurred in three, five and seven patients, respectively. Ten mutations were missense and one was a truncation mutation; *de novo* occurrence could be shown in 20 patients. Functional studies using a *Xenopus oocyte* two-microelectrode voltage clamp system revealed mutations with only loss-of-function effects (mostly dominant-negative current amplitude reduction) in eight patients or only gain-of-function effects (hyperpolarizing shift of voltage-dependent activation, increased amplitude) in nine patients. In six patients, the gain-of-function was diminished by an additional loss-of-function (gain-and loss-of-function) due to a hyperpolarizing shift of voltage-dependent activation combined with either decreased amplitudes or an additional hyperpolarizing shift of the inactivation curve. These electrophysiological findings correlated with distinct phenotypic features. The main differences were (i) predominant focal (loss-of-function) versus generalized (gain-of-function) seizures and corresponding epileptic discharges with prominent sleep activation in most cases with loss-of-function mutations; (ii) more severe epilepsy, developmental problems and ataxia, and atrophy of the cerebellum or even the whole brain in about half of the patients with gain-of-function mutations; and (iii) most severe early-onset phenotypes, occasionally with neonatal onset epilepsy and developmental impairment, as well as generalized and focal seizures and EEG abnormalities for patients with gain- and loss-of-function mutations. Our study thus indicates well represented genotype–phenotype associations between three subgroups of patients with *KCNA2* encephalopathy according to the electrophysiological features of the mutations.

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Abbreviations: ESES = encephalopathy related to status epilepticus during sleep; GTCS = generalized tonic-clonic seizures; K_v = voltage-gated potassium channel

Introduction

Epileptic encephalopathies comprise a heterogeneous group of severe neurological disorders with childhood onset often characterized by severe and pharmacoresistant epilepsy and progressive cognitive and neurological deficits. Many genes have been identified that cause the spectrum of epileptic encephalopathies, but there is a large phenotypic and genetic heterogeneity and the majority of genetic defects is still unknown. Genetic characterization and detailed genotype–phenotype correlations have contributed to the identification of specific forms of epileptic encephalopathies, for example those associated with mutations of genes encoding voltage-gated ion channels, such as *SCN1A*, *SCN2A*, *SCN8A*, *KCNQ2*, or *KCNT1*. Ion channels have a central role in neuronal excitability and neurotransmitter release and their altered function seems to be a key factor in the aetiology of genetic epilepsies (Claes *et al.*, 2001; Reid *et al.*, 2009; Carvill *et al.*, 2013*a*; Epi4K, 2013; Lerche *et al.*, 2013; McTague *et al.*, 2016; Møller *et al.*, 2016).

Recently, mutations in *KCNA2* encoding the voltage-gated K^+ channel $K_v1.2$, have been reported as a novel cause of epileptic encephalopathy (Pena and Coimbra, 2015; Syrbe *et al.*, 2015; Allen *et al.*, 2016; Corbett *et al.*, 2016; Hundallah *et al.*, 2016; Allou *et al.*, 2017). $K_v1.2$ belongs to the K_v1 family with eight members ($K_v1.1$–8), all of which are expressed in the CNS. K_v1 channels are composed of four subunits with six transmembrane segments each (S1–S6, Fig. 1A) (Jan and Jan, 2012). The S4 segments form the voltage-sensor and S5–S6 the pore region. Different K_v1 subunits can assemble in different combinations to form numerous heterotetrameric channels with different characteristics, such as different kinetics and voltage dependence of channel gating (Christie *et al.*, 1990; Sheng *et al.*, 1994). This heteromerization can also involve assembly with auxiliary proteins such as $K_v\beta$ subunits (Li *et al.*, 1992). Interestingly, the $K_v1.2$ channel forms heteromers with different K_v subunits depending on the neuronal cell type, suggesting distinct roles of $K_v1.2$ in different neuronal compartments (Sheng *et al.*, 1994). Mice carrying a *Kcna2* point mutation show motor incoordination, myoclonic jerks, tremor, and small body size (Xie *et al.*, 2010) and *Kcna2*-null animals have an increased seizure susceptibility (Brew *et al.*, 2007). Functional studies of so far four pathogenic *KCNA2* mutations were shown to cause either a dominant-negative loss-of-function, or a drastic gain-of-function (Syrbe *et al.*, 2015).

The aim of this study was to further characterize the phenotypic spectrum associated with novel or known *KCNA2* mutations, to characterize the functional effects of newly identified mutations, and to assess genotype–phenotype associations with special emphasis on the differentiation of phenotypes due to distinct or opposite effects on protein function.

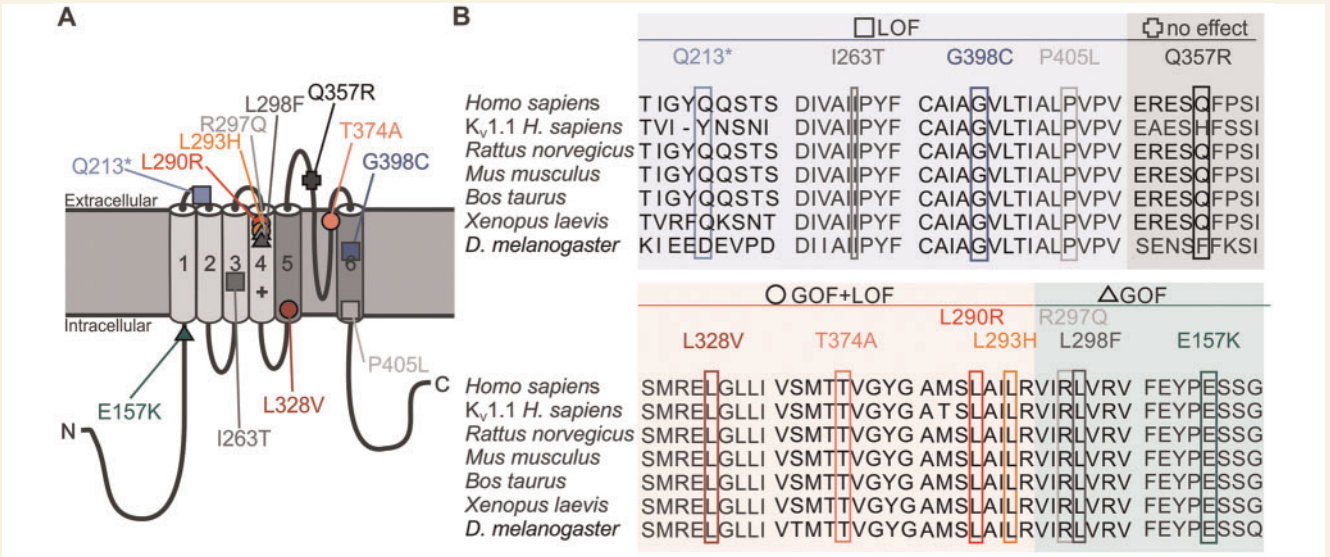

Figure 1 Mutations affecting the $K_v1.2$ potassium channel. (**A**) Structure of the voltage-gated potassium channel $K_v1.2$ with transmembrane segments S1–S4 forming the voltage sensor domain (light grey) and segments S5 and S6 forming the pore region (dark grey) with its pore-forming loop. All variants (except the truncation mutation Q213* in blue and Q357R in black) are localized to highly conserved regions in the N-terminus (E157K, green), the S3 segment (I263T, grey box), the S4 segment constituting the voltage sensor (L290R, red; L293H, orange; R297Q, light grey triangle; L298F, dark grey triangle), the S5 segment (L328V, dark red), the pore region (T374A, light red) and the S6 segment (G398C, blue box; P405L, light grey box). Loss-of-function (LOF) are shown as boxes in blue shades, gain-of-function (GOF) mutations as triangles in green shades, and gain- and loss-of-function mutations (GOF + LOF) as circles in red shades. Already published mutations are shown in grey with the corresponding symbol. (**B**) Mutant amino acid positions and their respective surrounding amino acids. All variants except Q357R show evolutionary conservation.

Materials and methods

Patients

Fifteen new, and eight previously reported patients (Pena Coimbra 2015; Syrbe *et al.*, 2015; Allen *et al.*, 2016) were included in this study. The previously unreported patients were collected through data sharing with Epilepsy and Genetic Centres in Europe, Latin and North America. Clinical data for each patient were collected and categorized by using a common database. The database was stored at the Danish Epilepsy Centre. Wakefulness and sleep EEG data, and MRI scans were obtained for all patients. Seizures and where possible, epilepsy syndromes were classified according to the latest International League Against Epilepsy (ILAE) classification proposal (Berg *et al.*, 2010). The study was approved by the local ethics committees of each participating clinical centre. Written informed consent was obtained by the parents or the legal guardian of each patient following local Institutional Review Board requirements.

Mutation analysis

All *KCNA2* variants were identified by routine genetic diagnostics performed in patients with epileptic encephalopathies either by targeted gene panels or whole exome sequencing, and verified by Sanger sequencing. The identified *KCNA2* variants were assumed to be pathogenic if they were absent in control samples (Exome aggregation consortium, ExAC set of ~61 000 exomes; exac.broadinstitute.org/) and fulfilled one or more of the following criteria: (i) had arisen *de novo*; (ii) found in a patient with a suggestive phenotype with additional functional studies showing a clear functional effect; or (iii) has previously been identified as disease-causing.

Functional studies

All methods have been previously described by Syrbe *et al.* (2015). Experiments were approved by the local Animal Care and Use Committee (Regierungspräsidium Tübingen). The human $K_v1.2$ in the pcDNA3.1 vector was kindly provided by Stephan Grissmer (Institute of Applied Physiology, Ulm University). Site-directed mutagenesis was performed using Quickchange™ (Agilent Technologies; primers are available on request). The mutated clones were fully resequenced. cRNA was prepared using the T7 mMessage kit from Ambion. *Xenopus laevis* oocytes were treated and stored as described. Fifty nanolitres of cRNA encoding wild-type or mutated $K_v1.2$ subunits (1 µg/µl) was injected using Roboocyte2 (Multi Channel Systems). Oocytes were stored for 2 days (at 17°C) prior to the experiment. Amplitudes of currents of wild-type and mutant channels recorded on the same day were normalized to the mean value of $K_v1.2$ wild-type on that day to pool normalized data from different experiments. Potassium currents in oocytes were recorded at room temperature (20–22°C) using two-electrode voltage-clamp with Roboocyte2. Electrode resistances were 0.4–1 MΩ (1 M KCl or 1.5 M KAc). The holding potential was −80 mV. Oocytes were perfused with a ND96 bath solution containing (in mM): 93.5 NaCl, 2 KCl, 1.8 $CaCl_2$, 2 $MgCl_2$, 5 HEPES (pH 7.6). Currents were sampled at 5 kHz. Standard voltage-clamp protocols and analysis methods were used as described in Syrbe *et al.* (2015). All data are reported as mean ± standard error of the mean (SEM). Statistical tests were one-way ANOVA with Bonferroni *t*-test as *post hoc* test (for normally distributed data) or one-way ANOVA on ranks with Dunn's *post hoc* test (for not-normally distributed data). For unpaired data sets, Student's *t*-test (normally distributed data) or Mann-Whitney rank-sum (not-normally distributed data) were used. Normality was tested using the Shapiro-Wilk test. Significance with respect to controls is indicated in the figures as follows: $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

Results

Genetics

We describe a cohort of 23 patients [11 females, 12 males; mean age at the last follow-up: 12.9 years (range: 4 months–37 years)] with a presumed pathogenic *KCNA2* mutation (Tables 1–3). An additional patient was excluded from our study as it was not possible to demonstrate the pathogenicity of his Q357R mutation (see below). Eight patients have been reported previously (Pena and Coimbra, 2015; Syrbe *et al.*, 2015; Allen *et al.*, 2016). Ten mutations were missense: E157K (found in one patient), I263T (one), L290R (one), L293H (one), R297Q (seven), L298F (one), L328V (one), T374A (three), G398C (one), P405L (five), and one (Q213*, S1/S2 loop), found in one patient, was a truncation mutation. The mutations occurred *de novo* in 20 patients; in three patients (Patients 8, 13 and 16) it was not possible to test the parents (because of ovodonation in Patient 13 and parental non-availability in the others). One patient with a *de novo* T374A mutation also had an abnormal karyotype with ring chromosome 21 (p11.1q22.3) (Table 3). All the identified mutations were absent in ExAC and predicted to be damaging by the prediction tools PolyPhen-2 and MutationTaster (Supplementary Table 1). The protein positions of the different *KCNA2* mutations are shown in Fig. 1A. Three recurrent mutations (R297Q, T374A, P405L) account for two-thirds of the pathogenic mutations.

Functional analysis

Ten of 11 detected *KCNA2* mutations were located in highly conserved and functionally important protein regions (Fig. 1A). Only the mutation found in Patient 18 (Q357R) affected a less conserved part of the pore region (Fig. 1B). The others were located in the N-terminus (E157K), the S3 segment (I263T), the voltage-sensor (L290R, L293H, R297Q and L298F), S5 (L328V), the pore region (T374A) or in the S6 segment (G398C and P405L) (Fig. 1A). If not indicated otherwise, the same total amount of cRNA encoding the wild-type $K_v1.2$ channel, mutants or their mixtures were injected and recordings were made in parallel 2 to 3 days after injection.

Table 1 Characteristics of patients with loss-of-function mutations

Patients (Reference)	Patient 1	Patient 2 (Syrbe *et al.*, 2015)	Patient 3	Patient 4 (Syrbe *et al.*, 2015)	Patient 5 (Syrbe *et al.*, 2015)	Patient 6 (Syrbe *et al.*, 2015)	Patient 7	Patient 8
Gender, age, origin	M, 8 y, French	M, 9 y, Turkish	M, 17 y, Irish	F, 9 y, German	F, 6 y, Spanish	M, 21 y, Danish	F, 8 y, Afro-American	F, 9 y, German
Mutation	c.637C > T p.Gln213* *de novo*	c.788T > C p.Ile263Thr *de novo*	c.1192G > T p.Gly398Cys *de novo*	c.1214C > T p.Pro405Leu *de novo*	c.1214C > T p.Pro405Leu *de novo*	c.1214C > T p.Pro405Leu *de novo*	c.1214C > T p.Pro405Leu *de novo*	c.1214C > T p.Pro405Leu
Age at epilepsy onset /seizure type	2 mo, focal seizure eye deviation, behavioural arrest, hypotonia	11 mo Generalized MC	2 mo Left hemiclonic	17 mo FS or afebrile seizures ± vomiting, eye deviation, hemiclonic jerks, postictal paresis	10 mo FS or afebrile seizures with staring, eye deviation, ±vomiting, hemiclonic jerks	9.5 mo Febrile SE (GTCS 45 min)	2 mo Hemiclonic seizure + sGTCS	14 mo Afebrile tonic-clonic seizure
Other seizure types	-	Generalized MC, MA	AS, MC, focal motor seizure, GTCS, SE, atonic	SE, FS, FDS, focal motor seizure ±, sGTCS	FDS, focal motor seizure, possible spasms	FS, afebrile focal motor seizure ± sGTCS; postictal paresis	MC, focal motor seizures, AS and GTCS, SE	AS, focal motor seizure (sometimes with vomiting)
Seizure outcome	Uncontrolled	Seizure free since 4 y	Uncontrolled	Seizure-free since 7.5 y	Seizure-free since 4 y	Seizure free since 16 y	Rare AS	1–3 AS/day
EEG at onset	Multifocal spike-waves and 'migrating' seizure	Sh-W and polySp in T-P-O regions	BG slowing; multifocal Sh-W	Sh-W and polySp in T-P-O regions	N	SW and polySp in T-P-O regions	Multifocal Sh-W	N
EEG in the evolution		Multifocal Sh-W and polySp Accentuation during sleep N since age 5 y	BG slowing, rare multifocal Sp and GSW with F predominance	Multifocal Sh-W > left Fr-C region ESES like (SWI > 80%)	T-P-O SW, C-T bilat polySp ESES like (SWI 80–100%)	C-T bilat polySp ESES like (SWI 70–75%) N since age 17 y	Left Fr-C sharp/ slow waves, multifocal Sh-W, GSW and polySp-wave	Multifocal Sp (bi-O, right C, left T); GSW since age 4 y ESES like (SWI > 70%)
Current AEDs	VPA, TPM, CLB	LTG, CLB	CLB, CBZ, LCM, KD	ACZ	VPA, CLB, TPM	LEV	LCM, LEV	VPA, STM CLB
Development before seizure onset	Poor visual contact	N	N	Mild coordination deficit	N	N	N	N
Neurological features (age of onset of the first symptom)	Psychom.dev. delay (3 mo) Hypotonia, very poor voluntary motricity, poor visual contact, head deviation to the right, impairment of coordination	Psychom.dev. delay (11 mo) Impairment of fine motor skills	Psychom.dev. delay (12 mo) Ataxia, dyskinesia, hypotonia, impairment of fine motor skills	Psychom.dev. delay (17 mo) Tremor, impairment of coordination and of fine motor skills, ataxia, dysarthria, mini-myoclonus	Psychom.dev. delay (10 mo) Language delay, impairment of coordination and of fine motor skills, ataxia, mini-myoclonus	Psychom.dev. delay (9.5 mo) Impairment of coordination and of fine motor skills, ataxia, dysarthria	Psychom.dev. delay (10 mo) Severe impairment of coordination and of fine motor skills, ataxia	Psychom.dev. delay and ataxia (18 mo) Impairment of coordination and of motor skills, tremor
Cognitive function; language	Severe ID, no language	Mild-moderate ID; language delay	Language delay	Mild-moderate ID; language delay	Learning disabilities; language delay	Moderate ID (TIQ 40); severe language delay	Severe ID, no language	Severe ID and language delayed
Behavioural features	Stereotypies	-	ASD and OCD	-	-	Aggressiveness, hyperactivity	Irritability, slight aggressiveness	ASD
Imaging	N	N	N	N	N	right P lacunar infarction	N	N
Additional features	Small feet	-	Osteoporosis	Small body size, GH deficiency; subclinical hypothyroidism	-	Severe scoliosis, pes planus, osteoarthritis, obesity	Fanconi syndrome (VPA), Stevens-Johnson syndrome (PHT), sensorineuronal hearing loss	-

ACZ = acetazolamide; AEDs = anti-epileptic drugs; AS = absence seizures; ASD = autism spectrum disorder; BG = background; C = central; CBZ = carbamazepine; CLB = clobazam; F = female; FDS = focal dyscognitive seizures; Fr = frontal; FS = febrile seizures; GH = growth hormone; GSW = generalized spike and waves; ID = intellectual disability; KD = ketogenic diet; LCM = lacosamide; LEV = levetiracetam; LOF = loss of function; LTG = lamotrigine; M = male; MA = myoclonic-atonic seizures; MC = myoclonic seizures; min = minutes; mo = months; N = normal; O = occipital; OCD = obsessive compulsive disorder; P = parietal; polySp = polyspikes; Psychom.dev. = psychomotor developmental; PTH = phenytoin; Sd = syndrome; SE = status epilepticus; sGTCS = secondary generalized tonic clinic seizures; Sh-W = sharp-waves; Sp = spikes; STM = sulthiame; SW = spike-waves; SWI = spike-wave index; T = temporal; TIQ = Total Intelligence Quotient; TPM = topiramate; VPA = valproic acid; y = years.

Table 2 Characteristics of the patients with gain-of-function mutations

Patients (Reference)	Patient 9	Patient 10 (Syrbe *et al.*, 2015)	Patient 11 (Pena and Coimbra, 2015	Patient 12	Patient 13	Patient 14	Patient 15	Patient 16	Patient 17 (Syrbe *et al.*, 2015)
Gender, age, origin	M ,15 y, European-American	M, 27 y, German	M, 8 y, Latin American	F, 37 y, Danish	F, 5 y, ovodonation	F, 32 mo, French-Spanish	F, 16 y, Hispanic and Caucasian	M 20 y, Arabic	M, 37 y, English
Mutation	c.469G > A	c.890G > A	c.890G > A	c.890G > A	c.890G > A	c.890G > A	c.890G > A	c.890G > A	c.894G > T
	p.Glu157Lys	p.Arg297Gln	p.Arg297Gln	p.Arg297Gln	p.Arg297Gln	p.Arg297Gln	p.Arg297Gln	p.Arg297Gln	p.Leu298Phe
	de novo	*de novo*	*de novo*	*de novo*		*de novo*	*de novo*		*de novo*
Age at epilepsy onset/seizure type	9 mo Febrile GTCS	5 mo Febrile SE	15 mo FS	10 mo FS	15 mo Typical AS	Infantile spasms Since birth	6 mo, GTCS	1 y GTCS	6 mo (febrile) GTCS
Other seizure types	Focal motor seizure, atypical AS	AS, GTCS, often febrile	MC, AS, GTCS	GTCS, MC	GTCS	AS ± myoclonia, GTCS	Atypical AS ± myoclonia	-	Atypical AS, GTCS, MC
Seizure outcome	Seizure free 3–14 y, breakthrough GTC and AS at age 14	1 GTCS/year	Seizure only after physical exertion	MC during sleep	Seizure free since age 18 mo	Daily AS	1–2 GTCS/months preceded by AS	Monthly GTCS	Monthly GTCS
EEG at onset	Rare left Fr Sh-W	NA	Slow background activity	NA	3 Hz SW	N	BG slowing, right O Sh-W	NA	NA
EEG in the evolution	BG slowing, GSW, right C Sp, bilat C ShW; sleep activation	GSW and polySp-W	Irregular GSW; sleep activation	GSW, theta-beta activity + Sp in the midline	GSW, bi O and right T SW and Sh-W	BG slowing, GSW, posterior SW	Disorganized BG; irregular 2H GSW	BG slowing, GSW, Multifocal EDs	BG slowing, GSW
Current AEDs	ACZ, VPA, LTG	LTG, ZNS	LEV	VPA, LTG, LEV	VPA	LTG, ESM	LTG, CLB, KD, VNS, ACZ	VPA, LTG	LEV, VPA, CBZ, ESM
Development before seizure onset	N	N	N	N	Delayed	Delayed	N	Delayed	N
Neurological features (age of onset of the first symptom)	Psychom.dev. delay (4 y) Hypotonia, mild ataxia, tremor, impaired fine motor skills, asterixis dysarthria	Psychom.dev. delay (2 y) Impaired fine motor skills, ataxia, dysdiadochokinesia, dys arthria, mild pyramidal signs	Psychom.dev. delay (17 mo) Tremor, impairment of fine and gross motor skills, mild ataxia and hypotonia	Psychom.dev. delay (13 mo) Tremor, impaired coordination of fine motor skills, ataxia,dysarthria, hand myoclonia, pyramidal signs	Psychom.dev. delay (15 mo) Impaired coordination of fine motor skills, ataxia, hypotonia, pyramidal signs	Psychom.dev. delay (since birth) Ataxia, finger tremor, impaired coordination	Psychom. dev. delay (8–9 mo) Tremor, ataxia, head titubation, axial hypotonia, pyramidal signs, impaired motor coordination	Psychom.dev. delay (1 y) Impaired incoordination, mild dysdiadochokinesia, mild-moderate ataxia, dysarthria	Psychom.dev. delay (6 mo) Severe ataxia, inability to walk unassisted
Cognitive status/ language	Moderate ID	Moderate ID (TIQ 42)	Moderate ID	Moderate ID, limited language	Moderate ID (TIQ 47), language delay	Moderate-severe ID and language delay	Severe ID, severely limited language	Learning difficulties	Severe ID, no language
Behavioural features	Moderate-severe behavioural problems and perseverations	Behavioural problems, stubbornness	Hyperactivity, stubbornness	Aggressiveness, stubbornness	Stubbornness, difficulty of concentration	-	ASD	-	-
Imaging	N	Severe cerebellar atrophy	N	Severe cerebellar atrophy, small hippocampi	N	N	Hyperintense subcortical white matter lesions,	Cerebellar atrophy	Severe cerebellar atrophy
Additional features	-	Mild facial dysmorphisms	Frequent respiratory infections	Knee valgus, thoracic kyphosis	Height and weight 90th percentile	-	Small nose and mouth, hepatic lesion of unknown origin	Microcephaly, 3rd centile for weight, cubitus valgus, scoliosis	Facial dimorphisms (wide forehead, bulbous nasal tip, deep-set eyes, synophris, full lips)

ACZ = acetazolamide; AEDs = anti-epileptic drugs; AS = absence seizures; ASD = autism spectrum disorder; BDZ = benzodiazepine; BG = background; bilat = bilateral; C = central; CBZ = carbamazepine; CLB = clobazam; CZP = clonazepam; EDs = epileptiform discharges; ESM = ethosuximide; F = female; Fr = frontal; FS = febrile seizures; GSW = generalized spike and waves; ID = intellectual disability; KD = ketogenic diet; LEV = levetiracetam; LTG = lamotrigine; M = male; MC = myoclonic seizures; mo = months; N = normal; NA = not available; O = occipital; OIRDA = occipital intermittent rhythmic delta activity; polySp = polyspikes; polySp-W = polyspike-waves; Psychom.dev. delay = psychomotor developmental delay; RFN = rufinamide; SE = status epilepticus; Sh-W = sharp-waves; Sp = spikes; SW = spike-waves; seizure = seizures; T = temporal; TIQ = Total Intelligence Quotient; VNS = vagus nerve stimulation; VPA = valproic acid; y = years; ZNS = zonisamide.

Table 3 Characteristics of patients with gain- and loss-of-function mutations

Patients (Reference)	Patient 19 (Allen *et al.*, 2016)	Patient 20	Patient 21	Patient 22	Patient 23	Patient 24
Gender, age, origin	F, 8 y, Irish	F, 28 mo, Israelian (Ashkenazy)	M, 12 y, German	F, 16 y, American	M, 5 y, American	M,4 mo, Caucasian
Mutation	c.869T > G p.Leu290Arg *de novo*	c.878T > A p.Leu293His *de novo*	c.982T > G; p.Leu328Val *de novo*	c.1120 A > G p.Thr374Ala, ring (21) p11.1q22.3 *de novo*	c.1120 A > G p.Thr374Ala *de novo*	c.1120 A > G p.Thr374Ala *de novo*
Age/seizure type at onset	7 wks Non-specific events	1 mo, GTCS	6 mo, Febrile SE	4 mo, IS, MC	Since birth, Tonic seizure	Since birth MC
Other seizure type	AS and atypical AS, GTCS	GTCS, MC	AS, atonic seizure, GTCS	Focal seizure, sometimes in clusters	Focal seizure	Focal seizure
Seizure outcome	Sporadic GTCS	Weekly GTCS, sporadic AS	Daily AS	Daily/weekly seizures incompletely characterized	Sporadic focal MC	Seizure response to TPM (follow-up very short)
EEG at onset	Right T-P Sp and polySp	Fr rhythmic abnormalities	NA	Diffuse 8–10 Hz activity. Polymorphic 2–3 Hz discharges during sleep	N	N
Course of EEG	BG slowing, GSW, focal Sh-W	BG slowing; sporadic multifocal SW	GSW and multifocal SW, sleep activation (SWI < 50%)	BG slowing; bi-occipital Sp and ShW	Multifocal Sh-W	Left focal/multifocal Sp
Current AEDs	STM, LTG, ESM, ACZ, CLB	ZNS, CZP, ACZ	LCM, LTG, KBR	CBD oil	-	TPM
Development before seizure onset	N	N	N	Delayed from birth	Delayed from birth	Delayed from birth
Neurological features (age of onset of the first symptom)	Ataxia and tremor (18 mo) Dysarthria	Severe psychom.dev. delay (1 mo) Hypotonia, ataxia, head titubation, tremor, hyperlaxity, chorea	Psychom.dev. delay (1 y) Moderate ataxia	Severe psychom.dev. delay (since birth) Progressive spastic quadriplegia	Severe psychom.dev. delay (since birth) Spastic tetraplegia, myoclonia, nystagmus, dystonia, choreoathetosis	Severe psychom. dev. delay (since birth) Hypotonia, no eye contact
Cognitive status	Mild ID (TIQ = 65). Learning support at school Language 6 years level	Moderate-severe ID, no language	Severe ID; language delayed	Profound ID; no language	Profound ID; no language	Profound ID
Behavioural features	ADHD	Hyperactivity	Hyperactivity	-	-	Not assessable due to young age
Imaging	N	N	Mild cerebellar atrophy	Cerebellar and cerebral atrophy	Cerebellar atrophy	N
Additional features	Hypermetropia	Mildly dysmorphic (slightly beaked nose and round forehead) Microcephaly	-	Bilateral optic atrophy, severe scoliosis, mitral valve prolapse and regurgitation, dilated aortic root, microcephaly	Bilateral optic atrophy, fair skinned, brachycephalic, occipital plagiocephaly, mild lumbar scoliosis, GERD	-

ACZ = acetazolamide; ADHD = attention deficit and hyperactivity disorder; AEDs = anti-epileptic drugs; AS = absence seizures; BG = background; CBD oil = cannabidiol oil; CLB; clobazam; CZP = clonazepam; ESM = ethosuximide; F = female; Fr = frontal; GERD = gastroesophageal reflux disease; GSW = generalized spike and waves; ID = intellectual disability; IS = infantile spasms; LCM = lacosamide; LTG; lamotrigine; M = male; MC = myoclonic seizures; mo = months; N = normal; NA = not available; P = parietal; polySp = polyspikes; Psychom.dev. delay = Psychomotor developmental delay; Pt = patient; SE = status epilepticus; Sh-W = sharp-waves; Sp = spikes; STM = sulthiame; SW = spike-waves; SWI = spike-wave index; seizure = seizures; T = temporal; TIQ = Total Intelligence Quotient; TPM = topiramate; wks = weeks; y = years; ZNS = zonisamide.

Mutations causing loss-of-function effects

We have previously shown that the I263T and P405L mutations are associated with a less severe phenotype and cause a loss-of-function (Syrbe *et al.*, 2015). The mutation Q213* identified in Patient 1 is predicted to either lead to nonsense-mediated mRNA decay or to truncate the channel early in the transmembrane region (S1/S2 loop), long before important phosphorylation sites in the C-terminus controlling $K_v1.2$ trafficking (Yang *et al.*, 2007, see also the 'Discussion' section). Therefore, it was assumed that it results in a loss-of-function. G398C, which was newly identified in Patient 3, was also predicted to have a loss-of-function effect as previously reported for $K_v1.1$ channels (Yifrach and MacKinnon, 2002; Upadhyay *et al.*, 2009). When we expressed G398C mutant $K_v1.2$ channels in *X. laevis* oocytes, the recorded K^+ currents were not significantly larger than background level, similar to those reported previously for I263T and P405L mutant channels (Syrbe *et al.*, 2015). However, in contrast to I263T and P405L, we did not detect a dominant-negative effect of G398C on wild-type channels in co-expression experiments (Fig. 2A and B).

Mutations causing gain-of-function effects

For the R297Q mutation, affecting the second arginine of the voltage sensor, and the neighbouring L298F, we have shown recently a dominant gain-of-function effect with up to 13-fold increased current amplitudes and a shift of steady-state activation by −40 to −50 mV compared with wild-type channels (Syrbe *et al.*, 2015). Here, we also identified the E157K mutation as gain-of-function: this mutation, located in the N-terminus of the channel, caused a dominant gain-of-function with a 5-fold increase in current amplitudes (Fig. 2A and D), a hyperpolarized resting membrane potential (Fig. 2C), and a less pronounced shift of steady-state activation by −12 mV (Fig. 2E).

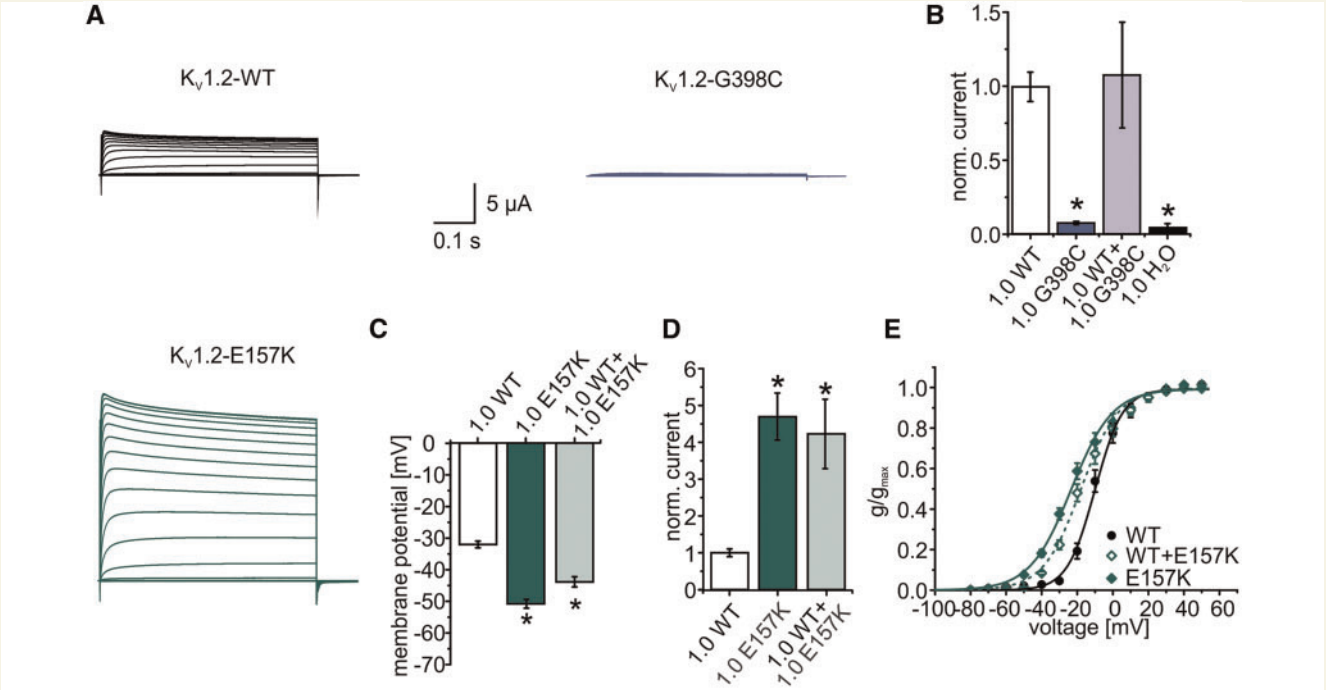

Figure 2 Functional effects of the loss-of-function-*KCNA2* mutation encoding G398C and the gain-of-function *KCNA2* mutation encoding E157K. (**A**) Representative current traces of $K_v1.2$ wild-type (WT, *left*), $K_v1.2$ G398C (*right*) and $K_v1.2$ E157K (*bottom*) channels recorded in a *X. laevis* oocyte during voltage steps (from −80 mV to +70 mV). (**B**) Mean potassium current amplitudes were significantly reduced for G398C mutants in comparison to the wild-type channel (WT, $n = 6$; G398C, $n = 6$). Current amplitudes of mutant channels were similar to those recorded in oocytes injected with water ($n = 5$). Co-expression of G398C and wild-type channel did not show any effect of the mutation on the wild-type (in a 1:1 ratio of cRNA amount injected into the oocytes; $n = 6$). Shown are means ± SEM. Statistically significant differences between wild-type channels and the tested groups were verified by ANOVA on ranks ($P < 0.001$) with *post hoc* Dunn's method (*$P < 0.05$). (**C**) Resting membrane potentials of oocytes injected with wild-type (1.0, $n = 15$), E157K (1.0; $n = 20$) or wild-type + E157K (1:1, $n = 14$). Shown are means ± SEM. Statistically significant differences between wild-type channels and the tested groups were verified by one-way ANOVA on ranks ($P < 0.001$) with *post hoc* Bonferroni *t*-test (*$P < 0.05$). (**D**) Mean current amplitudes of oocytes injected with wild-type (1.0, $n = 15$), E157K (1.0, $n = 20$) and wild-type + E157K (1.0:1.0, $n = 27$). Shown are means ± SEM. There was a statistically significant difference between wild-type channels and the tested groups [one-way ANOVA, ($P < 0.001$) with *post hoc* Bonferroni *t*-test (*$P < 0.05$)]. (**E**) Mean voltage dependence of $K_v1.2$ channel activation for E157K channels together with the activation curves for wild-type and co-expressed channels (1:1 ratio). Shown are means ± SEM. Lines represent Boltzmann functions fit to data points. The activation curves were significantly shifted to more hyperpolarized potentials for the mutation ($P < 0.05$).

In contrast to the gain-of-function effect of mutations located in highly conserved regions of the channel, the Q357R variant, located in a less conserved part of the pore region and for which a *de novo* status remained unclear, did not show any detectable functional changes (Supplementary Table 1). Due to the drastic changes of all other mutations, we rather consider this variant as a benign polymorphism that is not responsible for the clinical phenotype of the patient, although the phenotype fits well with a gain-of-function *KCNA2* mutation and we cannot exclude that we missed an alteration with our experimental system. Patient 18, carrying the Q357R variant, is not included in our analysis; however, his phenotype is described in the Supplementary material.

Mutations causing gain- and loss-of-function effects

We also found hyperpolarizing shifts of the activation curves for L290R and L293H, located in S4 (Fig. 1A and 3D), which predict a gain-of-function effect with permanently open mutant channels under physiological conditions. In contrast to E157K, R297Q and L298F, however, inactivation curves were also shifted to more hyperpolarized potentials predicting a loss-of-function effect with less steady-state availability for larger depolarizations. These shifts were less pronounced for L290R than for L293H, but there was a markedly decreased steepness of both activation and inactivation curves for L290R channels suggesting an enhancement of the gain-of-function effect on activation and a reduction of the loss-of-function effect on inactivation in the physiologically most relevant voltage range near the resting membrane potential (Fig. 3D, E and Supplementary Table 1). In addition, L290R mutant channels yielded significantly larger current amplitudes (Fig. 3A and C), another gain-of-function effect. Although the negative shifts of steady-state inactivation diminish the gain-of-function effect on activation and amplitude, resting membrane potentials were significantly more negative in oocytes injected with mutant compared to wild-type cRNA (Fig. 3B). This indicates a net and dominant gain-of-function effect at resting conditions for those two mutations.

Furthermore, we found mutations in S5 or the pore region of the channel that were predicted to have a gain-of-function effect from functional studies of $K_v1.1$, a highly conserved channel from the same family (L328V; Upadhyay *et al.*, 2009), and its *Drosophila* homologue *shaker* (T374A; Heginbotham *et al.*, 1994; Yool and Schwarz, 1995; Zheng and Sigworth, 1997). L328V (located in S5) caused a −20 mV shift of steady-state activation and more negative resting potentials in injected oocytes compared to the wild-type (Fig. 3F, G, I and Supplementary Table 1). However, current amplitudes were decreased in contrast to the S4 mutations, even exerting a slight dominant-negative effect on the wild-type, which should reduce the gain-of-function (Fig. 3H and Supplementary Table 1). The mutation T374A, which was found in three patients (Patients 22, 23 and 24) with the most severe phenotype (see phenotypic descriptions below), caused a more prominent combination of both gain- and loss-of-function effects. This mutation was located in a highly conserved part of the pore region, which has been shown to be essential for K^+ selectivity (Heginbotham *et al.*, 1994). It caused a gain-of-function effect, by a similar −20 mV shift of the activation curve as L328V; however, the resting potential of injected oocytes was much less negative than for L328V and there was a more prominent decrease in current amplitude with a dominant-negative effect (Fig. 3G, H and Supplementary Table 1).

Therefore, the functional analysis showed that in the group of gain-of-function mutations, some had prominent gain-of-function effects only (E157K, R297Q, L298F) whereas others displayed a combination of both gain- and loss-of-function effects (L290R, L293H, L328V, T374A).

Phenotypic characterization and genotype–phenotype associations

Our previous data indicate that the phenotypes associated with *KCNA2* mutations may be differentiated into two main groups, based on the severity of the encephalopathy and of the seizure disorder, with the milder phenotype correlating with loss-of-function mutations and more severe phenotypes with gain-of-function mutations (Syrbe *et al.*, 2015). To further explore this initial impression, we illustrate the phenotypic features of our patients with loss-of-function *KCNA2* mutations separately from the patients with gain-of-function *KCNA2* mutations. Since some of the gain-of-function mutations also showed some additional loss-of-function effects, we further subgroup those patients carrying mutations with similar electrophysiological properties.

Phenotypic features of patients with loss-of-function *KCNA2* encephalopathy

Eight patients presented with loss-of-function *KCNA2* mutations (Table 1). The mean age at seizure onset was 8.4 months (range: 2–17 months), with prior cognitive and motor development reported as normal in all patients. At onset, febrile seizures were reported in three of eight patients, one of them (Patient 6) presenting with prolonged convulsive febrile status epilepticus. Seizure semiology at onset was consistent with focal seizures in six patients, four of them presenting with hemiclonic seizures, which in two subjects (Patients 4 and 5) were preceded by eye-deviation and vomiting. Eye deviation as an ictal feature was reported also in Patient 1 (Q213* mutation). With increasing age, six of eight of them developed focal dyscognitive seizures and focal motor seizures with possible secondarily generalized tonic-clonic seizures (GTCS); in three of them (Patients 3, 7 and 8) generalized seizures were also

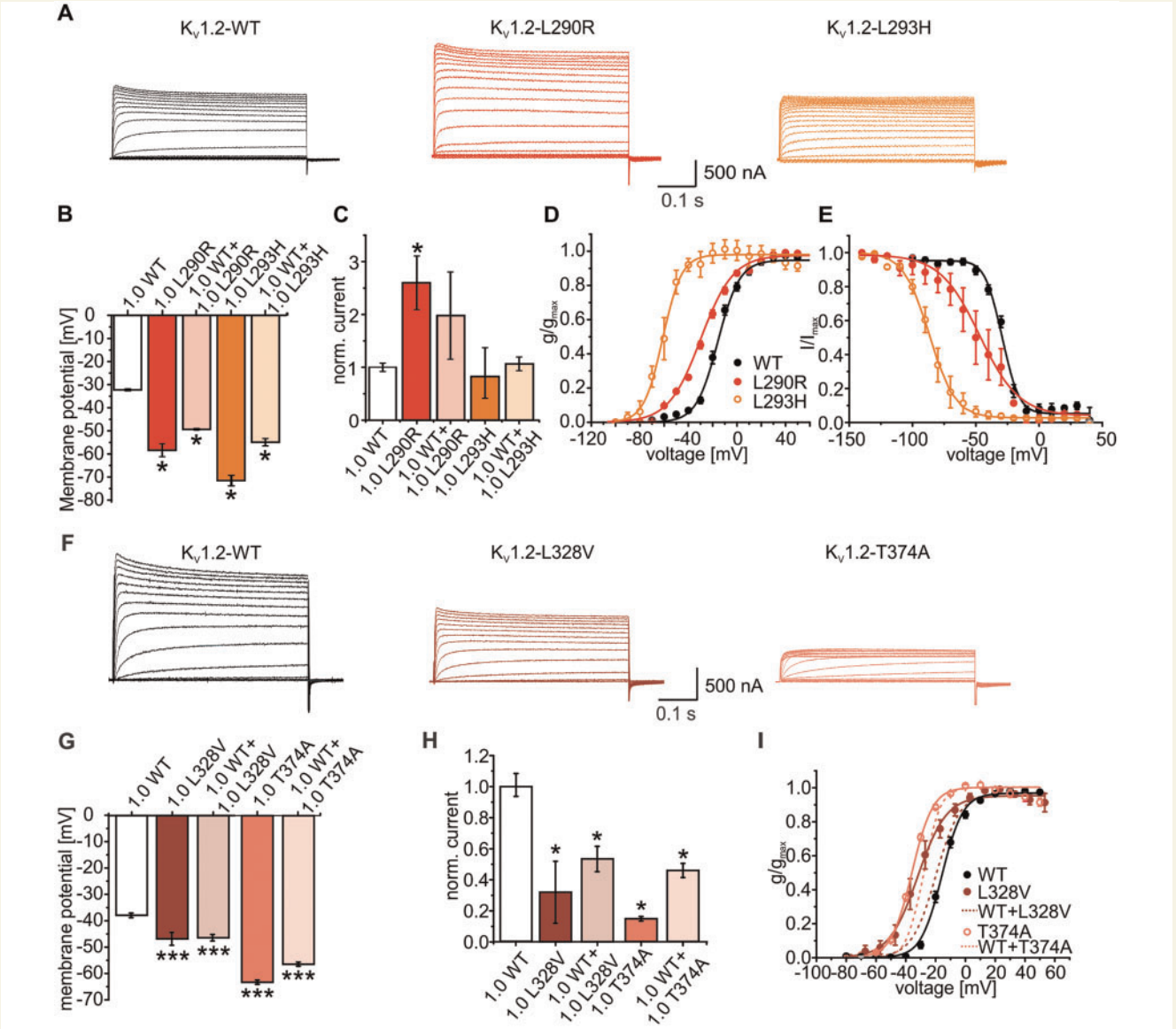

Figure 3 *KCNA2* mutations can cause gain- and loss-of-function. (**A**) Representative current traces of $K_v1.2$ wild-type (WT) (*left*), $K_v1.2$ L290R (*middle*) and $K_v1.2$ L293H (*right*) channels recorded in a *X. laevis* oocyte during voltage steps (from −100 mV to +70 mV). (**B**) Resting membrane potentials of oocytes injected with wild-type (1.0, $n = 33$), L290R (1.0, $n = 8$), wild-type + L290R (0.5:0.5, $n = 6$), L293H (1.0, $n = 6$) or wild-type + L293H (1:1, $n = 21$). Shown are means ± SEM. Statistically significant differences between wild-type channels and the tested groups were verified by one-way ANOVA on ranks ($P < 0.001$) with *post hoc* Bonferroni *t*-test (*$P < 0.05$). (**C**) Mean current amplitudes of oocytes injected with wild-type (1.0, $n = 33$), L290R (1.0, $n = 8$), wild-type + L290R (1.0:1.0, $n = 6$), L293H (1.0, $n = 6$) or wild-type + L293H (1.0:1.0, $n = 21$). (**D**) Mean voltage dependence of $K_v1.2$ channel activation for wild-type, L290R and L293H channels. Shown are means ± SEM. Lines represent Boltzmann functions fit to data points. The activation curves were significantly shifted to more hyperpolarized potentials for all mutations ($P < 0.05$). (**E**) Mean voltage dependence of $K_v1.2$ channel inactivation for wild-type, L290R and L293H mutants. Shown are means ± SEM fitted to a standard Boltzmann function. Inactivation curves of L290R and L293H channels are significantly shifted to more hyperpolarized potentials in comparison to the wild-type. Statistically significant differences between wild-type channels and the tested groups for **C** to **E** were verified by ANOVA on ranks ($P < 0.001$) with *post hoc* Dunn's method ($P < 0.05$). Shown are means ± SEM. (**F**) Representative current traces of $K_v1.2$ wild-type (WT) (*left*), $K_v1.2$ L328V (*middle*) and $K_v1.2$ T374A (*right*) channels recorded as described in Fig. 2A. (**G**) Resting membrane potentials of oocytes injected with wild-type (1.0, $n = 54$), L328V (1.0, $n = 12$), wild-type + L328V (1:1, $n = 20$), T374A (1.0, $n = 19$) or wild-type + T374A (1:1, $n = 23$). Shown are means ± SEM. Statistically significant differences between wild-type channels and the tested groups were verified by one-way ANOVA ($P < 0.001$) with *post hoc* Bonferroni *t*-test (*$P < 0.05$). (**H**) Mean current amplitudes of oocytes injected with wild-type (1.0, $n = 54$), L328V (1.0, $n = 12$), wild-type + L328V (1:1, $n = 20$), T374A (1.0, $n = 19$) or wild-type + T374A (1:1, $n = 23$). Shown are means ± SEM. (**I**) Mean voltage dependence of $K_v1.2$ channel activation for L328V (filled symbols) and T374A (open symbols) channels together with the activation curves for wild-type (black) and co-expressed channels (1:1 ratio, indicated as dotted lines) for each of the mutations. Shown are means ± SEM. Lines represent Boltzmann functions fit to data points. The activation curves were significantly shifted to more hyperpolarized potentials for all mutations ($P < 0.05$). Statistical significant differences between wild-type channels and the tested groups of **H** and **I** were tested using ANOVA on ranks ($P < 0.001$) with *post hoc* Dunn's method ($P < 0.05$).

observed. Only Patient 2 (carrying the I263T mutation) presented with generalized seizure types, including myoclonic seizures at onset and myoclonic-atonic seizures later on. Post-ictal hemiparesis was reported in two patients (Patients 4 and 6), both featuring hemiclonic or focal motor seizures with or without secondarily GTCS. The course of epilepsy was relatively favourable in most of the patients compared to those with gain-of-function or gain- and loss-of-function mutations. Four of eight patients became seizure-free (mean follow-up 4 years; range 1.5–6 years), and one patient (Patient 7) continued to have rare absences. Three patients continued to have daily atypical absences (Patient 8), multiple seizure types (Patient 3) or uncontrolled focal seizures (Patient 1). All patients are still on anti-epileptic medications, two of them on monotherapy.

Epilepsy onset was accompanied or followed by a delay or a stagnation of psychomotor development in all patients. The neurological picture worsened over time, mainly because of the appearance of impairment of fine motor skills (seven patients, $n = 7$), ataxia ($n = 6$), poor coordination ($n = 6$), or dysarthria ($n = 2$). Additional motor symptoms were fine continuous finger myoclonia ('polyminimyoclonus') ($n = 2$), hand tremor ($n = 2$), or dyskinesia ($n = 1$). All patients were cognitively impaired with mild-to-moderate intellectual disability in five patients, three of them (Patients 2, 4 and 6) presenting also with language problems. Patients 1, 7 and 8 had severe intellectual disability with delayed or absent language acquisition. Behavioural disturbances including aggressiveness, irritability or hyperactivity were reported in two patients (Patients 6 and 7). Autism spectrum disorders were diagnosed in Patients 3 (associated with obsessive compulsive disorder) and 8; Patient 1 was reported to have stereotypies. Variable additional symptoms such as endocrinological dysfunction (growth-hormone deficiency and subclinical hypothyroidism) ($n = 1$), scoliosis ($n = 1$), pes planus and osteoarthritis ($n = 1$), osteoporosis ($n = 1$), or sensori-neural hearing loss ($n = 1$) were observed. Overall the clinical pictures of Patients 2 and 4–6 (three carrying the P405L and one the I263T mutation) were relatively mild and homogeneous with similar age of onset, benign course of epilepsy, mild-to-moderate intellectual disability and neurological compromise, whereas Patients 1 (Q213*), 3 (G398C), and 7 and 8 (both with P405L) differed from the other patients due to more severe intellectual disability with behavioural disturbances, more prominent motor/coordination dysfunction, and incompletely controlled epilepsy.

EEG at onset in five patients (Patients 2, 4–6 and 8) showed a peculiar pattern characterized by focal, mainly central or posterior-temporo-occipital sharp-slow waves and clusters of polyspikes, that tended to spread to fronto-prefrontal regions (Fig. 4). Multifocal sharp waves combined with generalized paroxysms were observed in two patients (Patients 3 and 7), both presenting with focal and generalized seizures; Patient 1 had multifocal spike-waves. Dramatic activation of EEG abnormalities during sleep (up to 100% of non-REM sleep), featuring diffuse epileptic discharges with posterior predominance, was reported in four patients (Patients 4, 5, 6 and 8), associated with worsening of the cognitive status and deterioration of language in Patient 6, reminiscent of the syndrome of encephalopathy with status epilepticus during slow sleep (ESES) (Tassinari *et al.*, 2012) (Supplementary Fig. 1). In this latter patient, normalization of the EEG at the age of 17 years was associated with an improvement of language, further supporting a diagnosis of ESES during the active phase of the sleep EEG. In the other three patients (Patients 4, 5 and 8) the difficulties to ascertain a deterioration of the pre-existent cognitive status during the activation of epileptiform activity in sleep EEG, and the lack of a longitudinal neuropsychological evaluation did not allow us to conclude that these patients suffered from ESES. As a whole, in all subjects the EEG abnormalities were more abundant in the infantile and childhood age. MRI was unremarkable in all patients.

Phenotypic features of patients with gain-of-function *KCNA2* encephalopathy

Fifteen patients presented with gain-of-function mutations, including a subgroup showing gain- and loss-of-function effects. We here present the phenotypes of patients with mutations with gain-of-function effects only separately from those with gain- and loss-of-function effects. Patient 18 carrying the Q357R mutation without a functional effect is not included in this analysis and his phenotype is presented in the Supplementary material.

Phenotypic features of patients carrying mutations with gain-of-function effects only

Nine patients carried such gain-of-function mutations (Table 2). The mean age of seizure onset was 8.7 months (range: from 5 to 15 months, except Patient 14 starting at birth with episodes of extension or flexion of the limbs and head interpreted as infantile spasms). Epilepsy onset was characterized by febrile convulsive seizures or febrile status epilepticus in five of nine patients. The remaining four patients presented at onset with absences or afebrile GTCS, and Patient 14 with infantile spasms. During development, all patients presented with generalized seizure types, such as typical or atypical absences, myoclonic seizures, and GTCS. Epileptic seizures were not controlled in eight of nine patients; seizure frequency varied from daily absences or weekly GTCS to monthly or even more sporadic seizures (once per year in Patient 10). One patient (Patient 13) became seizure-free (the follow-up was at 3.5 years). The majority of patients were on polytherapy, whereas only two were on monotherapy; in none was medication stopped.

All patients had a developmental delay during the course of the disease, including patients with primary developmental delay and patients with developmental plateauing

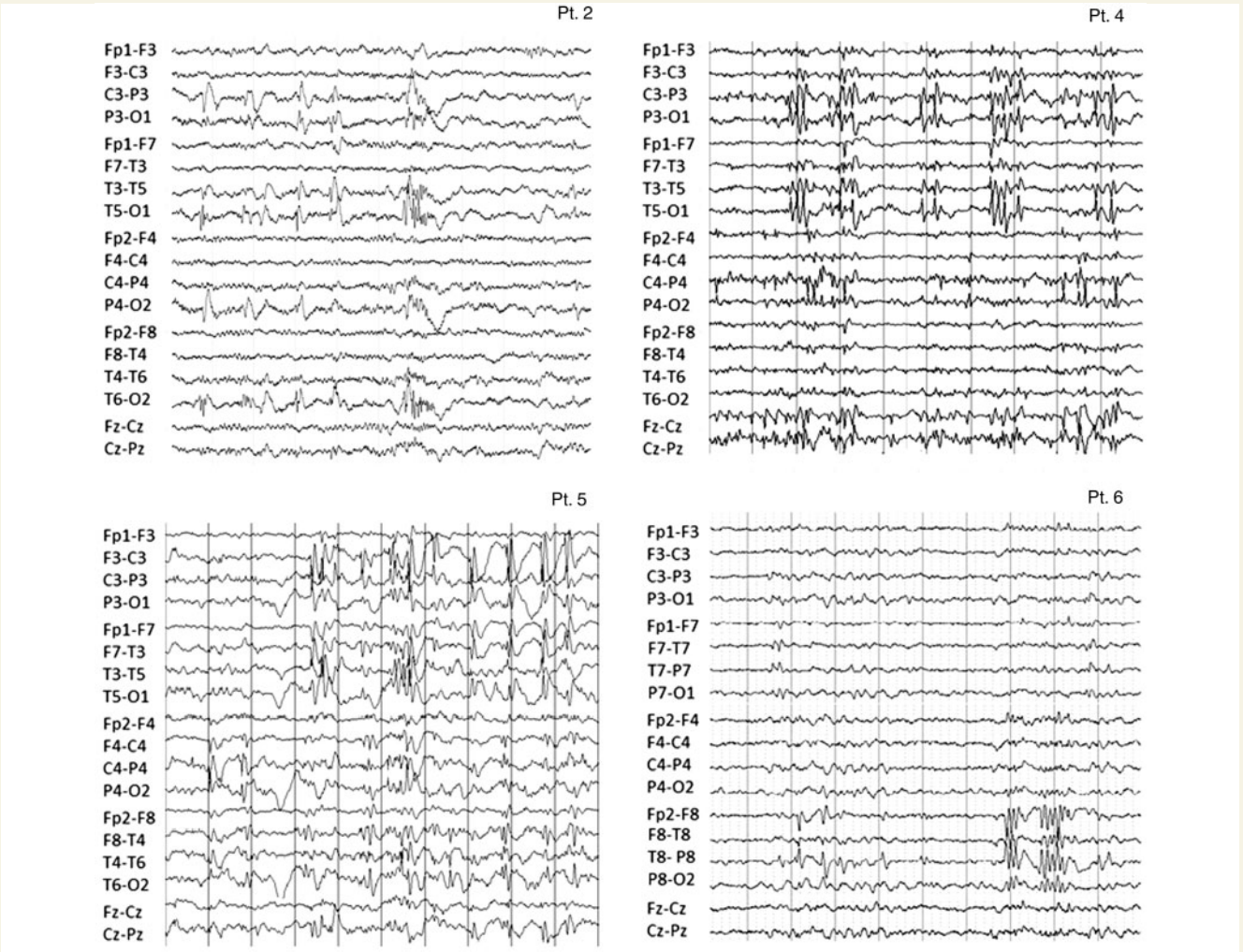

Figure 4 EEG features of four patients with *KCNA2* loss-of-function mutations (Patients 4–6 carry a P405L mutation). The interictal EEG showed a pattern of recurrent sharp and slow waves or spike and waves alternated with short bursts of polyspikes at ~8–10 Hz (in Patient 1 was ~18–20 Hz), lasting ~200 ms–1 s, over both temporo-parieto-occipital regions, synchronous or asynchronous in both hemispheres.

following an initial unremarkable development. Additional neurological features developed over time including ataxia ($n = 9$ patients), impairment of fine or gross motor skills ($n = 5$), tremor ($n = 5$), dysarthria ($n = 4$), hypotonia ($n = 4$), pyramidal signs ($n = 4$), dysdiadochokinesis ($n = 2$), or myoclonus ($n = 1$). The severity of ataxia ranged from mild-moderate (Patients 9, 11 and 16) to severe with inability to walk unassisted (Patient 17). Pyramidal signs were usually mild, such as a positive Babinsky sign or a modest impairment of fine motor skills. All patients had cognitive impairment ranging from moderate intellectual disability with delayed language acquisition (Patients 9–13 and 16) to severe intellectual disability (Patients 14 and 15) without language acquisition (Patient 17). Behavioural features such as hyperactivity, stubbornness, and aggressiveness were reported in five patients (Patients 9–13); autistic spectrum disorder was reported only in one patient (Patient 15).

Craniofacial dysmorphisms including a wide forehead, deep-set eyes with synophris, a bulbous nasal tip or beaked nose, or microcephaly were observed in two patients (Patients 16 and 17), kyphosis and genu valgum were reported in Patient 12. Patient 16 presented with scoliosis.

The main EEG features in all nine (100%) patients were background slowing with generalized spike-polyspike waves or generalized sharp and slow-waves. In the older patients, the bursts of generalized epileptiform discharges were less frequent and tended to be more prominent with highest amplitude over the midline (Fig. 5). Four patients (Patients 9, 12, 13 and 16) showed additional focal or multifocal epileptiform discharges.

MRI in adult patients showed mild-to-severe cerebellar atrophy ($n = 4$ patients) (Supplementary Fig. 2). Unremarkable MRIs were reported in childhood suggesting that cerebellar atrophy might appear later in the course of the disease.

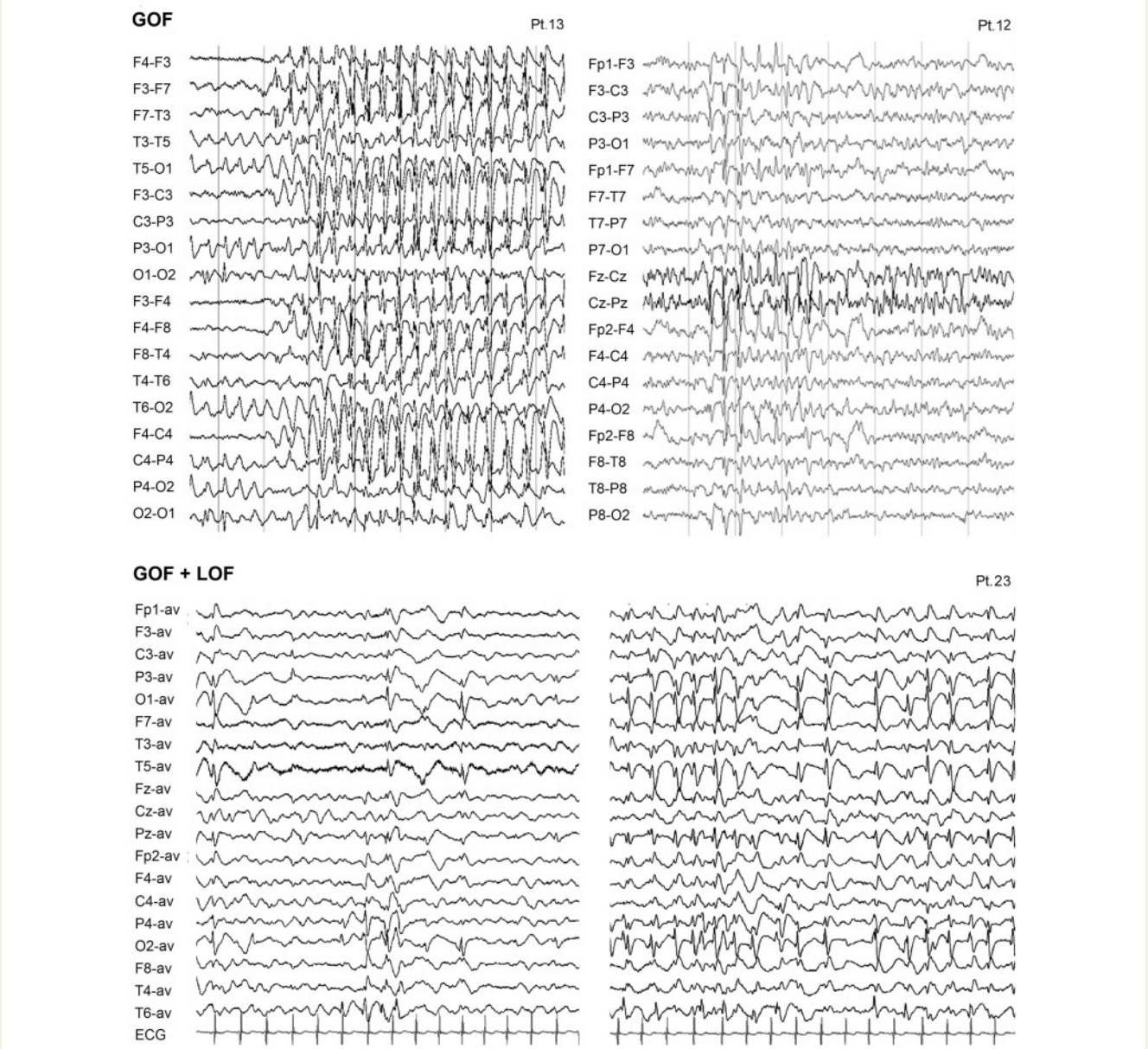

Figure 5 EEG features in patients with *KCNA2* gain-of-function, and *KCNA2* gain- and loss-of-function mutations. *Top row*: Interictal EEG of two patients carrying the same *KCNA2* gain-of-function mutation (R297Q) at different ages. In Patient 13 (5 years old), the EEG features were background slowing, delta activity and spikes and slow waves bilaterally in the occipital regions and bursts of generalized spike/polyspike-and-slow wave complexes. In Patient 12 (37 years old), the EEG shows subcontinuous theta and beta activity in the midline, intermixed with 3–5 Hz positive spikes, with accentuation and bilateral spreading during drowsiness and sleep. *Bottom row*: Interictal EEG features in a 5-year-old patient (Patient 23), carrying a *KCNA2* gain- and loss-of-function mutation (T374A). During wakefulness (*left*), the EEG shows multifocal abnormalities with predominance over both temporo-parieto-occipital regions, with striking accentuation during sleep (*right*).

Phenotypic features of patients carrying mutations with gain-of-function and loss-of-function effects

Six patients presented with mutations showing both gain- and loss-of-function effects (Table 3). The mean age at seizure onset was 2.1 months (range: from birth to 6 months). Fever sensitivity was reported only in one patient (Patient 21). Generalized seizures such as myoclonic seizures, tonic seizures or GTCS were reported at the onset and throughout the evolution in three of six patients, whereas the remaining three presented with focal seizures. Patient 22 presented also with episodes of extension or flexion of the limbs and head, and eye deviation that were diagnosed as infantile spasms. Seizures persisted with variable frequency ranging from sporadic or weekly GTCS to daily absences. The majority of patients were on polytherapy. None of the patients achieved seizure freedom. A prolonged period of seizure freedom was seen in a 4-month-old child (Patient 24) after the introduction of topiramate; however,

the follow-up period of 5 months was too short to establish whether he achieved long-standing seizure control. Developmental delay was reported in all patients, preceding the seizure onset in three of six patients. At the time of the last follow-up, five of six patients had severe or profound intellectual disability; three of them were non-verbal (Patients 20, 22 and 23). Additional neurological features included ataxia ($n = 3$), hypotonia ($n = 2$), tremor ($n = 2$), dysarthria ($n = 1$). Two patients (Patients 22 and 23) with a T374A pathogenic variant showed a more severe phenotype with profound intellectual disability, lack of language acquisition, spastic tetraplegia, optic atrophy, and severe scoliosis. A third patient (Patient 24) with this mutation was too young at the last follow-up (5 months old) to fully assess disease severity; however, he presented with neonatal epilepsy, primary severe developmental delay, hypotonia, choreoathetosis, myoclonus and lack of fixation. Behavioural features such as attention deficit hyperactivity disorder and hyperactivity were reported in three patients (Patients 19, 20 and 21). One of the three patients carrying the T374A mutation (Patient 22) was also found to have ring chromosome 21. However, considering the striking similarity of his phenotype with the other two patients carrying the same mutation, we concluded that the clinical picture was likely contributed primarily by the *KCNA2* mutation.

Craniofacial dysmorphisms such as microcephaly, or brachycephaly with occipital plagiocephaly were observed in Patients 20, 22 and 23. Scoliosis was reported in Patients 22 and 23. In all patients, EEG showed focal or multifocal spikes or sharp waves. Discharges were more frequent in the posterior regions (Fig. 5), associated with generalized spike-waves in two of six patients. Brain MRI showed cerebellar atrophy in three patients (Patients 21–23; in Patient 22 associated also with cerebral atrophy) (Supplementary Fig. 2) at a younger age as compared with patients carrying mutations with gain-of-function effects only.

In summary, the subgroup of patients carrying mutations with gain- and loss-of-function effects presents distinctive features compared to the subgroup with gain-of-function effects only, such as: (i) an earlier age of seizure onset, rarely triggered by fever; (ii) propensity to present either with focal or with generalized seizures; (iii) a higher incidence of focal EEG epileptic discharges; (iv) more severe neurological and more pronounced intellectual disability; and (v) MRI evidence of cerebellar atrophy at an earlier age.

Discussion

Our study including a large number of new patients and novel mutations has three major results for *KCNA2*-related encephalopathy. First, it detects a new class of mutations not exhibiting either a gain- or loss-of-function effect on channel function, but a combined gain- and loss-of-function. Second, it widens the clinical spectrum of this new disease entity and now distinguishes three groups of clinical phenotypes, which are related to the functional effects on protein function (loss-of-function only, gain-of-function only, and gain- and loss-of-function). Third, in each of these three categories, one mutation recurs with a strikingly homogeneous phenotype in most patients (P405L, R297Q and T374A). Two-thirds of all patients with *KCNA2* encephalopathy reported so far, 10 new patients from this study and seven from previous ones (Pena and Coimbra, 2015; Syrbe *et al.*, 2015; Corbett *et al.*, 2016; Hundallah *et al.*, 2016), carried one of these three mutations. Furthermore, *KCNA2* mutations arose *de novo* in all patients in whom it could be tested (20/23).

On one hand, the three phenotypic groups shared common clinical features, and on the other they showed several distinctive characteristics and different degrees of disease severity. Common phenotypic features were the early age of epilepsy onset, fever sensitivity, cerebellar involvement, cognitive and language impairment and behavioural disorders. Onset of epilepsy occurred within the first to second year of life, while the gain- and loss-of-function subgroup showed the earliest, often neonatal onset. Febrile seizures or febrile status epilepticus at onset occurred in a similar proportion in the loss- or gain-of-function groups, but less in the gain- and loss-of-function group. Cerebellar involvement was one of the prominent characteristics of *KCNA2* encephalopathy. Ataxia was reported in the majority of patients in both groups, although the degree of severity was much more pronounced in the gain-of-function group, in which some patients were unable to walk without support. Other cerebellar features observed in all patients were impaired coordination and dysarthria, whereas hypotonia and tremor were reported mainly in the gain-of-function subgroup. Intellectual disability was observed in all patients, but the cognitive impairment was much more severe in patients carrying gain-of-function mutations compared to patients with loss-of-function mutations. Various degrees of language impairment were reported in almost all patients, without overt differences between loss-of-function and both gain-of-function subgroups with regards to the proportion of non-verbal patients. Finally, behavioural features such as aggressiveness and irritability were reported in patients from both groups, whereas stubbornness and hyperactivity associated with moderate intellectual disability characterized mainly the gain-of-function group.

In addition to common symptoms with a different degree of severity, there were several distinctive features that differentiate the phenotypes associated with loss- or gain-of-function only, or with gain- and loss-of-function mutations, including seizure types, EEG features, epilepsy outcome, and neuroimaging. In the gain-of-function only group, the seven patients carrying the same R297Q mutation presented a homogeneous epilepsy phenotype characterized by generalized seizures, such as typical and atypical absence seizures, myoclonic seizures and GTCS, in agreement with the EEG data showing generalized epileptic discharges in all of them. Also Patient 17 carrying the pathogenic variant

L298F showed features of a generalized epilepsy; however, the neurological picture was more severe as compared to R297Q. Only Patient 9 with the E157R gain-of-function mutation presented with both focal (motor) and generalized (atypical absences) seizures, and a mixture of focal and generalized epileptic discharges on EEG.

In the gain- and loss-of-function subgroup, patients presented in equal proportion with either focal or generalized seizures. Interestingly, all three patients presenting with focal seizures (Patients 22–24) shared the same recurrent mutation (T374A) and the associated phenotypes were more severe than any of the other *KCNA2*-related phenotypes described so far, including profound intellectual disability, spastic tetraplegia, hypotonia, intractable epilepsy, choreoathetosis, microcephaly and optic atrophy. Hundallah *et al.* (2016) reported a patient with a similar phenotype and the same *KCNA2* mutation. In Patient 22, the contribution of ring chromosome 21 to the phenotype was difficult to assess, as the clinical picture associated with this chromosomal abnormality can be extremely variable, ranging from normal intellect to severe psychomotor retardation, with impaired speech, epilepsy, hypotonia, and craniofacial dysmorphisms including microcephaly (Specchio *et al.*, 2011). Some of these features are also shared by patients with *KCNA2* encephalopathy. The striking similarity of the phenotype of Patient 22 with that of Patients 23 and 24, carrying the same *KCNA2* mutation, suggest that the clinical phenotype of Patient 22 was mainly determined by the *KCNA2* mutation. The T374A mutation showed a gain-of-function with the strongest loss-of-function in combination, i.e. a dominant-negative amplitude reduction. This peculiar electrophysiological feature may thus be specific for a particularly severe subgroup of patients with *KCNA2* encephalopathy.

Most of the patients with loss-of-function mutations had focal seizures with the only exception of Patient 2 who presented with generalized (myoclonic and myoclonic-atonic) seizures. In addition, we found that three loss-of-function patients (Patients 4–6) with the same mutation (P405L) had similar focal seizures types including focal dyscognitive seizures and hemiclonic seizures, sometimes evolving to secondary generalization or even to status epilepticus, followed by post-ictal paresis in two of them. Moreover, two of these patients presented at epilepsy onset with seizures characterized by eye deviation, vomiting, prolonged hemiclonic jerks: these features may be consistent with a focal onset in posterior brain regions (Sveinbjornsdottir and Duncan, 1993), in agreement with the EEG finding of epileptic abnormalities in temporo-occipital regions. Only one patient with a loss-of-function mutation (Patient 7) suffered from both focal and generalized seizures with generalized and multifocal EEG epileptic abnormalities, in the context of a very severe phenotype that included severe intellectual disability, behavioural disturbances, and additional symptoms such as sensorineural hearing loss. It is worth noting that some phenotypic features of loss-of-function patients (i.e. infantile or early-childhood seizure onset, febrile and afebrile hemiclonic or myoclonic seizures, focal motor seizures, and status epilepticus) can overlap with Dravet syndrome, thus including *KCNA2* loss-of-function encephalopathy in the phenotypic spectrum of the Dravet-like conditions.

An EEG feature only seen in loss-of-function patients was the propensity for striking activation of the epileptiform activity during non-REM sleep (Patients 4, 5, 6 and 8). This finding and the concomitant further deterioration of language and cognitive/behavioural status, raises the concern for ESES in *KCNA2* loss-of-function patients (Tassinari *et al.*, 2012). This possibility is corroborated by Patient 6, in whom the improvement of the sleep EEG was associated with a partial recovery of language. Therefore, in *KCNA2* loss-of-function patients, further deterioration of the cognitive and behavioural status during the course of the disease warrants a proper electroclinical assessment to detect the possible occurrence of ESES. Further evidence is necessary to designate *KCNA2* mutations as a possible genetic cause of ESES. P405L was the most common loss-of-function pathogenic variant being associated with the typical features of a normal development before disease onset, focal motor and hemiclonic seizures, posterior EEG abnormalities, occurrence of an ESES-like EEG pattern during sleep, and response to treatment in four of five patients.

Epilepsy outcome also distinguishes the loss-of-function versus gain-of-function groups, with a relatively favourable course in patients with loss-of-function mutations with four of eight patients becoming seizure-free. Pharmaco-responsive epilepsy, associated with episodic ataxia, has been reported also in a novel *KCNA2* pathogenic variant (255_257del) with a loss-of-function effect (Corbett *et al.*, 2016). In contrast, only one of nine patients with gain-of-function mutations became seizure-free, and none with a gain- and loss-of-function mutation, even though the severity and frequency of seizures decreased in most patients over time.

The presence of cerebellar atrophy was a further distinguishing feature between the three subgroups of patients, with marked cerebellar atrophy in about half of patients with gain-of-function mutations. In contrast, brain MRI was unremarkable in patients with loss-of-function mutations. In the gain- and loss-of-function subgroup, the cerebellar atrophy was detected already in childhood, whereas in the gain-of-function only group it was observed only in adulthood. This suggests that the degree of cerebellar atrophy correlates with particular electrophysiological characteristics of the underlying *KCNA2* pathogenic variants. Spastic tetraplegia was observed only in patients with gain-of-function pathogenic variants; this feature has not been reported so far in *KCNA2* encephalopathy. However, spastic paraplegia has been recently associated with a specific *KCNA2* loss-of-function variant with a probable additional electrophysiological defect of a proton current through the gating pore of the voltage sensor (Helbig *et al.*, 2016). Finally, facial dysmorphism

(mostly broad forehead), microcephaly, and orthopaedic abnormalities (scoliosis, kyphosis, genu valgum) were detected mainly in the gain-of-function subgroup, whereas Patient 4 with a loss-of-function mutation had short stature and growth hormone deficiency.

There are specific mutations that were associated with distinct phenotypes, e.g. T374A, which caused the most severe phenotype with neonatal epilepsy onset with both generalized and focal features, R297Q, which was characterized by moderate/severe degrees of intellectual disability and generalized seizures, and the P405L mutation, which was associated with a milder phenotype with focal epilepsy. Thus, there is evidence emerging from our data that at least in some cases, the specific mutation itself is largely responsible for specific clinical symptoms. However, there were some patients deviating from this pattern suggesting that other environmental factors or the genetic background also influence the phenotype.

Our electrophysiological studies indicate that all mutations with any gain-of-function effect hyperpolarize the membrane potential of oocytes and may therefore inhibit the firing of neurons expressing these mutations ('electrical silencing'). It is tempting to speculate that inhibitory neurons may play a major role in this case to generate seizures. This hypothesis fits well with the observation that inhibitory neurons are primarily affected in many generalized epilepsies [GABA receptor mutations associated with generalized genetic epilepsies or generalized epilepsy with febrile seizures plus (GEFS +), Dravet syndrome and GEFS + associated with mutations in *SCN1A* encoding the main Na^+ channel in inhibitory neurons, *KCNC1* mutations in progressive myoclonic epilepsy, PTZ model of acute generalized epilepsy] (Reid *et al.*, 2009; Coppola and Moshe, 2012; Lerche *et al.*, 2013; Muona *et al.*, 2015). In contrast, loss-of-function mutations predict an impaired repolarization of an action potential and neuronal hyperexcitability (McNamara *et al.*, 1996; Robbins *et al.*, 2012), which may primarily affect the excitatory pathway. This would strengthen the hypothesis of a primarily glutamatergic impairment in many focal epilepsies, such as *SCN2A* mutations in benign familial neonatal-infantile seizures or NMDA receptor mutations in genetic focal epilepsies, or the kainate model of acute seizures (Liao *et al.*, 2010; Carvill *et al.*, 2013*b*; Lemke *et al.*, 2013; Lesca *et al.*, 2013; Lévesque and Avoli, 2013). Both gain-of-function and loss-of-function effects on $K_v1.2$ channels could also impact the expression of heteromeric $K_v1.2$-containing K_v1 channels, alter the excitability of specific neuronal compartments of different cell types (Sheng *et al.*, 1994; Manganas *et al.*, 2001) and thus cause a specific phenotype. For the truncation mutation $Q213^*$ two different scenarios could be possible, which cannot be predicted: the mutation could either (i) turn on nonsense-mediated mRNA decay leading to degradation of the mRNA; or (ii) a deleterious truncated protein can be translated. The truncated protein not only causes a complete loss-of-function of $K_v1.2$ channels (translated protein stops long before the C-terminal phosphorylation sites that are essential for trafficking), but could also impact trafficking of heteromeric $K_v1.2$-containing Kv1 channels to the cell membrane (Yang *et al.*, 2007). Therefore, further detailed studies in neurons and animal models are required to unravel the real consequences of *KCNA2* dominant loss-of-function and gain-of-function mutations in neurons and understand the pathophysiology on a network and behavioural level.

There are two mouse models that have been studied with genetic alterations in *Kcna2*, a spontaneous point mutation (Pingu mouse) causing cerebellar ataxia (Xie *et al.*, 2010), and a knock-out model (Brew *et al.*, 2007). $K_v1.2$ channels are highly expressed in the cerebellum. In Pingu mice, the $K_v1.2$ mutant channel reduces the spike output of Purkinje cells, which could explain the ataxia, and transgenic overexpression of *KCNA2* could rescue coordinated motor control (Xie *et al.*, 2010). Mice lacking $K_v1.2$ displayed increased seizure susceptibility and premature death (Brew *et al.*, 2007). However, these models are only insufficiently representing the effects of *KCNA2* mutations observed in our patients, since the dramatic biophysical consequences we showed on the channel level were much different from those described for the Pingu mutation (Xie *et al.*, 2010) or from a knock-out (Brew *et al.*, 2007).

In conclusion, our study provides evidence for specific symptoms and significant genotype–phenotype correlations in *KCNA2* encephalopathy. This suggests that different pathophysiological mechanisms correspond to distinct clinical presentations. Additional clinical, genetic and pathophysiological studies may further corroborate our findings and provide useful information to predict the disease course and to orient targeted treatments.

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Supplementary material

Supplementary material is available at *Brain* online.

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