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

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Running title

Clinical spectrum of *KCNA2* encephalopathy

Abstract

Recently, *de novo* mutations in the gene *KCNA2*, causing either a dominant-negative loss-of-function (LOF) or a gain-of-function (GOF) of the voltage-gated K$^+$ channel K$_V$1.2, were described to cause a new molecular entity within the epileptic encephalopathies (EEs). Here, we report a cohort of 23 patients (eight previously described) with EE 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* 2-microelectrode voltage clamp system revealed mutations with only LOF effects (mostly dominant-negative current amplitude reduction) in eight patients or only GOF effects (hyperpolarizing shift of voltage-dependent activation, increased amplitude) in 9 patients. In six patients, the GOF was diminished by an additional LOF (GOF+LOF) 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 (LOF) vs. generalized (GOF) seizures and corresponding epileptic discharges with prominent sleep activation in most cases with LOF 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 GOF 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 GOF+LOF 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.

Key words: *KCNA2,* encephalopathy, gain-of-function, loss-of-function, phenotype-genotype associations

Abbreviations: KCNA2 = potassium voltage-gated channel subfamily A member 2; K_v = voltage-gated potassium channel; WT = wild type; ADHD = attention deficit hyperactivity disorder

Introduction

Epileptic encephalopathies (EEs) 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 EEs, 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 EEs, 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 etiology of genetic epilepsies (Claes et al, 2001; Reid et al., 2009; Lerche et al., 2013; EpiK4, 2013; Carvill et al., 2013a; Møller et al., 2016; McTague et al., 2016).

Recently, mutations in *KCNA2* encoding the voltage-gated K^+ channel $K_V1.2$, have been reported as a novel cause of EE (Syrbe et al, 2015; Pena and Coimbra, 2015; Hundallah et al., 2016; Allen et al., 2016; Allou et al., 2016; Corbett et al., 2016). $K_V1.2$ belongs to the K_V1 family with eight members ($K_V1.1–8$), all of which are expressed in the central nervous system. K_V1 channels are composed of four subunits with six transmembrane segments each (S1-S6, see 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 at 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 is 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.

Materials and Methods

Patients. Fifteen new and eight previously reported patients (Syrbe et al., 2015; Pena Coimbra 2015; Allen et al., 2016) were included in this study. The previously unreported patients were collected through data sharing with Epilepsy and Genetic Centers 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 magnetic resonance imaging (MRI) scans were obtained for all patients. Seizures and where possible epilepsy syndromes were classified according to the latest ILAE classification proposal (Berg et al., 2010). The study was approved by the local ethics committees of each participating clinical center. Written informed consent was obtained by the parents or the legal guardian of each patient following local IRB requirements.

Mutation analysis

All *KCNA2* variants were identified by routine genetic diagnostics performed in patients with EEs 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, Tübingen, Germany). 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, USA; primers are available upon 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. 50 nl of cRNA encoding wildtype (WT) or mutated $K_V1.2$ subunits (1µg/µl) was injected using Roboocyte2 (Multi Channel Systems, Reutlingen, Germany). Oocytes were stored for two days (at 17°C) prior to the experiment. Amplitudes of currents of WT and mutant channels recorded on the same day were normalized to the mean value of $K_V1.2$ WT 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 ± SEM. Statistical tests were one-way ANOVA with Bonferroni t-test as posthoc test (for normally distributed data) or one-way ANOVA on ranks with Dunn's posthoc 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 using the following symbols: $*p<0.05$, $**p<0.01$, $***p<0.001$.

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, 2 and 3). An additional patient was excluded by our study since it was not possible to demonstrate the pathogenicity of his Q357R mutation (see below). Eight patients have previously been reported (Syrbe et al., 2015, Pena and Coimbra, 2015; Allen et al., 2016). Ten mutations were missense: E157K (found in one patient), I263T (1), L290R (1), L293H (1), R297Q (7), L298F (1), L328V (1), T374A (3), G398C (1), P405L (5), 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 (#8, # 13, # 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 had also 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 Polyphen2 and Mutation Taster (Supplementary Table). 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 out 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 WT $K_V1.2$ channel, mutants or their mixtures were injected and recordings were made in parallel two to three days after injection.

Mutations causing loss-of-function (LOF) 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 discussion). 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 LOF effect as previously reported for $K_V1.1$ channels (Upadhyay et al., 2009; Yifrach and MacKinnon, 2002). When we expressed G398C mutant $K_V1.2$ channels in *Xenopus 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 WT channels in co-expression experiments (Fig. 2A,B).

Mutations causing gain-of-function (GOF) effects: For the R297Q mutation, affecting the second arginine of the voltage sensor, and the neighboring L298F, we have shown recently a dominant GOF effect with up to 13-fold increased current amplitudes and a shift of steady-state activation by -40 to -50 mV compared with WT channels (Syrbe et al., 2015). Here, we also identified the E157K mutation as GOF: this mutation, located in the N-terminus of the channel, caused a dominant GOF

with a 5-fold increase in current amplitudes (Fig. 2A,D), a hyperpolarized resting membrane potential (Fig. 2C), and a less pronounced shift of steady-state activation by -12 mV (Fig. 2E).

In contrast to the GOF 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 (see Supplementary Table). Due to the drastic changes of all other mutations, we rather consider this variant as a benign polymorphism which is not responsible for the clinical phenotype of the patient, although the phenotype fits well with a GOF *KCNA2* mutation and we cannot exclude that we missed an alteration with our experimental system. The patient #18, carrying the Q357R variant is not included in our analysis, however, his phenotype is described in the Supplementary Note.

Mutations causing GOF+LOF effects: We also found hyperpolarizing shifts of the activation curves for L290R and L293H, located in S4 (Fig. 1A and Fig. 3D), which predict a GOF 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 LOF 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 GOF effect on activation and a reduction of the LOF effect on inactivation in the physiologically most relevant voltage range near the resting membrane potential (Fig. 3D,E, Supplementary Table). In addition, L290R mutant channels yielded significantly larger current amplitudes (Fig. 3A,C), another GOF effect. Although the negative shifts of steady-state inactivation diminish the GOF effect on activation and amplitude, resting membrane potentials were significantly more negative in oocytes injected with mutant compared to WT cRNA (Fig. 3B). This indicates a net and dominant GOF 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 GOF effect from functional studies of $K_V1.1$, a highly conserved channel from the same family (L328V; Upadhyay et al., 2009), and its drosophila homolog *shaker* (T374A; Yool and Schwarz, 1995; Heginbotham et al., 1994, 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 WT (Fig. 3 F,G,I and Supplementary Table). However, current amplitudes were decreased in contrast to the S4 mutations, even exerting a slight dominant-negative effect on the WT, which should reduce the GOF (Fig. 3H and Supplementary Table). The mutation T374A, which was found in three patients (#22, #23, #24) with the most severe phenotype (see phenotypic descriptions below), caused a more prominent combination of both GOF and LOF 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 GOF 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, Supplementary Table).

Therefore, the functional analysis showed that in the group of GOF mutations, some had prominent GOF effects only (E157K, R297Q, L298F) whereas others displayed a combination of both GOF and LOF effects (GOF+LOF) (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 LOF mutations and more severe phenotypes with GOF mutations (Syrbe et al., 2015). To further explore this initial impression, we illustrate the

phenotypic features of our patients with LOF *KCNA2* mutations separately from the patients with GOF *KCNA2* mutations. Since some of the GOF mutations also showed some additional LOF effects, we further subgroup those patients carrying mutations with similar electrophysiological properties.

Phenotypic features of patients with LOF KCNA2 encephalopathy (Table 1). Eight patients presented with LOF *KCNA2* mutations. 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 3/8 patients, one of them (#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 (#4 and #5) were preceded by eye-deviation and vomiting. Eye deviation as ictal feature was reported also in patient #1 (Q213* mutation). With increasing age, 6/8 of them developed focal dyscognitive seizures and focal motor seizures with possible secondarily generalized tonic-clonic seizures (sGTCS); in three of them (#3, #7, #8) generalized seizures were also 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 (#4 and #6), both featuring hemiclonic or focal motor seizures with or without sGTCS. The course of epilepsy was relatively favourable in most of the patients compared to those with GOF or GOF+LOF mutations. Four of eight patients became seizure-free (mean follow-up 4 years; range 1.5-6 years), and one patient (#7) continued to have rare absences. Three patients continued to have daily atypical absences (#8), multiple seizure types (#3) or uncontrolled focal seizures (#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 (7 patients), ataxia (6), poor coordination (6), or dysarthria (2). Additional motor symptoms were fine continuous finger myoclonia ("polyminimyoclonus") (2), hand tremor (2), or dyskinesia (1). All patients were cognitively impaired with mild-to-moderate intellectual disability (ID) in 5 patients, three of them (#2, #4, #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 (#6 and #7). Autism spectrum disorders were diagnosed in patient #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) (1 patient), scoliosis (1), pes planus and osteoarthritis (1), osteoporosis (1), or sensori-neural hearing loss (1) were observed. Overall the clinical pictures of patient #2, #4, #5, and #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 ID 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 ID with behavioral disturbances, more prominent motor/coordination dysfunction, and incompletely controlled epilepsy.

EEG at onset in 5 patients (#2, #4, #5; #6, #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 (#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 NREM sleep), featuring diffuse epileptic discharges with posterior predominance, was reported in four patients (#4, #5, #6, #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 (#4,#5,#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 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 GOF KCNA2 encephalopathy (Tables 2&3). Fifteen patients presented with GOF mutations, including a subgroup showing GOF+LOF effects. We here present the phenotypes of patients with mutations with GOF effects only separately from those with GOF+LOF 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 Note.

Phenotypic features of patients carrying mutations with GOF effects only (Table 2): Nine patients carried such GOF mutations. 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 5/9 patients. The remaining four patients presented at onset with absences or afebrile GTCS, and #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 8/9 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 (#13) became seizure-free (the follow-up was at 3.5 years). The majority of

patients was 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 following an initial unremarkable development. Additional neurological features developed over time including ataxia (9 patients), impairment of fine or gross motor skills (5), tremor (5), dysarthria (4), hypotonia (4), pyramidal signs (4), dysdiadochokinesis (2), or myoclonus (1). The severity of ataxia ranged from mild-moderate (patients #9, #11, #16) to severe with inability to walk unassistedly (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, #16) to severe intellectual disability (patients #14-15) without language acquisition (patient #17). Behavioral features such as hyperactivity, stubbornness, and aggressiveness were reported in five 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 (#16 and 17), kyphosis and genu valgum were reported in patient #12. Patient #16 presented with scoliosis.

The main EEG features in 9/9 (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 (#9,#12,#13,#16) showed additional focal or multifocal epileptiform discharges.

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

Phenotypic features of patients carrying mutations with GOF+LOF effects (Table 3). Six patients presented with mutations showing both GOF and LOF effects. The mean age at seizure onset was 2.1 months (range: from birth to 6 months). Fever sensitivity was reported only in one patient (#21). Generalized seizures such as myoclonic seizures, tonic seizures or GTCS were reported at the onset and throughout the evolution in 3/6 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 (#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 3/6 patients. At the time of the last follow-up, 5/6 patients had severe or profound ID; three of them were non-verbal (#20, #22, #23). Additional neurological features included ataxia (3), hypotonia (2), tremor (2), dysarthria (1). Two patients (#22 and #23) with a T374A pathogenic variant showed a more severe phenotype with profound ID, lack of language acquisition, spastic tetraplegia, optic atrophy, and severe scoliosis. A third 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 ADHD and hyperactivity were reported in three patients (#19, #20, #21). One of the three patients carrying the T374A mutation (#22) was found to have also 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 2/6 patients. Brain MRI showed cerebellar atrophy in three 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 GOF effects only.

In summary, the subgroup of patients carrying mutations with GOF+LOF effects presents distinctive features compared to the subgroup with GOF effects only, such as: a) an earlier age of seizure onset, rarely triggered by fever; b) propensity to present either with focal or with generalized seizures; c) a higher incidence of focal EEG epileptic discharges; d) more severe neurological and more pronounced intellectual disability, and e) 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 GOF or LOF effect on channel function, but a combined GOF+LOF. 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 (LOF only, GOF only, and GOF+LOF). 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 (Syrbe et al., 2015; Pena and Coimbra, 2015; Hundallah et al., 2016; Corbett 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 GOF+LOF subgroup showed the earliest, often neonatal onset. Febrile seizures or febrile status epilepticus at onset occurred in a similar proportion in the LOF or GOF groups, but less in the GOF+LOF 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 GOF 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 GOF subgroup. Intellectual disability was observed in all patients, but the cognitive impairment was much more severe in patients carrying GOF mutations compared to patients with LOF mutations. Various degrees of language impairment were reported in almost all patients, without overt differences between LOF and both GOF 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 GOF group.

In addition to common symptoms with a different degree of severity, there were several distinctive features that differentiate the phenotypes associated with LOF or GOF only, or with GOF+LOF mutations, including seizure types, EEG features, epilepsy outcome, and neuroimaging. In the GOF 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 GOF mutation presented with both focal (motor) and generalized (atypical absences) seizures, and a mixture of focal and generalized epileptic discharges on EEG.

In the GOF+LOF subgroup, patients presented in equal proportion with either focal or generalized seizures. Interestingly, all three patients presenting with focal seizures (#22, #23, #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 ID, spastic tetraplegia, hypotonia, intractable epilepsy, choreoathetosis, microcephaly and optic atrophy. Hundallah et al (2016) recently 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, since 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 GOF with the strongest LOF 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 LOF 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 LOF patients (#4, #5, #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 LOF mutation (#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 to note that some phenotypic features of LOF 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*-LOF encephalopathy in the phenotypic spectrum of the Dravet-like conditions.

An EEG feature only seen in LOF patients was the propensity for striking activation of the epileptiform activity during NREM sleep (pts #4, #5, #6, #8). This finding and the concomitant further deterioration of language and cognitive/behavioral status, raises the concern for ESES in *KCNA2*-LOF 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*-LOF patients, further deterioration of the cognitive and behavioural status

during the course of the disease warrants a proper electro-clinical assessment to detect the possible occurrence of ESES. Further evidences are necessary to designate *KCNA2* mutations as a possible genetic cause of ESES. P405L was the most common LOF 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 4/5 patients.

Epilepsy outcome also distinguishes the LOF vs GOF groups, with a relatively favorable course in patients with LOF mutations with 4/8 patients becoming seizure-free. Pharmacoresponsive epilepsy, associated with episodic ataxia, has been reported also in a novel *KCNA2* pathogenic variant (255_257del) with a LOF effect (Corbett et al., 2016). In contrast, only 1/9 patients with GOF mutations became seizure-free, and none with a GOF+LOF 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 GOF mutations. In contrast, brain MRI was unremarkable in patients with LOF mutations. In the GOF+LOF subgroup, the cerebellar atrophy was detected already in childhood, whereas in the GOF 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 GOF pathogenic variants; this feature has not been reported so far in *KCNA2* encephalopathy. However, spastic paraplegia has been recently associated with a specific *KCNA2*-LOF 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 orthopedic abnormalities (scoliosis,

kyphosis, genu valgum) were detected mainly in the GOF subgroup, whereas patient #4 with a LOF mutation had short stature and growth hormone deficiency.

There are specific mutations, which were associated with distinct phenotypes, such as the T374A which caused the most severe phenotype with neonatal epilepsy onset with both generalized and focal features, the 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 GOF 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 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, LOF 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 BFNIS or NMDA receptor mutations in genetic focal epilepsies, or the kainate model of acute seizures (Liao et al., 2010, Lemke et al., 2013; Lesca et al., 2013; Carvill et al.,

2013b; Levesque and Avoli, 2013). Both GOF and LOF effects on $K_V1.2$ channels could also impact the expression of heteromeric $K_V1.2$-containing Kv1 channels, alter the excitability of specific neuronal compartments of different cell types (Manganas et al., 2001, Sheng et al., 1994) 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 LOF 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 LOF and GOF mutations in neurons and understand the pathophysiology on a network and behavioral 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.

Acknowledgments

References

Allen NM, Conroy J, Shahwan A, Lynch B, Correa RG, Pena SD, et al.. Unexplained early onset epileptic encephalopathy: Exome screening and phenotype expansion. Epilepsia 2016; 57: e12-7.

Allou L, Julia S, Amsallem D, El Chehadeh S, Lambert L, Thevenon J, et al. Rett-like phenotypes: expanding the genetic heterogeneity to the KCNA2 gene and first familial case of CDKL5-related disease. Clin Genet 2016 doi: 10.1111/cge.12784.

Berg AT, Berkovic SF, Brodie MJ, Buchhalter J, Cross JH, van Emde Boas W, et al. Revised terminology and concepts for organization of seizures and epilepsies: report of the ILAE Commission on Classification and Terminology, 2005-2009. Epilepsia. 2010; 51: 676-85

Brew HM, Gittelman JX, Silverstein RS, Hanks TD, Demas VP, Robisnon LC et al. Seizures and reduced life span in mice lacking the potassium channel subunit Kv1.2, but hypoexcitability and enlarged Kv1 currents in auditory neurons. J Neurophysiol. 2007; 98: 1501-25.

Carvill GL, Heavin SB, Yendle SC, McMahon JM, O'Roak BJ, Cook J, et al. Targeted resequencing in epileptic encephalopathies identifies de novo mutations in CHD2 and SYNGAP1. Nat Genet. 2013a; 45: 825-30.

Carvill GL, Regan BM, Yendle SC, O'Roak BJ, Lozovaya N, Bruneau N, et al. GRIN2A mutations cause epilepsy-aphasia spectrum disorders. Nat Genet. 2013b; 45: 1073-6.

Claes L, Del-Favero J, Ceulemans B, Lagae L, Van Broeckhoven C, De Jonghe P. De novo mutations in the sodium-channel gene SCN1A cause severe myoclonic epilepsy of infancy. Am J Hum Genet. 2001; 68: 1327-32.

Christie MJ, North RA, Osborne PB, Douglass J, Adelman JP. Heteropolymeric potassium channels expressed in Xenopus oocytes from cloned subunits. Neuron. 1990; 4: 405-11.

Coppola A1, Moshé SL Animal models. In: H Stefan and WH. Theodore, editors. Handbook of Clinical Neurology, vol. 107. Amsterdam: Elsevier B.V.; 2012. p. 63-98.

Corbett MA, Bellows ST, Li M, Carroll R, Micallef S, Carvill GL, et al. Dominant KCNA2 mutation causes episodic ataxia and pharmacoresponsive epilepsy. Neurology 2016; 87: 1975-1984

Epi4K Consortium & Epilepsy Phenome/Genome Project. De novo mutations in epileptic encephalopathies. Nature 2013; 501, 217–21.

Heginbotham L, Lu Z, Abramson T, MacKinnon R. Mutations in the K+ channel signature sequence. Biophys J 1994; 66: 1061-7.

Helbig KL, Hedrich UB, Shinde DN, Krey I, Teichmann AC, Hentschel J, et al. A recurrent mutation in KCNA2 as a novel cause of hereditary spastic paraplegia and ataxia. Ann Neurol 2016 doi: 10.1002/ana.24762

Hundallah K, Alenizi A, AlHashem A, Tabarki B. Severe early-onset epileptic encephalopathy due to mutations in the KCNA2 gene: Expansion of the genotypic and phenotypic spectrum.

Eur J Paediatr Neurol. 2016; 20: 657-60.

Jan LY, Jan YN. Voltage-gated potassium channels and the diversity of electrical signalling. J Physiol. 2012 Jun 1;590(11):2591-9.

Lemke JR, Lal D, Reinthaler EM, Steiner I, Nothnagel M, Alber M, et al. Mutations in GRIN2A cause idiopathic focal epilepsy with rolandic spikes. Nat Genet 2013; 45: 1067-72.

Lerche H, Shah M, Beck H, Noebels J, Johnston D, Vincent A. Ion channels in genetic and acquired forms of epilepsy. J Physiol 2013; 591: 753-64.

Lesca G, Rudolf G, Bruneau N, Lozovaya N, Labalme A, Boutry-Kryza N, et al. GRIN2A mutations in acquired epileptic aphasia and related childhood focal epilepsies and encephalopathies with speech and language dysfunction. Nat Genet 2013; 45: 1061-6.

Lévesque M, Avoli M. The kainic acid model of temporal lobe epilepsy. [Review]. Neurosci Biobehav Rev 2013; 37: 2887-99.

Li M, Jan YN, Jan LY. Specification of subunit assembly by the hydrophilic amino-terminal domain of the Shaker potassium channel. Science. 1992; 257: 1225-30.

Liao Y, Deprez L, Maljevic S, Pitsch J, Claes L, Hristova D, et al. Molecular correlates of age-dependent seizures in an inherited neonatal-infantile epilepsy. Brain 2010; 133: 1403–14.

Manganas LN, Wang Q, Scannevin RH, Antonucci DE, Rhodes KJ, Trimmer JS. Identification of a trafficking determinant localized to the Kv1 potassium channel pore. Proceedings of the National Academy of Sciences of the United States of America. 2001; 98: 14055-9.

McNamara NM1, Averill S, Wilkin GP, Dolly JO, Priestley JV. Ultrastructural localization of a voltage-gated K+ channel alpha subunit (KV 1.2) in the rat cerebellum. Eur J Neurosci 1996; 8: 688-99.

McTague A, Howell KB, Cross JH, Kurian MA, Scheffer IE. The genetic landscape of the epileptic encephalopathies of infancy and childhood. [Review]. Lancet Neurol. 2016; 15: 304-16.

Muona M, Berkovic SF, Dibbens LM, Oliver KL, Maljevic S, Bayly MA, et al. A recurrent de novo mutation in KCNC1 causes progressive myoclonus epilepsy. Nat Genet 2015; 47: 39-46.

Møller RS, Larsen LHG, Johannesen KM, Talvik I, Talvik T, Vaher U, et al. Gene panel testing in epileptic encephalopathies and familial epilepsies. Mol Syndromol 2016; 7: 210-19.

Pena SD, Coimbra RL. Ataxia and myoclonic epilepsy due to a heterozygous new mutation in KCNA2: proposal for a new channelopathy. Clin Genet 2015: 87: e1–e3.

Reid CA, Berkovic SF, Petrou S. Mechanisms of human inherited epilepsies. [Review]. Prog Neurobiol. 2009; 87:41-57.

Robbins CA, Tempel BL Kv1.1 and Kv1.2: similar channels, different seizure models. Epilepsia 2012; 53 (Suppl 1): 134-41.

Sheng M, Tsaur ML, Jan YN, Jan LY. Contrasting subcellular localization of the Kv1.2 K+ channel subunit in different neurons of rat brain. The Journal of neuroscience: the official journal of the Society for Neuroscience 1994; 14: 2408-17.

Specchio N, Carotenuto A, Trivisano M, Cappelletti S, Digilio C, Capolino R, et al. (2011) Ring 21 chromosome presenting with epilepsy and intellectual disability: clinical report and review of the literature. Am J Med Genet 2011; 155A: 911–14.

Sveinbjornsdottir S, Duncan JS. Parietal and occipital lobe epilepsy: a review. Epilepsia. 1993 May-Jun;34(3):493-521.

Syrbe S, Hedrich UB, Riesch E, Djemie T, Muller S, Moller RS, et al. De novo loss- or gain-of-function mutations in KCNA2 cause epileptic encephalopathy. Nat Genet 2015; 47: 393-9.

Tassinari CA, Cantalupo G, Dalla Bernardina B, Darra F, Bureau M, Cirelli C, et al. Encephalopathy related to status epilepticus during slow sleep (ESES) including Landau-Kleffner syndrome. In: Bureau M, Genton P, Dravet C, Delgado-Escueta A, Tassinari CA, Thomas P, & Wolf P, editors. Epileptic Syndromes in Infancy, Childhood and Adolescence (5th ed). Montrouge: John Libbey Eurotext Ltd; 2012. p.255-75.

Upadhyay SK, Nagarajan P, Mathew MK. Potassium channel opening: a subtle two-step. J Physiol. 2009; 587: 3851-68.

Xie G, Harrison J, Clapcote SJ, Huang Y, Zhang JY, Wang LY, et al. A new Kv1.2 channelopathy underlying cerebellar ataxia. J Biol Chem. 2010; 285: 32160-73.

Yang JW, Vacher H, Park KS, Clark E, Trimmer JS. Trafficking-dependent phosphorylation of Kv1.2 regulates voltage-gated potassium channel cell surface expression. Proceedings of the National Academy of Sciences of the United States of America. 2007; 104: 20055-60.

Yifrach O, MacKinnon R. Energetics of pore opening in a voltage-gated K(+) channel. Cell 2002; 111:231-9.

Yool AJ, Schwarz TL. Interactions of the H5 pore region and hydroxylamine with N-type inactivation in the Shaker K+ channel. Biophys J 1995; 68: 448-58.

Zheng J, Sigworth FJ. Selectivity changes during activation of mutant Shaker potassium channels. J Gen Physiol 1997; 110: 101-17.

Figure legends

Fig. 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 gray) and segments S5 and S6 forming the pore region (dark gray) with its pore-forming loop.

All variants (except the truncation mutation Q213* in blue and Q357R in green) are localized to

highly conserved regions in the N-Terminus (E157K, yellow), the S3 segment (I263T, gray box),

the S4 segment constituting the voltage sensor (L290R, red; L293H, orange; R297Q, light gray

triangle; L298F, dark gray triangle), the S5 segment (L328V, dark red), the pore region (T374A,

yellow) and the S6 segment (G398C, blue box; P405L, light gray box). Loss of function (LOF) are

shown as boxes in blue shades, gain of function (GOF) mutations as triangles in yellow shades, and

GOF+LOF mutations as circles in red shades. Already published mutations are shown in gray with

the corresponding symbol. (B) Mutant amino acid positions and their respective surrounding amino

acids. All variants except Q357R show evolutionary conservation.

Fig. 2: Functional effects of the LOF-*KCNA2* mutation encoding G398C and the GOF-*KCNA2*

mutation encoding E157K. (A) Representative current traces of $K_V1.2$ wildtype (WT, left), $K_V1.2$

G398C (right) and $K_V1.2$ E157K (bottom) channels recorded in a *Xenopus* 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 WT channel (WT, n=6; G398C, n=6). Current

amplitudes of mutant channels were similar to those recorded in oocytes injected with water (n=5).

Coexpression of G398C and WT channel did not show any effect of the mutation on the WT (in a

1:1 ratio of cRNA amount injected into the oocytes; n=6). Shown are means ± s.e.m. Statistically

significant differences between WT channels and the tested groups were verified by ANOVA on

ranks ($P < 0.001$) with post-hoc Dunn's method ($P < 0.05$) (indicated by asterisks). (C) Resting

membrane potentials of oocytes injected with WT (1.0, n = 15), E157K (1.0; n = 20) or WT +

E157K (1:1, n = 14). Shown are means ± s.e.m. Statistically significant differences between WT

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) (indicated by asterisks). (D) Mean current amplitudes of oocytes

injected with WT (1.0, n = 15), E157K (1.0, n=20) and WT + E157K (1.0:1.0, n = 27). Shown are

means ± s.e.m. There was a statistically significant difference between WT channels and the tested

groups (one-way ANOVA, (P < 0.001) with post-hoc Bonferroni t-test (P < 0.05) (indicated by

asterisks)). (E) Mean voltage dependence of $K_V1.2$ channel activation for E157K channels together

with the activation curves for WT and coexpressed channels (1:1 ratio). Shown are means ± s.e.m.

Lines represent Boltzmann functions fit to data points. The activation curves were significantly

shifted to more hyperpolarized potentials for all mutations (P < 0.05).

Fig. 3: *KCNA2* mutations can cause gain and loss of function. (A) Representative current traces of

$K_V1.2$ WT (left), $K_V1.2$ L290R (middle) and $K_V1.2$ L293H (right) channels recorded in a *Xenopus*

oocyte during voltage steps (from −100 mV to +70 mV). (B) Resting membrane potentials of

oocytes injected with WT (1.0, n = 33), L290R (1.0; n = 8), WT + L290R (0.5:0.5, n = 6), L293H

(1.0; n = 6) or WT + L293H (1:1, n = 21). Shown are means ± s.e.m. Statistically significant

differences between WT 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) (indicated by asterisks). (C) Mean

current amplitudes of oocytes injected with WT (1.0, n = 33), L290R (1.0, n= 8), WT + L290R

(0.5:0.5, n = 6), L293H (1.0; n = 6) or WT + L293H (0.5:0.5, n = 21). (D) Mean voltage

dependence of $K_V1.2$ channel activation for WT, L290R and L293H channels. Shown are means ±

s.e.m. 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 WT, L290R and L293H mutants. Shown are

means ± s.e.m fitted to a standard Boltzmann function. Inactivation curves of L290R and L293H

channels are significantly shifted to more hyperpolarized potentials in comparison to the WT.

Statistically significant differences between WT 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 ± s.e.m. (F) Representative current traces of $K_V1.2$ 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 WT (1.0, n = 54), L328V (1.0; n = 12), WT + L328V (1:1, n = 20), T374A (1.0, n = 19) or WT + T374A (1:1, n = 23). Shown are means ± s.e.m. Statistically significant differences between WT channels and the tested groups were verified by one-way ANOVA (P < 0.001) with post-hoc Bonferroni t-test (P < 0.05) (indicated by asterisks). (H) Mean current amplitudes of oocytes injected with WT (1.0, n = 54), L328V (1.0; n = 12), WT + L328V (1:1, n = 20), T374A (1.0, n = 19) or WT + T374A (1:1, n = 23). Shown are means ± s.e.m. (I) Mean voltage dependence of $K_V1.2$ channel activation for L328V (closed symbols) and T374A (open symbols) channels together with the activation curves for WT (black) and coexpressed channels (1:1 ratio, indicated as dotted lines) for each of the mutations. Shown are means ± s.e.m. 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 WT 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).

Fig.4: EEG features of four patients with *KCNA2*–LOF mutations (pts #4, #5 and #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 about 8-10 Hz (in pt #2 was about 18-20 Hz), lasting about 200 ms-1 s, over both temporo-parieto-occipital regions, synchronous or asynchronous in both hemispheres.

Fig.5: EEG features in patients with *KCNA2*-GOF and *KCNA2*-GOF+LOF mutations

Upper panel: Interictal EEG of two patients carrying the same *KCNA2*-GOF mutation (R297Q) at different ages. In pt #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 pt #12 (37 years old), the EEG shows sub-continuous theta and beta activity in the midline, intermixed with 3-5Hz positive spikes, with accentuation and bilateral spreading during drowsiness and sleep.

Lower panel: Interictal EEG features in a 5-years old patient (# 23), carrying a *KCNA2* GOF+LOF mutation (T374A). During wakefulness (left), the EEG shows multifocal abnormalities with predominance over both temporo-parieto-occipital regions, with striking accentuation during sleep (right).