4-aminopyridine is a promising treatment option for patients with gain-of-function *KCNA2*-encephalopathy

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One Sentence Summary: 4-aminopyridine is a potential therapeutic option for a subgroup of patients with *KCNA2*-encephalopathy and gain-of-function (GOF) variants

Abstract:

Developmental and epileptic encephalopathies (DEE) are devastating disorders characterized by epilepsy, intellectual disability and other neuropsychiatric symptoms, for which available treatments are largely ineffective. Following a precision medicine approach, we show for *KCNA2*-encephalopathy that the K^+ channel blocker 4-aminopyridine can antagonize gain-of-function defects caused by variants in the $K_V1.2$ subunit in vitro, by reducing current amplitudes and negative shifts of steady-state activation, and increasing the firing rate of transfected neurons. In n-of-1 trials carried out in nine different centers, nine of 11 patients carrying such variants benefitted from treatment with 4-aminopyridine. All six patients experiencing daily absence, myoclonic or atonic seizures became seizure-free (except some remaining provoked seizures). Two of six patients experiencing generalized tonic-clonic seizures showed marked improvement, three showed no effect and one worsening. Nine patients showed improved gait, ataxia, alertness, cognition or speech. 4-aminopyridine was well tolerated up to 2.6 mg/kg/day. We suggest 4-aminopyridine as a promising tailored treatment in *KCNA2*-(gain-of-function)-encephalopathy and provide an online tool assisting physicians to select patients with gain-of-function mutations suited to this treatment.

Introduction

Whereas technological advances in gene discovery have brought precision medicine into view for severe neurological disorders (*1*), more vigorous steps to successful therapies based on underlying disease mechanisms for epilepsy are urgently needed and should now follow successful examples like ketogenic diet for Glut1 deficiency syndrome and sodium channel blockers in *SCN2A*/*SCN8A*–related syndromes (*2*). A diverse group of severe early-onset epilepsies combined with developmental delay (DD), intellectual disability (ID), ataxia, chorea, spasticity, hypotonia, or autism constitutes developmental and epileptic encephalopathies (DEE) (*3-11*).

Recently, we described clinically distinguishable groups of *KCNA2*-encephalopathy, in which the electrophysiological dysfunction caused by the variants in Kv1.2 (*KCNA2*)-channel subunits correlated with the clinical picture (*5, 11*). Loss-of-function (LOF) variants mainly cause milder phenotypes with focal seizures, focal epileptic discharges, normal MRI and better outcomes. In stark contrast, patients carrying gain-of-function (GOF) variants present with predominantly generalized seizures and epileptic discharges, severe cognitive deficits and cerebellar ataxia. Patients carrying variants with both GOF and LOF features (GOF+LOF) may present more similar to those with GOF, and also include the most severe phenotype.

Since the latter two categories of GOF or GOF+LOF variant carriers are relevant for this study, the results for those patients which were previously published (*5*) are summarized in more detail here. The age of seizure onset of nine patients carrying GOF variants (*5*) ranged from 5 to 15 months, often starting with febrile seizures. All showed generalized seizure types, such as typical or atypical absences, myoclonic, atonic, and generalized tonic-clonic seizures (GTCS), many of those uncontrolled, but often with rare seizures. More than half were on polytherapy (see also Fig. S1 for patients included in this study). All GOF variant carriers had a slow background and generalized spike- or polyspike-waves or generalized sharp and slow-waves as EEG features. Most of them (6/9) displayed additional focal or multifocal epileptic discharges. All patients had DD, most with developmental plateauing after initial early normal development. Ataxia and ID varied from moderate to severe. Language delay was universal or completely absent in some. Cerebellar atrophy was found on magnetic resonance imaging (MRI), often noted in adult patients.

Six patients with variants inducing both GOF and LOF characteristics included the most severe phenotype, with onset between birth and 6 months (*5*). They can be divided into two groups; similar to GOF variants or a more severe phenotype. Three patients were similar to the GOF patients described above. These patients presented with generalized seizures (absence, myoclonic, tonic or generalized tonic-clonic seizures (GTCS), all persisting with variable frequency ranging from sporadic or weekly GTCS to daily absences despite polytherapy. Electroencephalograms (EEGs) showed generalized or multifocal discharges, or both. All patients showed DD with severe or profound ID and additional neurological features including ataxia, hypotonia, tremor and delayed or no language. One juvenile patient had mild cerebellar atrophy. The three other patients all carried the recurring p.(Thr374Ala) variant. Here, the phenotype was most severe with neonatal onset, profound ID with little development, lack of language acquisition, spastic quadriplegia, optic atrophy, and severe scoliosis. MRI showed cerebellar or even global atrophy.

The affected $K_V1.2$ channel subunits belong to the group of *Shaker*-related slowly inactivating voltage-gated K^+ channels, which play an important role for action potential firing and neuronal excitability. K_V1 family members assemble to heteromeric channels with distinct characteristics (*12*). 4-aminopyridine (4-AP) is a known blocker of K_V1 and some other K_V channels (*13-15*). 4-AP has been shown to be effective in small but well-designed clinical controlled trials in patients with downbeat nystagmus and episodic ataxia type 2 (*16-18*), as well as in some other patients with ataxia (*19, 20*). Furthermore, it is a drug licensed for gait disturbance in adults with multiple sclerosis, with a well-established safety profile (*21-23*). We sought to use 4-AP to antagonize the functional effect of GOF and GOF+LOF variants in vitro, and to explore 4-AP clinically as a repurposed drug in n-of-1 trials. Over a period of four years, eleven patients were treated in nine different centers. N-of-1 trials are a possibility to test a medication that is licensed for another indication in a single patient for which a rationale exists that this drug could have beneficial effects (*24-27*). N-of-1 trials have existed for decades (*28*), but are now becoming increasingly important with improved understanding of genetic determinants and pathophysiological mechanisms of very rare diseases, since they enable initial trials of a putative personalized treatment based on a patient's specific genetic defect (*29*).

Results Functional studies using 4-AP in vitro.

To investigate the effects of 4-AP on recombinant $K_V1.2$ channels in *Xenopus oocytes* we used automated two-microelectrode voltage clamping (*5, 11*). 4-AP inhibited wildtype (WT) and mutant channels (p.(Glu157Lys), p.(Arg297Gln), p.(Leu298Phe), p.(Leu290Arg) and p.(Leu293His), Fig. 1A) by reducing current amplitudes and shifting the voltage-dependence of channel activation in a depolarizing direction in a dose-dependent manner with IC_{50} values ranging 0.39 – 1.21 mM (Figs. 1B-D, S2, S3; Table S1). In contrast, p.(Leu328Val) (P10) and p.(Thr374Ala) (P11) mutant channels, which caused both GOF by a relatively smaller hyperpolarizing shift of the activation curve and LOF by reduction of current amplitudes, were not blocked by 4-AP (Table S1). However, when these mutants were co-expressed with WT $K_V1.1$ or $K_V1.2$ channels, a clear blocking effect of 4-AP was observed (Fig. 1E and Fig. S4) and the activation curves were shifted to more depolarized potentials after application of 4-AP (Fig. S4 and Table S1).

Since high concentrations of 4-AP were needed to block WT and mutant $K_V1.2$ channels in *Xenopus* oocytes (possibly due to the vitelline layer of the cells), we also tested the effects of 4-AP in mammalian cells. We used transfected Chinese hamster ovary (CHO) cells and performed dose-response experiments for WT and mutant p.(Arg297Gln) channels. 4-AP blocked WT and mutant channels in concentrations similar to those required in *Xenopus* oocytes (Fig. S5). As shown before in oocytes (*5, 11*), resting membrane potentials of CHO cells expressing mutant $K_V1.2$ channels were much more hyperpolarized than those expressing WT channels (Table S1; Fig. S5). Application of 1 mM 4-AP led to a depolarization of resting membrane potentials for both populations of cells expressing either mutant or WT channels (Table S1, Fig. S5).

To better understand the pathophysiological effects of *KCNA2* variants, we next transduced primary hippocampal cultures with either WT or p.(Arg297Gln) mutant channels using adeno-associated viruses (AAV8-hSyn-*KCNA2*-WT-T2A-EGFP or AAV8-hSyn-*KCNA2*-R297Q-T2A-EGFP). We found that the expression of mutant p.(Arg297Gln) channels caused a dramatic reduction in the firing frequency of hippocampal pyramidal neurons compared to neurons overexpressing WT channels (Fig. 2A, E). In addition, the resting membrane potential was decreased, and higher current injections were needed to elicit single action potentials (APs) for neurons expressing mutant channels in comparison to those expressing the WT Fig. 2C, D). Application of 0.1 mM 4-AP to neurons expressing mutant channels reversed the observed firing deficit. Single APs were observed with lower current injections, comparable to neurons expressing WT channels (Fig. 2). In cultures with neurons expressing WT channels, application of 4-AP caused a broadening of APs resulting in a reduction of the firing frequency (Fig. 2A, E). We also observed spontaneous rhythmic activity in WT-overexpressing neurons under 4-AP, which was not seen without 4-AP, whereas cultures overexpressing p.(Arg297Gln) mutant channels only rarely showed rhythmic activity upon 4-AP application (Fig. 2B, D).

Clinical assessment before treatment with 4-AP

We describe the individual treatment response of 11 patients (five females, six males; age at treatment initiation ranging from seven months to 38 years) with pathogenic GOF (7 patients) and GOF+LOF (4 patients) *KCNA2 de novo* variants to 4-AP (Table 1). The clinical and genetic results of ten patients were previously reported (*5, 10, 11*), but not the treatment effects of 4-AP in this highly pharmacoresistant cohort, in which a mean number of 5.8 ± 1.0 ASM had been

tried before the initiation of 4-AP (Fig. S1). One patient was newly identified (P6), carrying a previously reported GOF variant (p.(Leu298Phe)) (*11*). Although his clinical condition improved markedly after administration of 4-AP (see below and Table 1), he died of acute cerebral hypoxia after food aspiration in the setting of esophageal atresia (type IIIb).

There were five other previously published patients (*5*) carrying GOF (p.(Arg297Gln), three patients) or GOF+LOF (p.(Thr374Ala), two patients) variants. To our knowledge, they have not undergone 4-AP treatment until now. Since this group of patients cover a broad spectrum of age and clinical characteristics, and therefore also of required clinical evaluations, we categorized them into three age groups, as outlined below.

Five preschool-aged patients (P2, P3, P6, P9, P11),ranging from 7 months (P11) to 5 years (P3) of age, all had generalized epilepsy with yearly to weekly GTCS (P6 in clusters). Four children also experienced myoclonic seizures (P2, P9, P11) and/or absence seizures (P2, P6, P9) each with seizure frequencies of approximately five to ten per day. Only two achieved seizure-freedom under conventional anti-seizure medications (ASMs) (P3, P11). EEG showed frequent multifocal or generalized epileptic discharges in all but P9. Motor function/milestones were delayed from the first year of life. When independent ambulation was achieved (P2, P3, P6; earliest at 20 months), gait was broad-based, ataxic with frequent falls. P9 did not achieve independent walking until treatment with 4-AP (start: 2 years 10 months). Regardless of age within this group, only single words were spoken. All patients suffered from at least moderate ID. The most severely affected patient (P11) carried the p.(Thr374Ala) variant. He had no head control or turning of the body aged seven months, with choreiform and dystonic movements of extremities. He only produced rare phonation and showed no eye contact. Altogether, the preschool group showed a dramatic slowing of natural development after the onset of epilepsy regarding motor function and speech, but also social and cognitive skills.

In the school-aged patients (P1, P8, P10), epilepsy began in the first months of life with febrile seizures (P1), febrile status epilepticus (P10) or atypical absences (P8). Monthly GTCS (P8) as well as frequent absence seizures (P1 and P10) were observed with up to 21 clusters per week (P1) or with 15 absences and 10-15 myoclonic seizures per day, and additional atonic seizures during febrile periods (P10). All seizure entities were highly pharmacoresistant, as more than eight conventional ASMs did not lead to seizure control for any seizure type in any of the school-aged patients. Occasional to frequent focal epileptic activity (all three) and frequent runs of generalized spike-and-slow-waves (P1, P10) were seen in regular EEGs. Ataxia was first noticed when P8 started to walk aged 18 months, and within three years for P1 and P10. All gained the ability to walk independently, but always with a broad-based ataxic gait, and with limitations, such as the inability to walk downhill or on uneven ground (P10). Although P1 was able to speak, he did almost not communicate probably because he was highly introverted, and his speech was not intelligible for people outside the family. P8 had dysarthria, excellent receptive language abilities and was able to count up to ten. P10 spoke only one- to two-word sentences which were difficult to understand. In conclusion, this group of school-aged children reached developmental plateauing for at least years without achieving any new milestones in natural development and suffered from ongoing uncontrolled seizures with conventional ASMs.

All adult patients (P4, P5, P7) experienced their first febrile seizures at the age of five, six or ten months. Subsequently, myoclonic seizures occurred in all three, with additional absence seizures in P4 and P7. P4 became seizure-free at the age of 24 years, whereas P5 and P7 had ongoing GTCS, P7 more than monthly, despite 12 different ASMs having been tried with up to four in

combination. EEG showed occasional multifocal epileptic discharges (P5) and generalized spike-and-slow-waves (all adults). Ataxia started in the second year of life. P7 is still completely wheelchair-bound, whereas P5 walks with help of a rollator or another person and P4 without help, but severe ataxia of gait and all extremities. On MRI, severe cerebellar atrophy was present in all patients. P7 lost language skills soon after acquisition, whereas P4 and P5 developed good speech comprehension with rather slow and scanning expressive speech. Behavior and social interaction in those two patients were with reduced engagement and restrained (P4) or irritable and aggressive behavior (P5). The adult patients showed similar constant features without any substantial improvements over several years. Natural history in this age group with the longest course of disease did therefore not suggest any progress of development.

General aspects of treatment with 4-AP

Patients were treated for three to 75 months using maximum daily dosages of 0.15-2.6 mg/kg body weight (mean: 1.31 mg/kg/d; range of absolute dosages 6.5-120 mg/d, Fig. S6, S7). The earliest treatment effects were noticed at dosages of 0.5 mg/kg/d. Dose escalation was stopped when no further benefit could be observed after the last dose increase (P3, P4), but also when seizure freedom (P1, P2, P6) or worsening (P7) occurred. Two patients (P10, P8) transiently received more than 2.0 mg/kg body weight/d (up to 2.6) and were doing well with that dosage over several months but then deteriorated so the dose was reduced (see below, section on dose-dependent effects).

To generate a first impression of treatment effects, we differentiated four categories of therapeutic responses (marked improvement, mild improvement, no effect, or worsening) for seizures, ataxia and cognition/speech (Table 1, Fig. S8, Table S2), based on medical examinations, histories taken by the parents, and videos before and after treatment. To better objectify any improvements upon treatment with 4-AP, we next used seizure counts and standardized scores for (i) overall clinical improvement, (ii) EEG recordings, (iii) motor function and cognition, as reported in the following paragraphs and illustrated in Fig. 3 (for methodological details seeMaterials and Methods and Supplementary Material).

First, for a standardized clinical evaluation, the overall change of patient's health state was determined by the 7-point Clinical Global Impression–Improvement scale (CGI-I) (*30*) as a reference measure for overall functioning in daily living (see Materials and Methods section). According to this rating, nine of the eleven patients improved upon treatment with 4-AP at least 'minimally'. Eight of the nine responders improved 'very much' or 'much', whereby the means of the preschool children were slightly better (mean: 1.5, that is between 'very much improved' and 'much improved') than for school children (mean: 1.7) and clearly better than for the adult group (mean: 2.5, that is between 'much' and 'minimally' improved). The numbers in each group were too low to differentiate the three groups in statistical tests (Fig. 3A). Effects on different symptoms (seizures, ataxia, cognition/speech) and EEG of those nine responders are described in detail in the following paragraphs. The two non-responders (P7, P11) are described separately thereafter.

Responders to 4-AP - Differential treatment effects (n=9; P1-P6 and P8-P10)

Our main finding is that all patients reached seizure-freedom upon treatment with 4-AP for all seizure types except GTCS, including previously pharmacoresistant daily absence, myoclonic, or atonic (including drop) seizures (summarized as non-GTCS) for the whole observation period ranging from three to 75 months (Table S2, Fig. S1, Fig. S7, Fig. S8). Only P1 and P2 experienced a few absences for several days provoked by relative dose reduction (see below, section on dose-dependent effects). In addition, the two preschool aged patients (P6 and P9) suffering from two to eight GTCS per month, had marked improvement in GTCS (> 50% reduction of seizure frequency, Fig.3C and Table S2). P1 and P2 only had two or three GTCS each before 4-AP initiation and were therefore disregarded in this group, but also remained seizure-free during treatment with 4-AP. At the start of 4-AP, two of the nine responders (P3 and P4) were seizure-free on ASM, but still showed epileptic EEG discharges (P3 and P4) or slow background activity (P4; for P7 and P11, see *Non-responders to 4-AP* section below). For P5, P10 and P8 who experienced exclusive GTCS in the years before starting 4-AP – neither improvement nor deterioration of GTCS frequency was observed.

All patients showed multifocal or generalized epileptic discharges, as is typical for the *KCNA2* GOF or GOF+LOF groups (*5*). We used the standardized computer-based organized reporting of EEG (SCORE) and counted epileptic discharges to quantify changes of the EEG upon treatment with 4-AP. EEGs before and after treatment with 4-AP were available for all responding patients (P1-P6 and P8-P10). Quantification could be done for all of them (Fig. 3C), except for P1 and P9. Long-term-EEGs of at least several hours could be analyzed and quantified in two patients (P2 and P10). Epileptic activity was markedly (P9 – only estimated from first inspection, no quantification – and P10) or mildly (P2, P3, P5, P6, P8) reduced, or unchanged (P1 and P4) in the responding patients (Figs. 3D, S6, S8). On average, the posterior dominant rhythm improved after start of 4-AP treatment (Fig. 3D). When excluding previously seizure-free patients (P3, P4), overall improvements in seizure control (marked or mild) went along with quantified improvements (marked or mild) in EEG in most of the responding patients (P2, P5, P6, P9, P10; Figs.3, S8), except P1 (seizures markedly improved, EEG unchanged) and P8 (seizures unchanged, EEG mildly improved, but epileptic discharges were occasional to uncommon and only routine EEGs were available).

To quantify individual improvements in motor function and ataxia in this highly heterogeneous cohort in terms of age and severity of symptoms, we used both a comprehensive and careful clinical evaluation (categorized as marked or mild improvement, no change or worsening) and standardized scores.

To capture the effects on overall motor function, we leveraged the standardized score WeeFIM, the responsiveness of which to clinical changes in neurologically impaired children has been proven (*31*). In addition, to capture more symptom-specific severity the SARA score (earliest valid $\geq$ 7 years), was performed for all responding school-aged children and adults (except P5, who still needs a rollator walker) to quantify ataxia severity. According to these scores, all nine responders showed a clinical improvement of their motor functioning. The WeeFIM overall motor function subscore was significantly improved (Fig.3B, $p<0.05$) in the preschool and school age groups (preschool age: at start of treatment: 28.3 ± 3.3; last follow-up: 60.7 ± 6.4; school-age: at start of treatment: 45.7 ± 7.8; last follow-up: 64.3 ± 14.8; adults: at start of treatment: 44.5 ± 28.5; last follow-up: 47.0 ± 26.0; Repeated Measures ANOVA on ranks with Tukey post-hoc test, $p<0.05$). SARA scoring for specific symptoms of ataxia could be performed in four patients (P1, P4, P8, P10).

On overall clinical evaluation, six patients were considered to have achieved marked improvements of gait. Four were preschool children (P2, P3, P6, P9) and two school children (P1, P8; Figs. 3, S6). Two adults (P4, P5) and one schoolboy (P10) improved mildly (Fig. 3, Movie S2). Targeted movements and fine motor functions improved markedly in four patients (P1, P2, P6, P8) and mildly in another five patients (Fig. 3, Table S2). Ataxia and motor function benefits were not sustained in P8 (see *Examples* below). Improvement in motor function went along in the majority of patients with seizure control: four of six patients showed marked improvement in motor function and seizure control (P1, P2, P6, P9) and in two of three patients mild improvement in motor function and seizure control was observed (P5, P10; Fig. 3).

In the following two paragraphs, we include specific examples, partially illustrated with movies, we start with examples for gait ataxia (P1, P2, P4, P9, P10; also with suppl. Movies S1, S2). P2, a 3.5-year-old girl, had problems walking without assistance and frequently fell before treatment. After initiation of 4-AP she showed markedly improved gait, less broad-based and more stable, and falls disappeared. Since treatment has started, the patient can jump, run and climb stairs independently. She learned to ride a bicycle with training wheels. These activities were not possible before starting treatment (Movie S1). Similarly, a teenager of 14 years (P10) learned how to use a bicycle as a balance bike, learned to walk downhill for the first time, jumping on the sofa or standing still with a bottle of water in his hands.

As an example of improvement of ataxia of extremities, P8, a girl at 10 years of age, suffered from severe appendicular ataxia and scored 10 points less in the SARA ataxia score after initiating 4-AP treatment, which reflects not only a faster and more stable gait, but also a markedly improved dysdiadochokinesia (Movie S3). After six months this effect became less prominent, which might have been associated with several viral illnesses and puberty onset with catamenial seizures, (4-AP was stopped, and re-started, however, no re-improvement was obtained). The teenaged boy P10 learned how to put the key in the lock and could eat with a knife and fork. Mild improvements like tying the shoelaces made life easier for P4.

In addition to clinical evaluation, we did a scoring of the WeeFIM for cognition and speech to judge treatment effects. All nine patients benefitted regarding alertness, motivation, concentration, and interaction with other people (Table S2). All responding patients showed mild or marked (P1, P2, P9) improvement regarding alertness, motivation and psychomotor speed, which was also the feature that parents reported mostly. Six patients improved their speech. Again, the highest yield was in the preschool and school aged children (WeeFIM subscore: preschool age: at start of treatment: 9.7 ± 1.8; last follow-up: 17 ± 0.6; school-age: at start of treatment: 11 ± 2.3; last follow-up: 18.3 ± 1.9; adults: at start of treatment: 19 ± 10; last follow-up: 20 ± 9). The adult group benefitted only mildly (P4), if at all (P5; see individual subscores for WeeFIM cognition, Table S5). Neurodevelopmental gains accelerated with 4-AP treatment (Table S5, Cognition-WeeFIM) and were much faster than we would have expected by age-dependent progress alone, particularly in young children. Furthermore, all patients showed a stable course between the last two follow-ups (at least three months) before starting 4-AP treatment (except P3, who improved minimally before 4-AP treatment; Table S5).

As an example for improvement of speech, P1, a 17 year old boy, had a slow, uneven speech cadence, unintelligible to non-family members. After six months of titrating 4-AP up to 0.8 mg/kg/day, he became much faster, more fluid and mostly intelligible. P2, a girl aged 3.5 years, only spoke single words before treatment and then talked increasingly in 3-5 word sentences

within a time period of three months. Very similarly, another girl (P3) first developed speech at three years and phrases of two to three words at almost five years of age. After starting 4-AP, a rapid growth of vocabulary was noticed by the parents with faster responses within several weeks. After one year on 4-AP treatment, she spoke in longer sentences with up to six words, and sustained improvements. Similarly, P9 (almost three years since commencing treatment) developed expressive language skills from few words within months to short sentences and larger vocabulary according to parents.

As examples for alertness and motivation, P6 was only treated for three months. In that short period of time, he was more awake and active, could better concentrate and focus during free playing and showed more initiative. Similar observations were reported by the parents of P10, who extended the range of occupying himself independently of the mother from only a few to more than 30 minutes, which was the most important improvement for the parents (more than freedom from absence seizures and mildly improved ataxia). P1 was treated with benzodiazepines due to uncontrolled seizures. After starting treatment with 4-AP, benzodiazepines could be stopped and he became much more alert, even more than before the medication with benzodiazepines, and could make first attempts on skis. In this case, we assume both the elimination of the sedative effect of the benzodiazepines and an additional stimulating effect on psychomotor speed of 4-AP as causative.

Non-responders to 4-AP (n=2, P7 and P11)

P11 (p.(Thr374Ala)) and P7 (p.(Leu298Phe)) did not respond to 4-AP. P11 was profoundly developmentally impaired with almost no progress at age seven months, despite seizure freedom on topiramate before starting 4-AP (Table S2). Treatment with 4-AP, maximized to a dose of 1.5 mg/kg/d, showed no beneficial effects on neurodevelopment, so that 4-AP was stopped after seven months of treatment. Quantification of epileptic discharges on EEG showed an increase in frequency after 4-AP (Fig. 3C), but seizures did not re-occur. In P7 (38 years), the frequency of GTCS increased from one to three per month after starting 4-AP at a relatively low dosage (10 mg/d, 0.15mg/kg/d) and therefore medication was stopped after three months. There was also no observable improvement of other symptoms on the low dosage used.

Tolerability and safety

Previous tolerability studies in people with multiple sclerosis identified dizziness, paresthesias, gait instability and nausea as most frequent adverse effects, with hepatitis and seizures also described at dosages below 0.5 mg/kg/d (*32*-*34*). The tolerability of 4-AP in our cohort was very good without any distinguishable side effects (except the aforementioned associated increase in seizure frequency in P7 at a very low dosage of 4-AP (0.15 mg/kg) and the dose-dependent deterioration of P10, see next paragraph). Particularly, we did not observe any cardiac side effects (for example a prolonged QTc interval) or previously described symptoms of overdosing, such as dizziness, insomnia, delirium or seizures (*35*). To our knowledge, descriptions of patients receiving dosages up to 120 mg/d without side effects are rare (*36*).

Dose-dependent clinical effects of 4-AP

We reduced 4-AP in three patients (P3, P4 and P10) for different reasons. In P3, 4-AP was gradually tapered off (from 50 mg/d to complete discontinuation over 11 days) to evaluate whether the favorable clinical course could be attributed to 4-AP treatment. Nine days after complete removal of the drug, a prolonged GTCS occurred (although she was seizure-free before 4-AP), as well as a marked deterioration of cognition (diminished psychomotor speed and attention deficit) and ataxia. Subsequent re-establishment of 4-AP in 10 mg steps per day up to 50 mg/d led to full recovery (seizure freedom, improvement of cognition and ataxia).

In P4, persistent evidence for benefit was doubted and we therefore reduced 4-AP from 20 to 10 mg/d; this resulted in frequent falls, sudden unexpected movements of the extremities (as a symptom of ataxia), and almost no communication. All effects were reversed after re-increasing the dosage to 20 mg/d. However, a second trial of 4-AP withdrawal about 2 years later did not lead to deterioration according to the father, so that the long-term effects of 4-AP in this patient remain questionable.

In P10, we first increased the dosage continuously in 10 mg steps up to 2.6 mg/kg body weight (120 mg/d) within two years. The parents and treating physicians had the impression of a slow but continuous improvement of this severely affected juvenile patient. After three months of treatment at a dose of 120 mg/d, P10 started to show instability of gait and an unusual accumulation of falls. To differentiate between effects of the co-medication and 4-AP, we first reduced lamotrigine by 100 mg/d, which did not improve the frequency of falls. We next reduced 4-AP to 100 mg/d, which markedly improved gait stability, but slowed psychomotor speed a bit. In addition, after one week a few absences and atonic seizures recurred, which however did not persist after the second week without further changes of the medication. After further reduction of 4-AP to 80 mg/d, the patient's gait deteriorated again, accompanied by aggressive and rejecting behavior, so that 100 mg/d (2.2 mg/kg body weight/d) seemed to be the optimum dose for P10.

P1 was switched from 40 mg/d of commercially available, sustained-release 4-AP to 45 mg/d immediate-release 4-AP prepared by a local pharmacy (to reduce costs of the medication, since treatment cost reimbursement was declined by the health insurance). This led to a serious deterioration with recurring absences, reduction of alertness and spontaneous speech (similar to the time before 4-AP). After changing back to sustained-release 4-AP, he re-gained seizure-freedom, and cognition and speech were again markedly improved as before.

In P2, episodes of cognitive and motor impairment and a recurrence of absences without an identifiable cause (except weight gain due to growth) were observed after several months on 15 mg/d. After increasing to 20 mg/d, the episodes and absences stopped.

Dosing of concomitant anti-seizure medications

Concomitant anti-seizure medication was not altered in six patients (P1, P4-P7, P9; see also Table 1) and even partially reduced in four patients (P2, P3, P10, P11). For P8, who did not respond to 4-AP regarding the effect on seizure frequency, the dose of lamotrigine (LTG) was increased. P10 experienced an increase of GTCS after an inpatient trial of dose reduction (LTG 200 mg/d to 150 mg/d, see detailed overview in Fig. S7). Consequently, LTG was increased again to 250 mg/d, when GTCS stopped again. However, following a gastrointestinal infection, he had another series of GTCS and LTG was increased to 350 mg/d. This aggravated his gait

disorder intolerably and LTG was decreased again to 200 mg/d with a subsequent seizure-free interval of seven months. He then received brivaracetam as an additional drug without a clear effect on the seizures (Fig. S7).

Online tool for selection of patients with GOF or GOF+LOF KCNA2 variants for treatment with 4-AP

The presented data suggest that treatment with 4-AP of patients with GOF or GOF+LOF variants improves their clinical outcome and overall functioning in everyday living. To select patients with GOF or GOF+LOF *KCNA2* variants for treatment and exclude those carrying LOF variants, who presumably should not benefit or could even deteriorate upon treatment with 4-AP, we generated an online support tool (www.kcna2-treatment.com, user: KCNA2, password: 4APtreatment). It is based on available electrophysiological results obtained from variants in the highly conserved K_V1.1 and K_V1.2 subunits and in the evolutionary conserved *shaker* channel of *Drosophila melanogaster*. Functional alterations (GOF, GOF+LOF or LOF) are listed in this regularly updated database and used to decide if 4-AP treatment could be an option for individual patients carrying *KCNA2* variants (Fig. S10). A user interface, in which physicians can insert the respective position and the amino acid exchange in *KCNA2*/K_V1.2, will indicate all functional changes that have been found in the three different channels, and whether treatment with 4-AP should be considered as a possibility for a n-of-1 trial according to our findings (that is for all variants showing GOF effects alone or GOF+LOF effects) or not (for variants with LOF effects alone) (Fig. S10). It is important to note, that all variants that we recorded in our previous studies in the K_V1.2 channel subunit (*5*, *11*) showed the same functional changes as in previous studies carried out in one of the closely-related channels.

Discussion

Our results suggest that nine of eleven patients with DEE due to GOF or GOF+LOF *KCNA2* variants experienced a clinical benefit in seizure frequency, cognition or ataxia from 4-AP administration. The most decisive factor for the best response was age at start of treatment based on the observation that effects were stronger in preschool- and school-aged children (see summaries in the text). This suggests that 4-AP treatment should be initiated as early as possible to maximize clinical response. In three patients, epileptic seizures could be controlled by conventional ASM before 4-AP was started. For the remaining eight pharmacoresistant patients, 4-AP treatment caused marked improvement in six patients, no effect in patient P8 and a seizure worsening in P7. All patients with non-convulsive seizure types (absence, myoclonic, atonic) became seizure-free, except some provoked seizures. Control of GTCS was markedly improved (>50% reduction) in all preschool-aged children, but not improved for all others. The remarkable decrease of epileptic EEG discharges observed in some patients went along with seizure control in most responding patients with ongoing seizures (except P1 and P8), which should be interpreted with some caution, as not all EEGs could be quantified and frequency of epileptic discharges is subject to natural fluctuation depending on many factors. Strikingly, nine patients had improvements of ataxia (gait and extremities), alertness/motivation and other abilities. Six of the nine responders showed improvement of speech. Additionally, the evaluation of the 4-AP treatment response and the patients' overall functioning in everyday living by using standardized scores (CGI and WeeFIM), supported a clear improvement of most patients on 4-AP treatment. We consider these favorable outcomes – at least to a large degree – to be due to treatment with 4-AP, since (i) the observed improvements in cognition, speech and ataxia were much faster than expected for the neurodevelopmental trajectory of each individual patient (based on the development before 4-AP was started) and (ii) improvements were also observed in adolescents and adults, in whom neurodevelopment has been largely completed, (iii) concomitant anti-seizure medication was kept constant (six patients) or reduced (four patients) (except P8, and some changes for P10), and (iv) we observed dose-dependent clinical effects of 4-AP in five patients. Two patients showed diminished effects over time (P8) or no deterioration after a second trial of removal of 4-AP (P4), raising questions about long-term beneficial effects of 4-AP. We did not find any general differences in the responses between patients bearing GOF versus GOF+LOF variants, except for P11 carrying the most severe variant p.(Thr374Ala).

As earliest treatment effects were noticed at dosages of 0.5 mg/kg/d, we suggest to aim for this dosage in a first approach (approximately 30 mg/d in adults; please see results section for a more detailed description of dose escalation), in case 4-AP will be considered in further patients. Dosages between 0.8 and 2.2 mg/kg body weight/d were effective as maintenance dosages in those patients, who showed marked improvements. Regular controls of ECG and EEG should be performed at least after every further increase of 10 mg/d. To our experience, it is also necessary to have sufficient time for valid and comprehensive clinical evaluations. Therefore, we suggest an observation period of several weeks after increases of every 0.5 mg/kg body weight/d. We suggest that further dose escalation should be stopped as soon as seizure freedom is achieved and no further improvement in ataxia or cognition can be observed. Upon seizure worsening, ECG abnormalities or occurrence of any other side effects (such as dizziness, insomnia (*35*)), the dosage should be reduced or 4-AP discontinued as appropriate.

Two patients (P7, P11) did not benefit from 4-AP. Although P11 was seizure-free before 4-AP treatment, we consider him as one of the most severely affected patients due to his severe clinical

syndrome (no speech, head control or turning of the body). He carries a variant with mixed GOF+LOF features (p.(Thr374Ala)), for which in vitro recordings had shown a LOF predominance (strong dominant-negative effect) (*5*). This might explain the lack of response to 4-AP of this variant in vitro and clinically. Nevertheless, it is remarkable that this patient did not experience clinical side effects up to a dose of 1.5 mg/kg body weight/d including no recurrence of seizures; however, his EEG deteriorated which might have been due to an enhancement of the LOF effect by 4-AP. P7 carries the same GOF variant as P6 (p.(Leu298Phe)), who showed marked clinical improvements. A different genetic background, the different age and state of neurodevelopment, or other unknown factors may explain this discrepancy.

There was a discrepancy between the clinical response of P10 to treatment with 4-AP and the absence of a blocking effect in vitro, when p.(Leu328Val) mutant subunits were expressed alone. The failure of 4-AP blocking p.(Leu328Val) mutant channels could be explained by the localization of Leu328 near the 4-AP binding site (*37*). However, co-expression of mutant with WT K_V1 subunits re-established the sensitivity to 4-AP, so that the clinical benefit of the drug can be explained, since mutant channels will most probably not be expressed alone in vivo *(38)*.

Previous studies of patients with common epilepsies of different etiologies have shown that patients treated with the first ASM achieve seizure freedom in about 50% of cases, with the second ASM only in 10-15% of cases, whereas with the third or even more regimens of ASM only a few percent of the initial cohort reach seizure freedom (defined as a seizure-free interval of at least twelve months after start of the medication) (*39-41*). In our cohort, a mean number of 5.8 ± 1.0 ASM was tried before the initiation of 4-AP and seizure freedom could be gained for non-GTCS in six patients, as well as a marked improvement of GTCS in two of five patients. This highlights that treatment with 4-AP can adequately control seizures, especially non-GTCS, and has a clear additional benefit even in our highly pharmacoresistant group of patients.

In previous non-epilepsy studies, severe side effects were reported with wrongly dosed tablets or intoxications through overdoses (*42-44*). In our cohort, only P7 carrying a clear GOF variant (same as P6) experienced more GTCS at a very low dosage of 4-AP (0.15mg/kg body weight/d). None of the nine other patients reported any of the described side effects or toxicity signs (*35, 45*), such as dizziness, insomnia, delirium or seizures, up to dosages of $\geq$1.0 mg/kg in several patients. To our knowledge, descriptions of patients receiving dosages up to 120mg/d are rare (*36*). Although 4-AP has not been reported to induce clinically relevant cardiac effects, such as arrhythmias or alterations in ECG morphology in a thorough QT/QTc trial with healthy subjects up to 60 mg/d (*46*) and in spinal cord injury patients up to 30 mg/d (*47*), we carefully monitored each increase of 4-AP, since $K_V1.2$, $K_V1.5$ and $K_V2.1$ channels are expressed in human ventricle (*48*) and 4-AP has been shown to prolong action potentials (APs) in a canine ventricular cell model (*49*). Serial ECGs were also performed in our cohort with each 4-AP increase, and no ECG alterations or cardiac side effects (such as a prolonged QTc interval) were observed. In previous studies, severe side effects were reported with intoxications through overdoses (for example due to incorrectly formulated medication) (*7, 42-44*). Although commonly associated with overdose, seizures have been described at lower doses of 4-AP (even after 2 single doses of 5 mg (*32*)), with both immediate and extended release formulations (*50, 51*). Although numbers are small, the excellent tolerability of 4-AP in our patients, even in high dosages (0.6 to 2.6 mg/kg/d in the responders), is remarkable, given that side effects are reported in the literature at even lower dosages. This could be attributed to the GOF of mutant $K_V1.2$ channels, so that 4-AP

may reduce the activity of K_V1 channel complexes towards physiological amounts but not below – as would be expected in other individuals with normal K_V1 channel activity.

There are clear limitations of our study: (i) Due to the rarity of *KCNA2*-encephalopathy, our cohort was small and highly heterogeneous in terms of age and severity of symptoms. (ii) This is not a controlled trial, but an open, observational, longitudinal series of n-of-1 studies without a blinded evaluation of clinical data. (iii) For the younger patients, some gains may also have been related to their intrinsic developmental trajectory. (iii) Especially young patients experience improvements also due to their natural development. (iv) 4-AP is a blocker of the K_V1 subfamily and some other K_V channels (*13*-*15*), it is not specific for the $K_V1.2$ subunit. Therefore, although it is intriguing to assume that our clinically observed treatment effects are due to a reduction of the genetically-induced GOF on $K_V1.2$ channels, which are ubiquitously expressed in the central nervous system (*52*), other non-$K_V1.2$-specific effects could play a role for the clinical improvement (*53*). In addition, the observed beneficial effects of 4-AP might be attributed to an increase of presynaptic Ca^{2+} influx, which could explain short-term seizure cessation after administration of 4-AP in an absence epilepsy mouse model caused by mutant P/Q-type Ca^{2+} channels with decreased network excitability (*54*).

To provide guidance to treating physicians to select patients carrying GOF *KCNA2* variants for personalized treatment with 4-AP, we designed an online tool providing information whether *KCNA2* variants induce GOF or LOF effects. The tool is based on all available electrophysiological results obtained from variants in $K_V1.2$, $K_V1.1$ and *shaker* channel subunits (>300 variants collected from >30 publications; www.kcna2-treatment.com). This platform correctly predicted the functional effects of all electrophysiologically characterized *KCNA2* variants described to date. Therefore, it offers a publicly accessible support system for selecting suitable patients and to save precious time before initiation of 4-AP treatment, especially in young patients. This online tool thus aims to integrate precision medicine for epilepsy patients into a standard clinical workflow. However, although we could reproduce all results of previous studies in other channel subunits when we engineered the five variants in the $K_V1.2$ channel subunit (*5*, *11*), this is no guarantee that there will not be any differences caused by distinct subunits or experimental conditions. So care should be taken on initiating treatment with 4-AP, particularly regarding a possible worsening of seizures, observed in one of our patients with a clear GOF variant.

In summary, our report suggests a well-tolerated new therapeutic option – based on disease mechanism – for an otherwise largely pharmacoresistant, untreatable, severe developmental and epileptic genetic disorder.

Materials and Methods

Study design.

This study aimed to evaluate 4-AP as a precision therapy in individual patients carrying *KCNA2* GOF and GOF+LOF variants. To study the effects of 4-AP on different variants, *Xenopus laevis* oocytes, mammalian CHO cells and murine hippocampal neurons expressing either WT or mutant channels were used. We used two-microelectrode voltage clamping and the patch-clamp technique to determine changes in K^+ currents and firing behavior, respectively, due to the application of 4-AP. The methods for these experiments have been partially reported previously *(5, 11)* and are all reported in detail in the Supplementary Material and Methods Section.

N-of-1 trials with 4-AP were performed in nine different centers worldwide according to the discretion of the treating physicians following recommendations for dosage that we obtained (i) from previous experiences with patients suffering from other forms of ataxia and (ii) from the first patients treated in this study (see results, section on treatment with 4-AP and Table 1 for final dosing). Data on seizure counts per type of seizure, routine or long-term EEGs, gait performance, ataxia of extremities, cognition and language were collected as best as possible from the treating physicians and reports from parents before and after treatment with 4-AP, depending on the patients' individual situations and performance. They were evaluated retrospectively using seizure diaries, standard EEG measures, video recordings and established clinical scales for global performance, cognition and motor behavior, as outlined in detail in the Supplementary Material and Methods Section ("*Assessment of treatment effects with 4-AP")*.

Since this was not a controlled trial, but a summarized series of n-of-1 trials in nine different centers, there was no fixed study protocol, but rather an adaptation of dosing schemes from case to case depending on previous experiences with this drug and the observed effects in individual patients. Accordingly, we did not perform any size calculations, there was no randomization, and no blinding.

Data and statistical analysis.

Data were analyzed offline using Roboocyte2+ software (Multi Channel Systems) for oocyte data and Clampfit 10 (Molecular Devices). 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. Two-way ANOVA was used to compare the effects of 4-AP application between WT and mutant channels. Repeated Measures ANOVA on ranks with Tukey posthoc test was used for the WeeFIM score. Normality was tested using the Shapiro-Wilk test. Alpha-level was 0.05, only two-sided testing was used. To test the effect of 4-AP on seizures and EEG data, the Wilcoxon signed rank test for not-normally distributed data was used. Significance with respect to controls is indicated in the figures as follows: *P<0.05, **P<0.01, ***P<0.001.

Supplementary Materials

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Author contributions: U.B.S.H., S.L. and H.L. designed the study and wrote the manuscript, all authors revised the manuscript for intellectual content and approved the final version. S.L., M.W., M.S., A.B., J.M.S., P.M.U., N.M.A., M.D.K., K.G., B.B.Z., M.T., L.W.-K., D.M., G.R., S.M.S., C.B., M.F. and H.L. contributed to the recruitment and treatment of patients and quantification of clinical data. U.B.S.H., H.P.A. and K.T. designed, performed, and analyzed the experiments. N.S. designed and produced viral vectors. U.B.S.H., P.Y. and F.L. collected electrophysiological data from variants in $K_V1.2$, $K_V1.1$ and shaker channel subunits and designed the online tool.

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Data and materials availability: All data associated with this study are present in the paper or the Supplementary Materials. See data file S1 for primary data for main text and supplemental figures, in which experiments had an $n < 20$.

Figure legends:

Fig. 1: 4-aminopyridine affects variants altering properties of the K_V1.2 channel in *Xenopus* oocytes. (A) Structure of the voltage-gated K^+ 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 are localized within highly conserved regions in the N-terminus (E157K, green), the S4 segment constituting the voltage sensor (L290R, red; L293H, orange; R297Q, light green; L298F, dark green), the S5 segment (L328V, dark red) and the pore region (T374A, dark yellow). Gain-of-function (GOF) variants are shown as triangles, gain- and loss-of-function (GOF+LOF) variants with dominant GOF effects are shown as circles, GOF+LOF variants with dominant LOF effects are shown as boxes. **(B)** Representative current traces of K_V1.2 wild-type (WT, top) and K_V1.2 R297Q (bottom) channels recorded in a *Xenopus laevis* oocyte during voltage steps (from -80mV to + 70 mV) before (left traces) and after application of 1 mM 4-AP (right traces). **(C)** Mean voltage dependence of K_V1.2 channel activation for R297Q channels together with the activation curves for WT before and after application of 1 mM 4-AP. Shown are means ± SEM. Lines represent Boltzmann functions fit to the data points. *P<0.05). **(D)** Dose-response curve for WT, GOF (E157K (green triangle), R297Q (light green triangle), L298F (dark green triangle)), GOF+LOF (L290R (red circle), L293H (orange circle), L328V (dark red box), T374A (yellow box)) mutant channels recorded upon application of different 4-AP concentrations (in mM: 0.01, 0.1, 1, 5, 10, 30) and normalized to the mean values of the three pulses before 4-AP application for each cell. Mann-Whitney rank-sum and Dunnet's post hoc test. **(E)** Dose-response curve for WT K_V1.2 (black circle), WT K_V1.1 (black triangle), L328V (dark red box), coexpression of WT K_V1.2 and WT K_V1.1 (grey circle), K_V1.2 together with mutant L328V (dark red circle) and K_V1.1 together with mutant L328V (dark red triangle) recorded upon application of 1 mM and 5 mM 4-AP. Values are normalized to the mean values of the three pulses before 4-AP application for each cell (see Supplementary Materials).

Fig. 2: 4-aminopyridine affects primary hippocampal neurons overexpressing WT or mutant R297Q K_V1.2 channels. (A) Representative voltage traces of evoked action potentials (APs) recorded in the current clamp mode for neurons transduced with wild-type (black traces) or R297Q channels (green traces) before (left) and after (right) 0.1mM 4-AP application. **(B)** Spontaneous activity of neurons transduced with WT (black traces) or mutant R297Q (green traces) channels before (left) and after (right) application of 0.1 mM 4-AP. **(C)** Box-dot-plots of resting membrane potential of neurons transduced with WT or mutant R297Q channels before (closed symbols) and after (open symbols) 4-AP application. (two-way ANOVA,* p<0.05). **(D)** Minimal injected current (5 ms pulse) needed to elicit a single action potential for transduced wild-type and mutant channels. **(E)** Number of action potentials plotted over injected current. Shown are means ± SEM for each data point (WT, n=8; R287Q, n=12). The corresponding values for the area under the curve were: WT: before 4-AP: 2.4 ± 0.4; after 4-AP: 1.0 ±0.3; before 4-AP: 0.2 ± 0.2; after 4-AP: 0.5 ±0.3).* p<0.05. **(F)** Percentage stacked bar chart of spontaneous rhythmic activity in neurons transduced with wild-type (filled/unfilled grey bars, left) or R297Q channels (filled/unfilled green bars) before (left) and after (right) 0.1 mM 4-AP application. Filled bars inside the stacked columns show presence of spontaneous

activity. Cochran-Mantel-Haenszel test was used to show that the conditions were significantly different.

Fig. 3: 4-AP treatment improved clinical outcome of patients with *KCNA2*-GOF encephalopathy. (A) Bar graphs of CGI improvement of all eleven patients. Interval of last follow-up to one day before treatment was at least three months. Ataxia and motor function benefits were not sustained in P8. **(B)** WeeFIM subscores for motor function (bottom), cognition (middle) and total WeeFIM (top) at last follow-up before treatment (last F-u), start of treatment (0 d) and next follow-up at stable dose (next F-u). WeeFIM scoring was not done for patients, where treatment was very short due to sudden death for other reasons (P6) or non-response (P7, P11). For P9 no data was available for last F-u before treatment. (Repeated measures ANOVA on Ranks with Tukey posthoc test, * $p<0.05$). **(C)** Number of non-GTCS (absence, myoclonic or atonic seizures) per day and GTCS per month at last follow-up before treatment (last f-u), start of treatment (day 0) and next follow-up at stable dose (next f-u). For P5 frequency of non-GTCS could not be specified since they occurred occasionally, probably a few per month. (Wilcoxon signed-rank test, P=0.03) but not for GTCS (Wilcoxon signed-rank test, P=0.75). **(D)** Number of epileptic discharges per 20 min of EEG recordings (top) and posterior dominant rhythm (PDR, bottom) for patients P2, P5, P6, P8, P10, P11 before and after 4-AP treatment. For all patients except P2 and P5, two EEGs have been quantified after start of 4-AP treatment and showed similar outcome and are therefore not presented here. Quantification was not possible for patients P1 due to short treatment, for P7 due to lack of EEG recordings in our center after non-response, and non-availability of recordings for P9. "#" indicates a 10 h-recording (before 4-AP treatment) and a 4 h-recording (after 4-AP treatment) of EEG. * indicates 24 h EEG recordings. Wilcoxon signed-rank test was used.

Table 1: Treatment effects of 4-AP. Patients are listed according to the variants and their functional consequences (gain-of-function [GOF] or gain- and loss-of-function [GOF+LOF]), as well as their age at start of treatment. Alterations of the co-medication are indicated with an arrow (increase ↑, reduction ↓, → no change). Effects on clinical symptoms following treatment with 4-AP are indicated in three different categories (on seizures, ataxia and cognition & speech). Marked improvement is indicated as '++', mild improvement as '+', no effect as '○ '. Furthermore, patients are overall categorized according to the strength of improvement (marked improvement: '+' in all three categories and at least one '++' required; mild improvement: '+' in at least two categories required (compare also Tables S2-S5 for a detailed description of symptoms before and after 4-AP). **Abbreviations:** bw: body weight, d: day, F: female, M: male, m: months, sz: seizure, y: years; anti-seizure medication: ACZ: acetazolamide, KBr: bromide, CBZ: carbamazepine, CLB: clobazam, ESM: ethosuximide, LEV: levetiracetam, LTG: lamotrigine, PHT: phenytoin, TPM: topiramate, VPA: valproic acid, ZNS: zonisamide.
* initial dramatic effect (++) attenuated after 6 months and became gradually '+', then o

GOF variant	*Patient #*	*Sex*	*Age at onset (months)*	*Age at start of treatment (years.months)*	*Treatment duration (months)*	*Max. dosage (mg/d; mg/kg bw/d)*	*Alterations in co-medication since start of 4-AP treatment*	*Effect on seizures*	*Effect on ataxia*	*Effect on cognition & speech*
p.(Glu157Lys)	P1	M	9	17	29	70; 1.1	→ (VPA, LTG, ACZ)	++	++	++
p.(Arg297Gln)	P2	F	1	3.5	19	20; 1.0	↓ (ESM), → (LTG)	++	++	++
p.(Arg297Gln)	P3	F	10	4.10	73	55; 1.375	↓ (LCM, VPA)	sz free before	++	+
p.(Arg297Gln)	P4	M	5	26	75	40; 0.73	→ (LTG, ZNS)	sz free before	+	+
p.(Arg297Gln)	P5	F	10	37	65	30; 0.6	↓ (LEV), → (VPA, LTG)	+	+	+
p.(Leu298Phe)	P6	M	10	4.3	6	20; 1.0	→ (VPA)	++	++	+
p.(Leu298Phe)	P7	M	6	38	3	10; 0.15	→ (LEV, VPA, CBZ, ESM)	sz worsening	○	○
GOF+LOF var.										
p.(Leu290Arg)	P8	F	2	10	46	60; 2.61	↑ (LTG); → (CLB, ESM, ACZ)	○	++*	+*
p.(Leu293His)	P9	F	3	2.10	53	30; 1.76	→ (PHT, CLB)	++	++	++
p.(Leu328Val)	P10	M	6	12	60	120; 2.6	↓ (KBr, LCM); → (LTG); ↑ (BRV)	+	+	+
p.(Thr374Ala)	P11	M	1	0.7	14	12.5; 1.5	↓ (TPM)	sz free before	○	○