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FULL-LENGTH ORIGINAL RESEARCH

The molecular and phenotypic spectrum of *CLCN4*-related epilepsy

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Abstract

Objective: This study was undertaken to expand the phenotypic and genetic spectrum of *CLCN4*-related epilepsy and to investigate genotype–phenotype correlations.

Methods: We systematically reviewed the phenotypic and genetic spectrum of newly diagnosed and previously reported patients with *CLCN4*-related epilepsy. Three novel variants identified in four patients reported in this study were evaluated through in silico prediction and functional analysis by Western blot, immunofluorescence, and electrophysiological measurements.

Results: Epilepsy was diagnosed in 54.55% (24/44) of individuals with *CLCN4*-related disorders and was drug-resistant in most cases. Of 24 patients, 15 had epileptic encephalopathy and four died at an early age; 69.57% of patients had seizure onset within the first year of life. Myoclonic seizures are the most common seizure type, and 56.25% of patients presented multiple seizure types. Notably, seizure outcome was favorable in individuals with only one seizure type. All patients showed intellectual disability, which was severe in 65.22% of patients. Additional common features included language delay, behavioral disorders, and dysmorphic features. Five patients benefitted from treatment with lamotrigine. Most variants, which were mainly missense (79.17%), were inherited (70.83%). Whereas frameshift, intragenic deletion, or inherited variants were associated with milder phenotypes, missense or de novo variants led to more severe phenotypes. All evaluated *CLCN4* variants resulted in loss of function with reduced ClC-4 currents. Nonetheless, genotype–phenotype relationships for *CLCN4*-related epilepsy are not straightforward, as phenotypic variability was observed in recurrent variants and within single families.

Significance: Pathogenic *CLCN4* variants contribute significantly to the genetic etiology of epilepsy. The phenotypic spectrum of *CLCN4*-related epilepsy includes drug-resistant seizures, cognitive and language impairment, behavioral disorders, and congenital anomalies. Notably, the mutation type and the number of seizure types correlate with the severity of the phenotype, suggesting its use for clinical prognosis. Lamotrigine can be considered a therapeutic option.

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KEYWORDS

behavior disorders, Cl^-/H^+ exchanger, ClC-4, intellectual disability, seizure

Key Points

- All individuals with pathogenic *CLCN4* variants should be evaluated for epilepsy due to the high prevalence of seizures
- *CLCN4*-related epilepsy has a wide phenotypic spectrum, varying from mild brief absence seizures to severe EE
- Mutation type and number of seizure types correlate with phenotype severity, but genotype–phenotype relationships are not straightforward
- Some patients benefited from treatment with lamotrigine, which should be considered as a therapeutic option in the future
- All evaluated *CLCN4* variants resulted in loss of function

1 | INTRODUCTION

The CLC gene family contains nine members in mammals, four of which encode plasma membrane chloride channels (ClC-1, ClC-2, ClC-Ka, ClC-Kb) and five intracellular $2Cl^-/H^+$ exchangers (ClC-3–7).[1,2] Dysfunction of some of these leads to severe neurological disorders, including leukodystrophy, neurodegeneration, intellectual disability (ID), epilepsy, and lysosomal storage disease for ClC-2, ClC-3, ClC-4, ClC-6, and ClC-7,[3–16] highlighting the pivotal role of CLC proteins in the central nervous system. ClC-4, which is encoded by the *CLCN4* (Online Mendelian Inheritance in Man database # 302910) gene located on human chromosome Xp22.2, is a voltage-dependent $2Cl^-/H^+$ exchanger.[17–19] Its precise physiological function is unclear, but ClC-4 is likely to be involved in the ion homeostasis of endosomes and intracellular trafficking.[20–23] ClC-4 is broadly expressed across tissues, including brain, skeletal muscle, liver, kidney, intestine, and heart. In brain, it is prominently found in hippocampus and cerebellum.[11,24] Mutations in the *CLCN4* gene have been found to underlie X-linked ID, epilepsy, behavior disorder, and dysmorphic features.[6,8,12,15] In 2013, a de novo loss-of-function mutation (p.Gly544Arg) in *CLCN4* was first reported to cause early onset epileptic encephalopathy (EE) and severe developmental delay (DD).[12] Subsequently, five new *CLCN4* variants were detected in five families with ID and/or epilepsy.[6] As massive parallel sequencing has expanded, increasingly more variants in this gene have been reported in association with supplementary phenotypic features and a range of severity. To date, there is a lack of systematic investigation of the spectrum of seizure semiology and genotype–phenotype correlations in subjects with *CLCN4*-related epilepsy. In this study, we characterized the functional effects of newly identified variants. Furthermore, we assessed the clinical data of four unreported and all previously reported patients with *CLCN4*-related epilepsy to delineate the clinical features and molecular genetic findings and to assess genotype–phenotype relationships.

2 | MATERIALS AND METHODS

2.1 | Subjects

Four subjects from four unrelated families with epilepsy of unknown etiology were analyzed. All patients were clinically evaluated and underwent whole exome sequencing (WES). This study was approved by the Ethics Committee of Xiangya Hospital of Central South University, China (human study/protocol # 201603205) and performed in accordance with the ethical standards laid down in the Declaration of Helsinki. Written informed consent was obtained from the parents of all subjects.

2.2 | Sequencing and bioinformatic analysis of novel *CLCN4* variants

The sequencing of genomic DNA from peripheral blood and the bioinformatic analysis of the *CLCN4* variants are described in detail in the Supplementary Materials. Clinical significance of novel variants was determined using the American College of Medical Genetics and Genomics (ACMG) standard guidelines.[25]

2.3 | Systematic literature review

We conducted a systematic literature review focused on *CLCN4*-related epilepsy by searching the PubMed database using the keywords "*CLCN4*", "ClC-4", and "epilepsy". All publications were reviewed for case series or cohort studies that contained clinical data on patients with *CLCN4* variants.

Individuals were included if they had a pathogenic *CLCN4* variant and their phenotype included epileptic seizures. We rechecked all the variants with nucleotide and amino acid numbering according to the *CLCN4* reference transcript NM_001830.3 (reference protein NP_001821.2). We systematically reviewed the detailed clinical, molecular genetics, and functional characterization of the previously reported and newly identified individuals with *CLCN4*-related epilepsy.

2.4 | In vitro functional assessment of novel *CLCN4* variants

To assess the effect of the three novel ClC-4 variants, we performed Western blot analysis of expression levels, immunofluorescence imaging of subcellular localization, and electrophysiological measurements upon heterologous expression, as previously described[6,26,27] and detailed in the Supplementary Materials.

3 | RESULTS

3.1 | Identification of *CLCN4* variants in four families

Using trio-based WES, we identified three novel (p.Val455Ile, p.Thr532Lys, p.Leu625Phe) and one previously reported (p.Arg718Trp) *CLCN4* variants. All probands suffered from severe ID and intractable EE. No pathogenic variation was found in other epilepsy or ID candidate genes (detailed clinical information is given in the Supplementary Materials). The p.Thr532Lys variant affects a highly conserved residue of the helical intramembrane domain , the p.Leu625Phe variant substitutes a conserved residue in the first cystathionine-β-synthase (CBS) domain, and the p.Val455Ile variant alters a less conserved residue of the helical transmembrane domain (Figure 1 and Figure S1). The p.Thr532Lys and p.Leu625Phe variants were not present in any database (Table 1). The pathogenicity of p.Thr532Lys and p.Leu625Phe variants was classified as likely pathogenic in accordance with the ACMG guideline (Table 1). Although the p.Val455Ile variant had a very low frequency in gnomAD, ExAC, 1000 Genomes, and ESP6500, it is predicted to be benign (Table 1). The p.Arg718Trp variant was recently reported in two patients.[8,15]

3.2 | Phenotypic spectrum of patients with *CLCN4* variants

To date, a total of 40 individuals with *CLCN4*-related disorders have been reported.[6,8,12,15,28] Twenty of them had epilepsy and are the subjects of this study. Therefore, with our four patients, the total number of patients with *CLCN4*-related epilepsy known to date is 24. Thus, epilepsy was

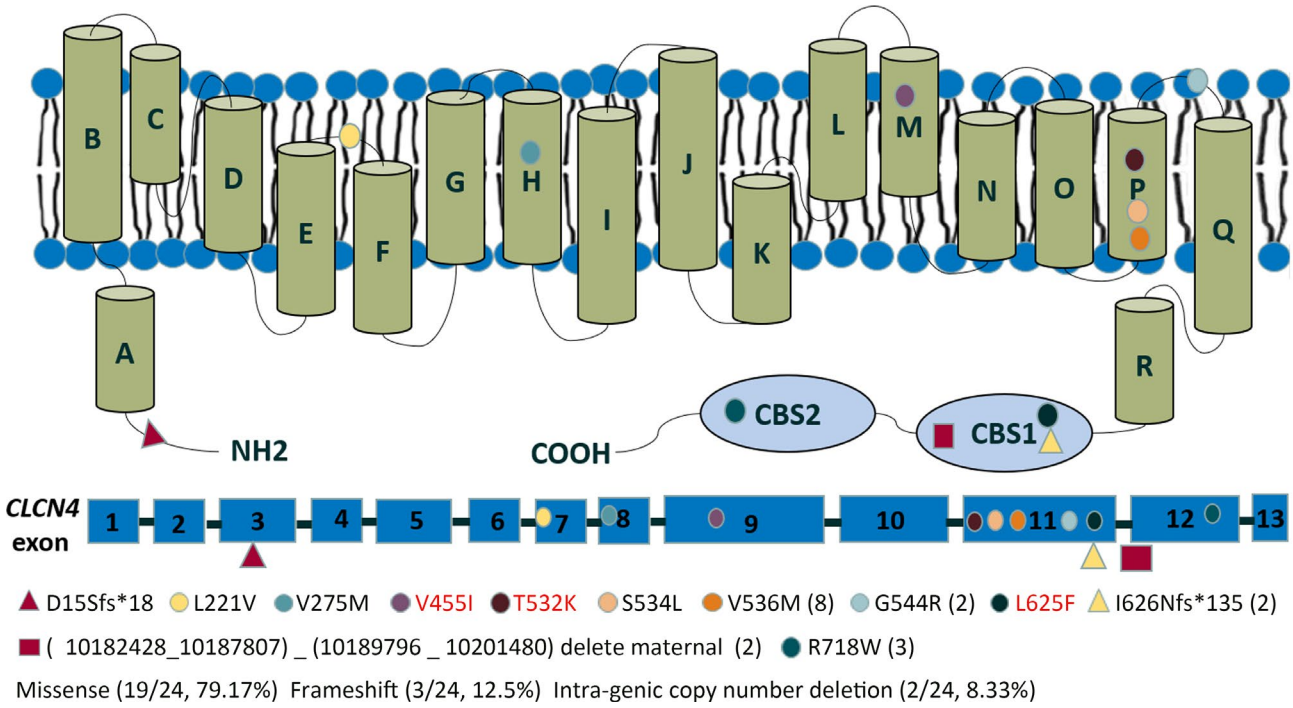

FIGURE 1 Schematic representation of ClC-4 topology and gene exon structure. The position of previously published and three novel *CLCN4* variants identified in *CLCN4*-related epilepsy are indicated. Triangles indicate frameshift, circles missense, and squares intragenic copy number deletion. The numbers in parentheses refer to the number of respective patients

TABLE 1 In silico analysis of novel *CLCN4* variants

Variants (NM_001830.3)	AA conservation	gnomAD	ExAC	1000 Genomes	ESP6500	SIFT	PolyPhen-2	CADD	MutationTaster
p.Val455Ile	Semiconserved	.00029.364e-05	.0003	.000264901	.0001	Tolerable (1.0)	Benign (.0)	Tolerable (8.498)	Polymorphism (1.0)
p.Thr532Lys	Highly conserved	–	–	–	–	Damaging (.0)	Probably damaging (.998)	Damaging (34)	Disease-causing (1.0)
p.Leu625Phe	Highly conserved	–	–	–	–	Damaging (.003)	Probably damaging (.915)	Damaging (24.1)	Disease-causing (1.0)

Abbreviations: AA, amino acid; CADD, Combined Annotation Dependent Depletion; ExAC, Exome Aggregation Consortium; gnomAD, Genome Aggregation Database; PolyPhen-2, Polymorphism Phenotyping v2; SIFT, Sorting Intolerant From Tolerant.

diagnosed in 54.55% (24/44) of individuals with *CLCN4*-related disorders at the time of reporting. The clinical features of the 24 patients with epilepsy are summarized in Table 2. As expected, males are more often affected than females, with 87.5% (21/24) of individuals being male and 12.5% (3/24) female. However, female patients can be as severely affected as the male counterparts. The seizure onset, ranging from neonatal to 14 years (data available for 23), was detected within the first year of life in 69.57% of patients. The majority (62.5%, 15/24) of affected individuals developed EE. Of the 15 patients with EE, four developed infantile spasms (IS), and one developed epilepsy of infancy with migrating focal seizures (EIMFS). The seizure types were available for review in 16 patients. Seven (43.75%) individuals had only one type of seizure, and nine (56.25%) patients showed multiple seizure types. Seizure semiology is variable, including myoclonic, absence, IS, tonic, complex partial, tonic–clonic, focal, and secondary generalized seizures. Myoclonic seizures, occurring in seven patients, were the most common seizure type.

In addition to the variability of the epilepsy phenotype, seizure outcome varied. One patient had brief absence seizures that did not require treatment. Twenty-three patients received antiseizure therapy. Thirteen of 23 (56.52%) patients were drug-refractory with at least two drugs failing that were appropriately applied.[29] Only 30.43% (7/23) were controlled on monotherapy, and three (13.04%) remitted after polytherapy. Seizures were refractory in all individuals with EE, and four patients died at an early age (2.75, 12, 16, and 19 years). In the EE group, four had IS, and three of them received adrenocorticotropic hormone therapy, which showed transient effectiveness in two of the three patients. Four patients were treated with a ketogenic diet in combination with other antiepileptic drugs (AEDs), which showed transient or no effectiveness on seizures. Of note, seizure outcome was favorable in individuals with only one seizure type, as seizures were controlled well with monotherapy or even without pharmacotherapy in all patients except one, whose seizures were controlled on polytherapy. Conversely, all nine individuals with multiple seizure types required two or more AEDs, and only two patients remitted. There is no established therapy for *CLCN4*-related epilepsy, and no superior medication was identified. Two (p.Val455Ile, p.Leu625Phe) patients with IS in this study had a trial of lamotrigine with good clinical response. One patient became seizure-free, and one showed dramatic improvement after other drugs had failed. Three previously reported individuals (one p.Gly544Arg, two p.Val536Met)[8] also showed significant improvement with the addition of lamotrigine. One patient was well controlled on carbamazepine monotherapy. By contrast, treatment with valproic acid was not effective for our four patients.

3.3 | Developmental, behavioral, neurologic, and facial features

Cognitive development has been monitored in all 24 cases with *CLCN4*-related epilepsy, and five patients showed developmental regression after seizure onset. Cognition before seizure onset varied from normal to severely delayed, and was abnormal during follow-up in all patients. Fifteen of 23 patients (65.22%) for whom information on cognition was available had severe ID; ID was moderate in six (26.08%) and mild in two cases (8.7%). Cognitive outcome was worse in patients with epilepsy than in those without epilepsy. Severe ID was observed in 15 of 23 (65.22%) patients with *CLCN4*-related epilepsy and in five of 20 (25%) patients carrying pathogenic *CLCN4* variants without epilepsy. Moreover, patients with severe seizures showed more severe cognitive impairment. However, cognition did not improve in most patients after seizures were controlled. Language delay was observed in 10 patients (41.67%, 10/24), and 25% (6/24) were nonverbal.

Neurologic examination showed anomalies in most patients. Microcephaly, defined as occipitofrontal circumference in the 3rd percentile or smaller, was observed in seven individuals (30.43%, 7/23). Six of them (85.71%, 6/7) developed EE. Infantile hypotonia, often more prominent in the limbs, was found in seven patients (29.17%). Additional neurologic features included hyperreflexia, nystagmus, spastic quadriplegia, extrapyramidal signs, and cerebellar symptoms. Other growth parameters, such as weight and height, were in the normal range for most patients. Behavioral disorders occurred in 10 patients (43.48%, 10/23), including aggression (30.43%, 7/23), autistic features (8.7%), hyperactivity (8.7%), self-abusive behavior (4.35%), irritability (4.35%), depressive disorder (4.35%), apathy and social disinhibition (4.35%), and bipolar disorder (4.35%). A variety of subtle facial dysmorphic features were noted in 15 patients (65.22%, 15/23). Notably, although most patients showed facial dysmorphisms, and the common facial features were long face, broad nasal bridge, and prominent chin, there was no recognizable facial pattern nor any facial features typical for pathogenic *CLCN4* variants.

3.4 | Electroencephalographic and magnetic resonance imaging features

Electroencephalography (EEG) and neuroimaging were also variable with no consistent findings. EEG data were available for 14 patients; 11 individuals (78.6%, 11/14) displayed epileptiform discharges, and three of them remitted. Three patients without epileptiform discharges were controlled on monotherapy. The spectrum of EEG abnormalities was wide, and no specific EEG abnormalities were found. Slow background activity and multifocal spike-and-slow waves were the most common patterns observed on EEG. Three patients with IS showed an hypsarrhythmia EEG, and one patient showed IS without hypsarrhythmia. Neuroimaging was available for review in 15 patients and showed brain structural abnormalities in 12 (80%, 12/15) individuals. The most common abnormalities were corpus callosum hypoplasia (46.67%, 7/15) and white matter changes (46.67%). Ventriculomegaly was observed in four cases. Cerebellar atrophy was evident in three cases and cerebral atrophy in two cases. Delayed myelination was evident in two patients who were imaged after 3 months of age. Other findings include small perivascular spaces, small hippocampus, enlarged subarachnoid spaces, and subcortical fluid-attenuated inversion recovery/T2 hyperintensity.

3.5 | Molecular genetics

Nine different variants were reported previously for the 20 patients with *CLCN4*-related epilepsy. In this study, we identified three novel variants (p.Val455Ile, p.Thr532Lys, p.Leu625Phe), and one previously reported variant (p.Arg718Trp). Thus, a total of 12 distinct *CLCN4* variants have been identified in 24 patients from 15 independent families (Table 3). Most variants (70.83%, 17/24) were inherited, and seven variants arose de novo, including in one patient who was mosaic. The most common types of variants are missense variants (79.17%, 19/24), followed by frameshift variants (12.5%, 3/24) and a small intragenic copy number deletion (8.33%, 2/24). In total, only two variants were recurrent, including p.Gly544Arg in two and p.Arg718Trp in three patients. The ClC-4 protein is a dimer with two identical subunits, each of which contains a pore responsible for the selective coupling of the Cl^- flux to H^+ countertransport.[1] Each subunit contains 18 α-helices and two CBS domains. Four variants mapped to the helical intramembrane domain (H-IM) (p.Val275Met, p.Thr532Lys, p.Ser534Leu, p.Val536Met), one to the helical transmembrane domain (H-TM) (p.Val455Ile), one to the cytoplasmic region (p.Asp15Serfs*18), two to the loop domain (p.Leu221Val, p.Gly544Arg), and four to the CBS domain (p.Leu625F, p.Ile626Asnfs*135, chrX: g.[10182428_10187807]_[10189796_ 10201480] delete maternal, p.Arg718Trp; Figure 1). The *CLCN4* gene is composed of 13 exons, and variants were identified in Exons 11 (50%), 12 (16.67%), 3 (8.33%), 7 (8.33%), 8 (8.33%), and 9 (8.33%), whereas no variants were reported for Exons 1, 2, 4, 5, 6, and 10. To date, functional studies have been performed for six missense variants (p.Leu221Val, p.Val275Met, p.Ser534Leu, p.Val536Met, p.Gly544Arg, p.Arg718Trp). All evaluated *CLCN4* variants resulted in loss of function with significantly reduced current amplitude.[6,8]

TABLE 2 Clinical details of patients with *CLCN4*-related epilepsy

Variants	Country of origin	Gender	Age at case report	Affected individuals	Affected families	Head circumference
Asp15Serfs*18	Belgian	Male	52 years	1	1	50th–97th centile
Leu221Val	Anglo-Australian	Male	16 years	1	1	50th centile
Val275Met	Anglo- American	Female	10 years	1	1	25th centile
Val455Ile	Chinese	Male	3 years 7 months	1	1	50th–75th centile
Thr532Lys	Chinese	Male	2 years 9 months	1	1	<3rd centile
Ser534Leu	Northern European	Male	3 years	1	1	<3rd centile
Val536Met	Anglo-Australian	7 male, 1 female	12 years, 16 years, 19 years, 42 years, 66 years, NR (3)	8	1	50th–98th centile
Gly544Arg	Dutch (1), North American (1)	2 male	14 months, 10 years	2	2	2nd centile
Leu625Phe	Chinese	Male	5 years	1	1	75th–98th centile
Ile626Asnfs*135	Kurdish	Male	24 years, 22 years	2	1	2nd−50th centile
chrX: g (10189796_10201480) del mat	Hispanic	2 male	14 years, 15 years	2	1	3rd–>98th centile
Arg718Trp	Scottish (1), Chinese (2)	2 male, 1 female	1 year 6 months, 3 years, 15 years	3	3	<3rd−75th centile (2), NR (1)

Variants	Level of ID	Regression	Infantile hypotonia	Hypertonia
Asp15Serfs*18	Moderate	No	No	No
Leu221Val	Moderate	No	Yes	No
Val275Met	Severe	Yes	No	Yes
Val455Ile	Severe	No	Yes	No
Thr532Lys	Severe	No	No	Yes
Ser534Leu	Severe	No	Yes	Yes
Val536Met	Mild (1), moderate (2), severe (5)	Yes (1), no (7)	Yes (1), no (7)	No (8)
Gly544Arg	Severe (2)	No (2)	Yes (1), no (1)	No (2)
Leu625Phe	Severe	Yes	No	No
Ile626Asnfs*135	Moderate (2)	No (2)	No (2)	No (2)
chrX: g.(10182428- 10187807) _(10189796_10201480) del mat	Severe (1), mild (1)	Yes (1), no (1)	Yes (1), no (1)	No (2)
Arg718Trp	Severe (2), NR (1)	Yes (1), no (2)	Yes (1), no (2)	Yes (1), no (2)

Height	Weight	Seizure onset	Seizure types	EE	Seizure-related death	Speech abilities
25th–>98th centile	75th–90th centile	NR	Absence	No	No	LD
25th–50th centile	25th−50th centile	Infancy	CP	No	No	Severe LD
25th–50th centile	75th centile	13 months	GS, M, CP	Yes	No	NV
<3rd centile	<3rd centile	Neonatal	IS, M, GT, FS	Yes, IS	No	NV
<3rd centile	<3rd centile	2 months	IS, M, GT	Yes, IS	Yes, 2 years 9 months	NV
75th–90th centile	75th–90th centile	3 months	IS, M, T, absence	Yes, IS	No	LD
25th–>98th centile	3rd−75th centile	Infancy/early childhood (8)	CP, GTC, atonic	Yes (5), no (3)	Yes (3/8), 12 years, 16 years, 19 years	LD (4), normal (4)
<1st centile (1), NA (1)	<1st centile (1), NA (1)	4 months (1), <1 year (1)	Absence, GTC (1), CP, secondary GS (1)	Yes (2)	No (2)	NV (1), NR (1)
3rd−15th centile	15th–25th centile	5 months	IS, M, GTC	Yes, IS	No	Severe LD
10th–50th centile	50th–75th centile	Infancy (2)	GS (2)	No (2)	No (2)	Normal (2)
50th–95th centile	50th centile	6 months (1), 14 years (1)	FS, GS, M (1), GTC (1)	Yes (1), no (1)	No (2)	Severe LD (2)
85th–75th centile (1), NR (2)	15th–98th centile (2), NR (1)	6 months (1), 1 month (2)	Absence (1), CP (1), M, GT, FS (1)	Yes (2), EIMFS (1), no (1)	No (3)	NV (2), NR (1)

Other neurological features	Behavioral issues	Facial dysmorphism	Other issue
Shuffling gait	Aggression	Long face, PC	No
Clumsiness and fatigue on walking	Depressive disorder	Long face	Gastroesophageal reflux, asthma
Increased peripheral tone, brisk patella reflexes, ABS	No	No	No
Tendon hyporeflexia, ABS	Repetitive patting on the head	Long face, BSF, DNB	No
Nystagmus	No	Long face	Right inguinal hernia, congenital laryngeal chondrodysplasia
CVI, evolving upper limb hypertonia and spasticity	No	Round face, small pointed chin	FD, recurrent aspiration, capillary hemangioma
PS, unsteady gait (2), diplegia (2), ataxia (1)	Aggression (3), hyperactivity (1), bipolar disorder (1), no (5)	Long face, PC, slightly "coarse" facial features, DNB (4), no (4)	No
Dystonic posturing (1), PS, unsteady gait (1)	Apathy, social disinhibition, motor stereotypies (1), no (1)	Round face, downsloping palpebral fissures, open mouth (1), NR (1)	FD (1), NR (1)
Unsteady gait	Aggression, hyperactivity, irritability	Congenital left upper eyelid ptosis	No
No (2)	Aggression (2)	Long face, PC, lean body habitus (2)	No (2)
Gait abnormality, CVI, nonprogressive upper limb choreiform movements (1), no (1)	No (2)	High arched palate (1), no (1)	No (2)
ABS (1), NR (2)	Drooling, some self-abusive behavior (1), no (1), NR (1)	BSF, deep set eyes, wide philtrum, full lower lip (1), no (2)	FD, scoliosis (1), no (2)

(Continues)

TABLE 2 (Continued)

Variants	EEG
Asp15Serfs*18	No epileptiform activity
Leu221Val	Mildly abnormal due to SB
Val275Met	High-amplitude discharges with excessive generalized SB and absence of posterior rhythm
Val455Ile	Multifocal spike wave, SSW, PSSW, AH, SB
Thr532Lys	Multifocal spike wave, SSW, PSSW, AH
Ser534Leu	Focal activity evolving to intermittent multifocal spikes and generalized SB
Val536Met	Potentially epileptogenic activity throughout recording (1), not tested (7)
Gly544Arg	SB, temporal spikes (1), high-amplitude spikes, generalized spike and polyspike waves (1)
Leu625Phe	Multifocal or generalized sharp waves, spike waves, SSW, polyspike waves IH
Ile626Asnfs*135	Not tested (2)
chrX: g.(10182428_10187807)_(10189796_10201480) del mat	Bioccipital spikes, generalized spikes, generalized SB (1), normal (1)
Arg718Trp	SB, IH, multifocal spike waves, PSSW, SSW (1), multifocal discharges (1), not tested (1)

Abbreviations: ABS, abnormal Babinski signs; AH, atypical hypsarrhythmia; BSF, broad square forehead; CA, cerebellar atrophy; CCH, corpus callosum hypoplasia; CP, complex partial; CVI, cortical visual impairment; DM, delayed myelination; DNB, depressed nasal bridge; EE, epileptic encephalopathy; EEG, electroencephalogram; EIMFS, epilepsy of infancy with migrating focal seizures; FD, feeding difficulties; FLAIR, fluid-attenuated inversion recovery; FS, focal seizures; GS, generalized seizures; GT, generalized tonic; GTC, generalized tonic–clonic; ID, intellectual disability; IH, intermittent hypsarrhythmia; IS, infantile spasms; LD, language delay; M, myoclonic; NA, not available; NR, not reported; NV, nonverbal; PC, prominent chin; PS, progressive spasticity; PSSW, polyspike and slow waves; SB, slow background; SSW, spike and slow waves; T, tonic; TCC, thin corpus callosum; WML, white matter loss.

3.6 | Genotype–phenotype correlations

Frameshift or intragenic deletion variants are associated with milder epileptic phenotypes, and seizures were easily controlled compared with most individuals with missense variants. The majority (4/5) of individuals with frameshift or intragenic deletion variants were controlled on monotherapy, whereas only 21.05% (4/19) of patients with missense variants achieved this with monotherapy or no treatment, and four of them died before the age of 19 years. In addition, the degree of ID was greater in individuals with missense variants. Severe ID was observed in 20% (1/5) of cases with frameshift or intragenic deletion variants and in 77.78% (14/18) of cases with missense variants. Hence, phenotypes of individuals with frameshift or intragenic deletion variants were relatively mild compared with the majority of individuals with missense variants. In comparison to inherited variants, de novo variants were more prone to cause more severe phenotypes. All patients with de novo variants had severe ID, and 85.71% (6/7) of these had intractable EE. In contrast, inherited variants are less frequently found among patients with severe ID (52.94%, 9/17) and EE (52.94%, 9/17). We observed no definite correlation between the location of the variants and the severity of the associated phenotype. Even with the same variant, a large phenotypic variance is observed. This is exemplified by the recurrent p.Arg718Trp variant, which has now been described in three unrelated patients, two in previous studies[8,15] and one here. Of the three patients, one had brief absence seizures with onset at 8 years of age and no need of treatment. In contrast, two suffered from intractable EE starting at the age of 1 month. This variability was illustrated by a considerable intrafamilial variability in disease severity. For example, eight patients from one family carrying the same variant (p.Val536Met) had distinct phenotypes.[6,8] Drug-resistant epilepsy was present in six individuals and early death occurred in three individuals, possibly because of limited antiepileptic treatment options.[8] However, seizures were controlled on monotherapy in two individuals. In addition, they also showed different degrees of ID, which was evaluated as severe in five patients, moderate in two, and mild in one.

3.7 | Functional findings of three novel *CLCN4* variants

To investigate the functional consequences of the three novel ClC-4 variants, we first tested their protein levels by Western blot analysis. The three mutant proteins were expressed at levels similar to wild-type (WT) ClC-4-FLAG in HEK293

Neuroimaging	Response to treatment
Not tested	Well controlled on monotherapy, medication ceased by 7 years
Small perivascular spaces seen in the inferior aspects of the putamen bilaterally	Well controlled on carbamazepine monotherapy
Persistently enlarged 3rd ventricle, nonspecific subcortical FLAIR/T2 hyperintensity left frontal region	Refractory to polytherapy
Periventricular leukomalacia	Refractory to polytherapy
CCH white matter reduction in posterior horn of lateral ventricle	Refractory to polytherapy
Periventricular WML with compensatory dilation of lateral and third ventricles	Refractory to polytherapy
Marked supra- and infratentorial atrophy, TCC, CA (2), ventriculomegaly (1), cortical atrophy, WML (1), not tested (6)	Well controlled on monotherapy (2), refractory to polytherapy (6)
CCH, increased FLAIR signal in white matter (1), normal (1)	Refractory to polytherapy until lamotrigine added (1), refractory (not fully responsive to medications) at 14 months (1)
Normal (2 years), CA (4 years)	Refractory to polytherapy until lamotrigine added
Not tested (2)	Controlled on monotherapy medication ceased by 7–10 years of age (2)
DM, TCC, global cerebral atrophy, particularly affecting the cerebral white matter (1), normal (1)	Refractory to polytherapy (1), controlled on levetiracetam monotherapy (1)
TCC (2), white matter reduction, small left hippocampus, left ventriculomegaly, DM (1), normal (1)	Refractory to polytherapy, but one finally remitted (2), not treated (1)

cells (Figure 2A). To test for the functionality of the ClC-4 variants, we performed electrophysiological recordings of WT and mutant ClC-4 in HEK293 T cells. All three novel variants yielded drastically reduced outwardly rectifying currents compared to WT, demonstrating loss of function (Figure 2B,C). We next assessed the subcellular localization of the ClC-4 variants upon heterologous expression in HEK293T cells. In agreement with previous reports,[23,30] WT ClC-4 localized predominantly to the endoplasmic reticulum (ER), which was positive for the coexpressed marker calnexin (Figure 2D). ClC-4, seemingly devoid of endosomal localization signals,[31] can heterodimerize with its paralogue ClC-3,[30] enabling the transport of ClC-3/ClC-4 dimers from the ER to endosomal–lysosomal compartments.[23,30,32] When we coexpressed WT ClC-4 with ClC-3b in HEK293T, both proteins expectedly colocalized to slightly enlarged endosomal compartments (Figure 2D). The three ClC-4 variants colocalized with ClC-3b, demonstrating that the ability to heterodimerize was not impaired. However, in addition to the vesicular localization, we observed a slightly more pronounced residual reticular pattern of ClC-3b/ClC-4 for ClC-4 variants than for WT (Figure 2E). This might be due to stronger ER retention of mutant ClC-4 and contribute to the loss of function observed in the electrophysiological measurements.

4 | DISCUSSION

Pathogenic *CLCN4* variants have been reported in patients with X-linked ID, epilepsy, behavioral disorder, and dysmorphic features. As expected for an X-linked disease, females are less often affected. However, the affected females can develop as severe phenotypes as males. The underlying complexity of X-chromosome inactivation in cells and tissues[33] hinders a prediction of the outcome. So far, *CLCN4* variants have been reported in 40 individuals, and 20 of them presented with epilepsy.[6,8,12,15] Here, we identified three novel and one previously reported *CLCN4* variant. All four patients displayed global DD and EE. Bioinformatics analysis and functional study supported the deleterious effect in p.Thr532Lys and p.Leu625Phe variants. For p.Val455Ile, the prediction tools suggest a benign variant. However, this variant segregated in the family, and WES showed no other likely pathogenic variants. Importantly, electrophysiological measurements showed that this variant caused a marked reduction of ClC-4 currents, supporting the pathogenicity of this variant. Combining our series with previously published cases, epilepsy was diagnosed in 54.55% of the subjects with *CLCN4* variants, suggesting that all individuals with pathogenic *CLCN4* variants should be evaluated for epilepsy due to the high prevalence of seizures in this population. Our

TABLE 3 The three novel variants found in this study and the nine reported variants are summarized

Location	Nucleotide change	AA change	Exon	Mutation type	Inheritance	Protein domain	Functional effect	Consequence	References
chrX: 10153111-10153123	del13 bp	Asp15Serfs*18	3	Frameshift	Inherited	Cytoplasmic region	NA	LOF	Palmer et al. 2018[8]
chrX: 10174503	c.661C>G	Leu221Val	7	Missense	Inherited	Loop	Reduced current	LOF	Hu et al. 2016,[6] Palmer et al. 2018[8]
chrX: 1017479	c.823G>A	Val275Met	8	Missense	De novo	H-ID	Reduced current	LOF	Palmer et al. 2018[8]
chrX: 10176604	c.1363G>A	Val455Ile	9	Missense	Inherited	H-TD	Reduced current	LOF	This study
chrX: 10181739	c.1595C>A	Thr532Lys	11	Missense	De novo	H-ID	Reduced current	LOF	This study
chrX: 10181745	c.1601C>T	Ser534Leu	11	Missense	De novo	H-ID	Reduced current	LOF	Palmer et al. 2018[8]
chrX: 10181750	c.1606G>A	Val536Met	11	Missense	Inherited	H-ID	Reduced current	LOF	Hu et al. 2016,[6] Palmer et al. 2018[8]
chrX: 10181774	c.1630G>C or G>A	Gly544Arg	11	Missense	De novo	Loop	Reduced current	LOF	Veeramah et al. 2013,[12] Hu et al. 2016,[6] Palmer et al. 2018[8]
chrX: 10182017	c.1873C>T	Leu625Phe	11	Missense	Inherited	CBS1	Reduced current	LOF	This study
chrX: 10182019-10182020	Insert A	Ile626Asnfs*135	11	Frameshift	Inherited	CBS1	NA	LOF	Palmer et al. 2018[8]
chrX: g.(10182428_10187807)_(10189796_10201480)	Delete maternal	NA	12	Intragenic copy number deletion	Inherited	CBS1	NA	LOF	Palmer et al. 2018[8]
chrX: 10188877	c.2152C>T	Arg718Trp	12	Missense	De novo or inherited	CBS2	Reduced current	LOF	Zhou et al. 2018,[15] Palmer et al. 2018,[8] this study

Abbreviations: AA, amino acid; CBS, cystathionine-β-synthase domain; H-ID, helical intramembrane domain; H-TD, helical transmembrane domain; LOF, loss of function; NA, not applicable.

results further indicate that *CLCN4* variants are associated with a wide phenotypical spectrum varying from mild brief absence seizures to severe EE. The seizure onset was within the first year of life in 69.57% of individuals, indicating an infantile onset epilepsy. EE was diagnosed in 62.5% of patients, including IS, EIMFS, and unclassifiable EE. All patients presented ID, which was more severe in individuals with more severe seizure phenotypes. Moreover, cognitive outcome was worse in patients with *CLCN4*-related epilepsy than in those without epilepsy. This suggests that ID is a common comorbidity of *CLCN4*-related epilepsy and severe epilepsy may negatively influence cognitive outcome. However, cognition and development did not improve in most patients after seizure control. This suggests that cognitive impairment was partially irreversible, possibly due to the negative effect of ClC-4 dysfunction on cognition. Our data also underline that epilepsy outcome is highly variable between patients with *CLCN4*-related epilepsy. Only one patient did not undergo any treatment, and 43.48% of patients remitted. Seizure-related death was observed in four patients, suggesting a relevant risk for premature and unexpected death with *CLCN4*-related epilepsy. Seizure semiology was variable, including myoclonic, absence, tonic, complex partial, tonic–clonic, focal, and secondary generalized seizures and IS, notably at the time of reporting, as it is not possible to capture the full seizure spectrum. Of note, there was a trend for patients with multiple seizure types to be more pharmacoresistant than those with a single seizure type. To date, there are few data on treatment response in *CLCN4*-related epilepsy. Causal treatment is not available. The limited data suggest that *CLCN4*-related epilepsy is difficult to treat but has responded relatively well to lamotrigine, a blocker of voltage-sensitive sodium channel that reduces neuronal excitability, suggesting that the targets of lamotrigine and ClC-4 are coexpressed in the same brain areas. Interestingly, our patients did not react to valproic acid, which exerts various pharmacodynamic effects, for example on ionic currents and γ-aminobutyric acid levels.[34] Lamotrigine abrogated or dramatically reduced seizures in five patients. Therefore, lamotrigine should be considered as a therapeutic option for *CLCN4*-related epilepsy in the future. However, these beneficial effects were seen in few patients, and larger prospective studies are needed to identify more effective therapeutic regimens. We also observed behavioral problems in almost half of the cases, with aggression being the most common. Neurological disturbances included microcephaly and infantile hypotonia as the most prominent features. The facial dysmorphisms found in most patients did not provide a recognizable pattern or any facial features suggestive for *CLCN4* variants. In addition, most individuals demonstrated structural brain abnormalities as detected by neuroimaging, but with no apparent pattern among the affected individuals. Nevertheless, corpus callosum abnormalities and white matter change were recurrent findings. The relationship of both white matter impairment and callosal abnormalities to epilepsy or DD will be important for future studies. However, no obvious changes were observed in white matter or corpus callosum of ClC-4-deficient mice.[8,23,35]

Although mutations were found throughout the gene or the protein, a significant number of mutations were identified in Exon 11 of the *CLCN4* gene, or located in the H-IM and CBS domains of the ClC-4 protein, underlining the crucial importance of these regions. However, no obvious correlation between the position of mutations on the gene or protein and severity of the phenotype was observed. We found two recurrent missense variants, p.Gly544Arg and p.Arg718Trp, suggesting these areas are possible mutational "hot spots" in *CLCN4*. Remarkably, all *CLCN4* variants lead to loss of function, whereas diseases associated with *CLCN6* and *CLCN7* can also be caused by gain-of-function mutations that increase the current amplitude or alter voltage-dependent activation kinetics.[10,36–39] For *CLCN4*, it is notable that frameshift or intragenic deletion variants cause rather mild phenotypes, whereas missense variants cause more severe phenotypes. Moreover, early death occurred in four patients with missense variants, whereas it did not occur in patients with frameshift or intragenic deletion variants, which may suggest that perturbation of normal ClC-4 protein function is more deleterious than a reduction in its abundance or a complete absence. Such reduced protein levels are expected for the frameshift and intragenic deletion variants, because the alteration is located before the last exon (Figure 1) and even production of a truncated ClC-4 version will be largely prevented through nonsense-mediated decay. A possible explanation is that defective proteins caused by missense mutations could be more deleterious, whereas loss of function due to reduced protein may be compensated by other proteins. In addition, ClC-4 mutants within ClC-3/ClC-4 heteromers may impinge on ClC-3, for example, by affecting the common subcellular trafficking or ion transport by the other subunit, as shown for ClC-7 dimers.[40,41]

Whereas de novo variants were associated with more severe ID and epileptic phenotype, patients with inherited variants had milder ID and epileptic phenotype. Notably, phenotypic variability was seen in both recurrent variants and within single families. A remarkable phenotypic variability was found for the recurrent p.Arg718Trp variant, with one carrier showing brief absence seizures without the need of treatment, whereas others had more severe EE. Particularly noteworthy were the discrepancies in seizure phenotype within single families in which different individuals presented with either well-controlled seizures on monotherapy or severe EE and early death. *CLCN4*-related epilepsy thus displays a remarkable phenotypic heterogeneity, indicating that the genotype–phenotype relationship remains complex. This presents challenges for genetic counseling. The

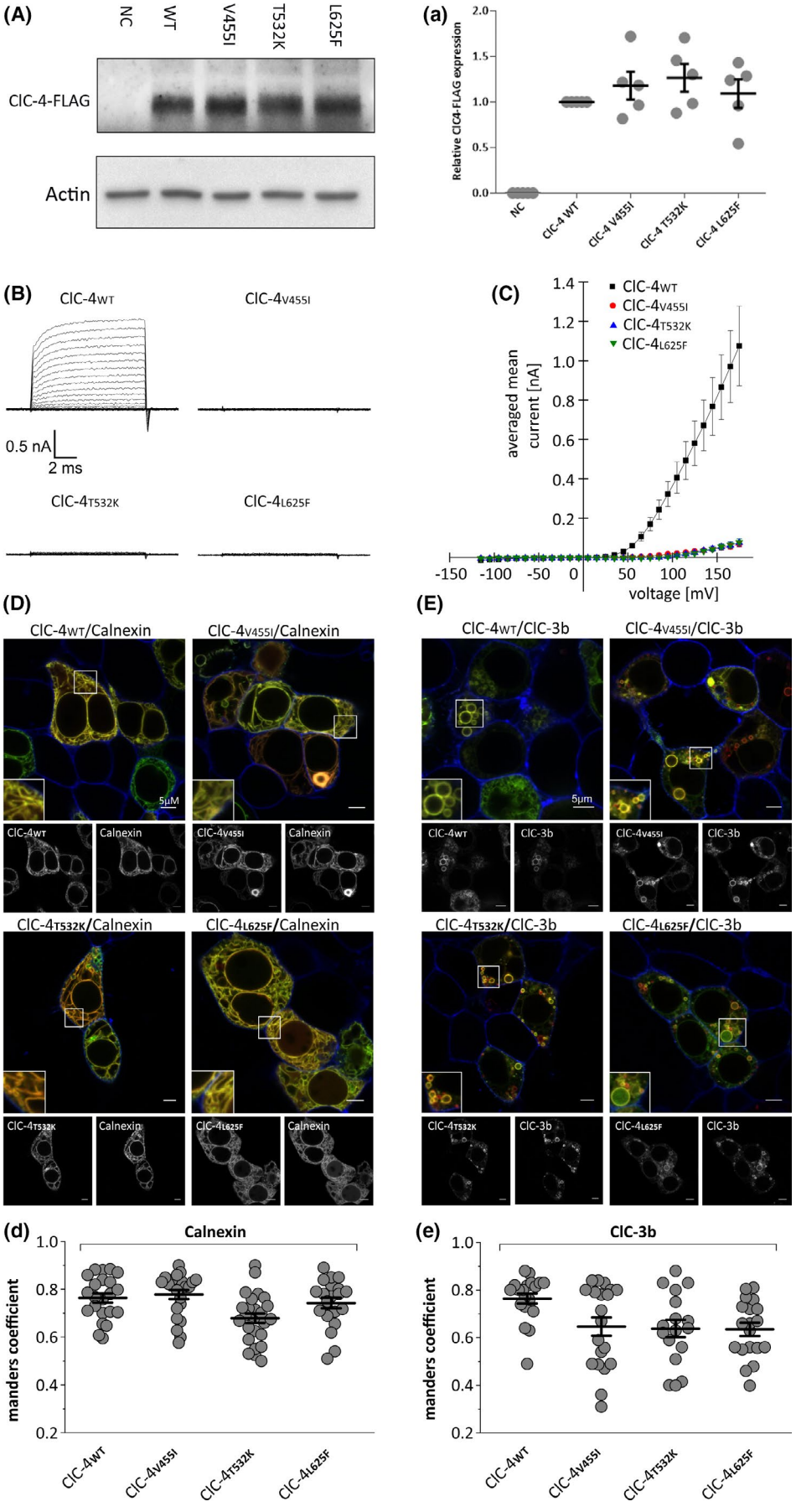
(A)
NC
WT
V455I
T532K
L625F
ClC-4-FLAG
Actin
(a)
Relative ClC4-FLAG expression
2.0
1.5
1.0
0.5
0.0
NC
ClC-4 WT
ClC-4 V455I
ClC-4 T532K
ClC-4 L625F
(B)
ClC-4WT
ClC-4V455I
0.5 nA
2 ms
ClC-4T532K
ClC-4L625F
(C)
ClC-4WT
ClC-4V455I
ClC-4T532K
ClC-4L625F
averaged mean current [nA]
1.4
1.2
1.0
0.8
0.6
0.4
0.2
-150 -100 -50 0 50 100 150
voltage [mV]
(D)
ClC-4WT/Calnexin
ClC-4V455I/Calnexin
5μM
ClC-4WT
Calnexin
ClC-4V455I
Calnexin
ClC-4T532K/Calnexin
ClC-4L625F/Calnexin
ClC-4T532K
Calnexin
ClC-4L625F
Calnexin
(E)
ClC-4WT/ClC-3b
ClC-4V455I/ClC-3b
5μm
ClC-4WT
ClC-3b
ClC-4V455I
ClC-3b
ClC-4T532K/ClC-3b
ClC-4L625F/ClC-3b
ClC-4T532K
ClC-3b
ClC-4L625F
ClC-3b
(d)
Calnexin
manders coefficient
1.0
0.8
0.6
0.4
0.2
ClC-4WT
ClC-4V455I
ClC-4T532K
ClC-4L625F
(e)
ClC-3b
manders coefficient
1.0
0.8
0.6
0.4
0.2
ClC-4WT
ClC-4V455I
ClC-4T532K
ClC-4L625F

FIGURE 2 Functional findings of the three novel *CLCN4* variants identified in this study. (A) Western blot analysis of HEK293 cells expressing wild-type (WT) ClC-4 and ClC-4 variants. WT and mutant ClC-4 proteins showed similar protein expression levels. β-actin was used as loading control. NC, negative control (mock-transfected). Values are expressed as mean ± SEM of five independent experiments. (B) Representative current traces recorded from HEK293T cells expressing either WT or mutant ClC-4. (C) Comparison of current–voltage relationships between WT (black solid squares, $n = 12$) and the three *CLCN4* variants (p.V455I [red solid circles, $n = 11$], p.T532K [blue solid triangles, $n = 13$], L625F [inverted green solid triangles, $n = 12$]). (D) Subcellular localization of human ClC-4 variants heterologously expressed in HEK293T cells. ClC-4WT and the respective mutant proteins localize mainly to the endoplasmic reticulum (ER) and show strong colocalization with the coexpressed ER marker calnexin (WT ClC-4, $n = 22$; p.V455I ClC-4, $n = 23$; p.T532K ClC-4, $n = 26$; or L625F ClC-4, $n = 20$). (E) Representative images of HEK293T cells heterologously coexpressing ClC-4 variants with ClC-3b. Colocalization of ClC-3b and WT or all the three ClC-4 mutants in cytoplasmic vesicles of HEK293T cells (WT ClC-4, $n = 20$; V455I ClC-4, $n = 20$; T532K ClC-4, $n = 17$; or L625F ClC-4, $n = 19$). Scale bars = 5 μm

mechanisms underlying phenotypic heterogeneity are unclear but may be explained by individual genetic background, genetic modifiers, epigenetic effects, or environmental factors. Further studies are required to precisely define the correlation between genotype and phenotype.

Electrophysiological studies revealed that all these missense variants reduced or abolished ClC-4 currents,[6,8,12] consistent with loss of function underlying the epilepsy and cognitive defects. The number of dendritic branches and dendritic length are remarkably reduced in primary hippocampal neurons from *Clcn4*-null mice and in *Clcn4* knockdown mouse hippocampal or cortical neurons.[6,42] Therefore, ClC-4 showed a significant effect on neuronal differentiation, which corroborates the notion that loss of ClC-4 function may be the major pathogenic mechanism. However, this conclusion appears to conflict with an apparent lack of neurological or behavioral phenotype, or altered brain morphology in $Clcn4^{-/-}$ mice.[8,23,35] These findings raised the possibility that another vesicular CLC protein with exactly the same properties might compensate for the loss of ClC-4 in $Clcn4^{-/-}$ mice. ClC-4 colocalizes with ClC-3, with which it can form functional heteromeric ClC-3/-4 complexes.[23,30,32] Hence, the lack of an obvious phenotype in $Clcn4^{-/-}$ mice may be explained by ClC-3 compensating for the loss of ClC-4.[43] On the other hand, additional depletion of ClC-4 augments the neurodegeneration observed in $Clcn3^{-/-}$ mice.[23,44] It remains unclear how loss of ClC-4 function leads to epilepsy in human. Additional cases and further functional studies are required to clarify the mechanism in *CLCN4*-related epilepsy.

In conclusion, this study expands the number of reported patients with *CLCN4*-related epilepsy and describes three novel loss-of-function variants. It provides an overview of all *CLCN4*-related epilepsy cases, comparing the epilepsy and developmental findings in 24 subjects. The phenotypic spectrum of *CLCN4*-related epilepsy includes medication-resistant seizures, ID, behavioral disorders, and congenital anomalies. Notably, patients with multiple seizure types, or missense or de novo variants showed more severe phenotype, whereas single seizure type, frameshift or intragenic deletion, or inherited variants were associated with milder phenotypes. However, genotype–phenotype relationships for *CLCN4*-related epilepsy are not straightforward. Phenotypic variability was observed in recurrent variants and within single families. Loss of ClC-4 function is the most likely underlying disease mechanism. Further elucidation of disease mechanisms may facilitate the development of targeted treatments, which are much needed for this drug-resistant genetic epilepsy.

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CONFLICT OF INTEREST

None of the authors has any conflict of interest to disclose.

AUTHOR CONTRIBUTIONS

Conceptualization: Hailan He, Jing Peng, and Tobias Stauber; investigation: Hailan He, Raul E. Guzman, and Juan Sierra-Marquez; resources: Dezhi Cao, Fei Yin, and Jing Peng; collecting and analyzing data: Hailan He, Raul E. Guzman, Juan Sierra-Marquez, and Christoph Fahlke; writing–original draft preparation: Hailan He; writing–review and editing: Raul E. Guzman, Christoph Fahlke, Jing Peng, and Tobias Stauber.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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